# BRAIN CANCERS: NEW PERSPECTIVES AND THERAPIES

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PUBLISHED IN: Frontiers in Neuroscience and Frontiers in Pharmacology

frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88974-732-0
DOI 10.3389/978-2-88974-732-0

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# BRAIN CANCERS: NEW PERSPECTIVES AND THERAPIES

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**Citation:** Bottone, M. G., Roda, E., eds. (2022). Brain Cancers: New Perspectives and Therapies. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-732-0

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frontiers in Neuroscience

EDITORIAL
published: 14 February 2022
doi: 10.3389/fnins.2022.857408

# Editorial: Brain Cancers: New Perspectives and Therapies

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**Keywords: brain tumors, glioblastoma, novel therapies, chemotherapeutic medicines, tumor resistance**

## Editorial on the Research Topic

## Brain Cancers: New Perspectives and Therapies

Brain diseases come in many different forms. It is estimated that these pathologies affect the lives of 1 in 6 people, and cost over a trillion dollars in annual treatment. The major categories of brain diseases include diverse brain cancers. Brain tumors are the most primitive, invasive and malignant in humans with poor survival after diagnosis (Mckinney, 2004; Laquintana et al., 2009). Although in recent years, numerous studies have been carried out to identify novel therapeutic protocols and tumor molecular markers capable to predict survival and response to treatment, the life expectancy of neuro-oncological patients is still very limited (24–36 months) (Aldape et al., 2019; Liang et al., 2020).

About 33% of all brain tumors are gliomas, accounting for about 80% of the total malignant central nervous system (CNS) tumors in adults (Hanif et al., 2017). Glioma is a broad category of glial brain and spinal cord tumors which originate in the glial cells that surround and support neurons in the brain, including astrocytes, oligodendrocytes, and ependymal cells. Among these, glioblastoma (GBM) is one of the most common and aggressive primary brain tumors (van Tellingen et al., 2015; Davis, 2016; Taylor et al., 2019; Birzu et al., 2021), characterized by diffuse infiltration of the adjacent brain parenchyma and development of drug resistance to standard treatment (Chen et al., 2018; Shergalis et al., 2018). So far, GBM remains associated with an extremely aggressive clinical course, and only 0.05–4.7% of patients survive 5 years from diagnosis (Ostrom et al., 2018). Cellular pleomorphism with nuclear atypia, high mitotic activity, and microvascular proliferation distinguish GBM from other lower-grade gliomas (Hambardzumyan and Bergers, 2015). In addition, the inter- and intra-patient tumor heterogeneity causes several obstacles, limiting the improvement of an early diagnosis and treatment protocols.

The tumor microenvironment (TME) plays a crucial role in mediating tumor progression and invasiveness, contributing to brain tumor aggression and poor prognosis (Di Cintio et al.; Yekula et al., 2020). Recent studies showed that differentiated tumor cells may have the ability to dedifferentiate acquiring a stem-like phenotype in response to microenvironment stresses such as hypoxia. Acidic extracellular pH and nitric oxide were also shown to be involved in stemness preservation (Dahan et al., 2014). Currently, the standard of care consists of surgical resection followed by radiotherapy (RT) and concomitant and adjuvant chemotherapy. Despite this aggressive treatment regimen, the median survival is only around 15 months, and the 2-year survival rate is only 26.5% (von Neubeck et al., 2015; Chen et al., 2018). Indeed, due to the location

OPEN ACCESS

***Edited and Reviewed by:***
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***Specialty section:***
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

***Received:*** *18 January 2022*
***Accepted:*** *24 January 2022*
***Published:*** *14 February 2022*

***Citation:***
*Roda E and Bottone MG (2022) Editorial: Brain Cancers: New Perspectives and Therapies. Front. Neurosci. 16:857408. doi: 10.3389/fnins.2022.857408*

of gliomas origin and infiltrative growth (Urbańska et al., 2014), complete surgical resection of the tumor is often not possible other than with a high risk of neurological damages for the patient (Goldbrunner et al., 2018). Treating patients with primary brain tumors and brain metastases can be challenging. This is primarily due to the presence of the blood–brain barrier (BBB), posing an obstacle to overcome for most systemic treatments (van Tellingen et al., 2015; Brahm et al., 2020). Despite initial benefits, chemotherapy, using conventional agents, e.g., alkylating agents such as temozolomide, platinum-based drugs, or VEGF inhibitors (Dasari and Tchounwou, 2014; Pérez et al., 2019; Senbabaoglu et al.; Strobel et al., 2019), is often associated with severe systemic toxicity, which occurs especially after long-term treatment (Karasawa and Steyger, 2015; Chovanec et al., 2017). Among these adverse side effects, neurotoxicity assumed increasing clinical importance as it is dose-cumulative and becomes limiting in long-lasting therapies, and also to the severe side effects (Chovanec et al., 2017; Staff et al., 2019). Therefore, high-grade gliomas or GBM are currently considered incurable and all patients inevitably experience and succumb to tumor recurrence, highlighting the urgent need to identify, validate and apply new therapeutic options (Ravanpay et al., 2019; Taylor et al., 2019; Maggs et al.; Ghouzlani et al., 2021).

This Frontiers Research Topic Proposal on "*Brain Cancers: New Perspectives and Therapies*" joined contributions from scientists and physicians who investigate on etiopathogenesis and treatment of brain cancers. In fact, studies exploiting the existing link between enhancing the knowledge of cellular and molecular pathways involved in the onset/progression of these pathologies and the development of innovative therapies, improving patient prognosis and quality of life, need further in-depth investigations.

The published articles are based on neuro-oncological research and deal with proposing novel effective therapeutic strategies, focusing on different targets and aspects typical of brain tumors: tumor heterogeneity and microenvironment, cancer cell response to new chemotherapeutics and innovative radiotherapy treatments settings (often tested in combined protocols), immune-mediated gene therapies, which may involve blockade of immune checkpoint inhibitors, and other targeted therapies such virotherapy, CAR-T cells, dendritic cells' vaccines, or nanoparticle-mediated vaccination technologies (Alghamri et al.; Brandalise et al.; Chen et al.; Di Cintio et al.; Ferrari et al.; Lange et al.; Maggs et al.; Pasi et al.; Senbabaoglu et al.).

The joint mechanisms of neuro-inflammation, tumor microenvironment and BBB leakage status, which have been shown to trigger the tumor onset, invasion and progression, often mediated by the deregulation of a number of channel proteins and ion pumps (Brandalise et al.), have been also explored as promising targets for personalized pharmacological interventions (Alghamri et al.; Di Cintio et al.; Lee et al., 2020). Another exploited key mechanism is cell death, a crucial multifaceted process dependent on signal transduction pathways, in which several Hsp90 client proteins, frequently abnormally expressed, may be involved (Cao et al.; Chen et al.). It widely accepted that in cancer cells, particularly in gliomas cells, cell death pathways can be deactivated or defective for various causes, thus promoting cancer formation, proliferation, invasiveness, and even the induction of resistance to the drugs treatment. Particular effort has been devoted to the repositioning of old drugs as potent therapeutics for GMB and/or to exploit the combined effects of novel drugs in synergism with different irradiation protocols (Chen et al.; Lange et al.; Ferrari et al.; Pasi et al.).

In summary, clinical evidences highlights the urgent medical need to further comprehend and delineate the complex mechanisms/interactions between cancer cells, immune cells, tumor stroma, resident healthy brain cells, and tumor vasculature, to develop innovative effective treatment strategies through the identification of novel targets. A multidisciplinary approach, taking into consideration all brain tumors aspects, including the modulation of the communication processes between cancer niche and tumor microenvironment and also the potential reactivation of defective cell death mechanisms, can currently be considered as a promising strategy.

This Frontiers Research Topic had the ultimate goal to apply new knowledges coming from multitiered approaches, to identify novel effective therapeutic strategies to be used in the field of clinical neuro-oncology, to improve the patient prognosis and quality of life, also reducing adverse side effects due to conventional treatments, in view of a focused, personalized medicine. The published contributions may play a crucial role, laying the groundwork to translate the experimental findings to clinical setting, turning them into new clinical therapeutic protocols, facing the challenges in this field and developing new healing perspectives.

## AUTHOR CONTRIBUTIONS

Both authors equally contributed to the work, giving a substantial, direct, and intellectual contribution, and they both approved the work for publication.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 30 June 2020
doi: 10.3389/fphar.2020.00974

# A Brain-Penetrating Hsp90 Inhibitor NXD30001 Inhibits Glioblastoma as a Monotherapy or in Combination With Radiation

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**Specialty section:**
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

**Received:** 30 March 2020
**Accepted:** 15 June 2020
**Published:** 30 June 2020

**Citation:**
Chen H, Gong Y, Ma Y, Thompson RC, Wang J, Cheng Z and Xue L (2020) A Brain-Penetrating Hsp90 Inhibitor NXD30001 Inhibits Glioblastoma as a Monotherapy or in Combination With Radiation. Front. Pharmacol. 11:974. doi: 10.3389/fphar.2020.00974

Glioblastoma multiforme (GBM) is a highly heterogeneous disease, which is initiated and sustained by various molecular alterations in an array of signal transduction pathways. Heat-shock protein 90 (Hsp90) is a molecular chaperone and is critically implicated in folding and activation of a diverse group of client proteins, many of which are key regulators for glioblastoma biology. We here assessed the anti-neoplastic efficacy of a novel brain-penetrating Hsp90 inhibitor NXD30001 as a monotherapy and combined with radiation *in vitro* and *in vivo*. Our results demonstrated that NXD30001 potently inhibited neurosphere formation, growth, and survival of CD133+ GBM cells with the half maximal inhibitory concentration at low nanomolar range, but CD133– GBM cells were less sensitive to NXD30001. NXD30001 also increased radio-sensitivity in glioblastoma stem cells (GSCs) at suboptimal concentrations. Moreover, NXD30001 dose-dependently decreased phosphorylation levels of multiple Hsp90 client proteins which play key roles in GBM, such as EGFR, Akt, c-Myc, and Notch1. In addition, NXD30001 could impair DNA damage response and endoplasmic reticulum stress response after radiotherapy by alteration of the related proteins expression. In a murine orthotopic model of human glioblastoma, NXD30001 marvelously induced tumor regression and extended median survival of tumor-bearing mice by approximately 20% when compared with the vehicle group (37 d vs 31 d, $P<0.05$). Radiotherapy solely increased median survival of tumor-bearing mice from 31 d to 38 d ($P<0.05$), while NXD30001 combined with radiation further extended survival to 43 d ($P<0.05$). We concluded that GSCs are more sensitive to NXD30001 than non-stem GBM cells, and NXD30001 in combination with radiation exerts better inhibitive effect in GBM progression than monotherapy.

**Keywords: glioblastoma, radiation resistance, Hsp90 inhibitor, molecular pharmacology, DNA damage response, ER stress**

## INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most common malignant form of gliomas that with a median survival period of 12–15 months (Gittleman et al., 2018). Radiation therapy, the clinical application of ionizing radiation, is one crucial treatment option in modern GBM therapy apart from surgical resection and the chemotherapeutic alkylating agent (Stupp et al., 2005; Hathout and Pope, 2016). However, GBM is well documented for its rapid recurrence following radiotherapy, due to inadequate killing of cancer stem cells, which is known as tumor-initiating cells or tumor-propagating cells. CD133 is a pentaspan transmembrane protein and was commonly used as a glioblastoma stem cells (GSCs) biomarker, it has been showed that CD133 positive (CD133+) glioblastoma cells contributes to glioma radioresistance and tumor progress through preferential checkpoint response and DNA repair (Beier et al., 2007; Norollahi et al., 2019). *In vivo* and *in vitro* studies have also verified the fraction of CD133+ glioma cells were significantly increased after radiation treatment (Ahmed et al., 2018). Therefore, compromising the resistant mechanisms in these cells may significantly improve the efficacy of radiotherapy for GBM (Sheehan et al., 2010). However, the mechanisms implicated in GSCs radiation resistance remain poorly defined, which may involve combinatorial alterations in signaling networks that regulate DNA damage checkpoints, DNA repair, cellular survival, etc. (Skvortsova et al., 2015). As GBM is a highly heterogeneous disease, the radio-resistant mechanisms of GSCs drastically varied among tumors with distinguished molecular background. Therefore, the efficacy of single-targeted radio-sensitizing approaches is likely to be constrained to a small subset of patients.

The 90-kDa heat-shock protein (Hsp90) is a highly abundant molecular chaperone that is responsible for the maintenance of protein homeostasis under basal conditions and during stress response (Den and Lu, 2012). Hsp90 client proteins regulate a large number of cellular functions, including signal transduction, protein trafficking, chromatin remodeling, autophagy, cell proliferation, and survival (Zuehlke and Johnson, 2010). Many of these client proteins are frequently abnormally expressed in cancer cells and therefore inhibition of Hsp90 may be a rational approach to target cancer cells. Currently, several Hsp90 inhibitors have been examined in preclinical and clinical settings for different human cancers (Sidera and Patsavoudi, 2014). The Hsp90 inhibitors simultaneously target multiple radio-resistant pathways and thereby have preferential effects for GBM therapy (Camphausen and Tofilon, 2007). 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a benzoquinone antibiotic derived from geldanamycin, is an Hsp90 inhibitor that has been shown to inhibit tumor growth in GBM either as a single agent or in combination with radiation (Sauvageot et al., 2009). However, 17-AAG and most other Hsp90 inhibitors cannot cross the blood-brain barrier (BBB) effectively, which greatly limited their potential efficacy for gliomas treatment. NXD30001, a novel radicicol-based series of Hsp90 inhibitors, has a more favorable brain pharmacokinetic profile and has been reported to inhibits Hsp90 potently than 17-AAG (Cha et al., 2014). NXD30001 could easily crosses the BBB and accumulates in the brain and would not cause liver or ocular toxicity *in vivo*, made it be an attractive therapeutic candidate for gliomas by downregulating Hsp90 client oncoproteins to inhibit tumor cell proliferation and induce cell apoptosis. Furthermore, previous studies depicted that NXD30001 could induce tumor regression in GBM model (Zhu et al., 2010) and neurofibromatosis type 2 model (Tanaka et al., 2013), which provides a compelling rationale for its use as an attractive therapeutic candidate in treatment for central nervous system tumors.

However, targeting Hsp90 solely faces several challenges. It has been reported that the anticancer efficacy of Hsp90 inhibitors as monotherapy in clinical trials was often less significant than predicted by preclinical models (Messaoudi et al., 2011). For example, Hsp90 may not be adequately inhibited in GBM or can be compensated by induction of co-chaperons, such as Hsp27 and Hsp70 (van Ommeren et al., 2016). Also, tumors *in vivo* may be less dependent on Hsp90 than *in vitro* (Neckers and Workman, 2012). As a preliminary study demonstrated that Hsp90 inhibitor could enhance the radiotherapy in a variety of human cancer cell lines, including GBM (Piper and Millson, 2011), we here explored the anti-neoplastic efficacy and mechanisms of NXD30001 as a monotherapy or in combination with radiation in GSCs and GBM orthotopic animal model.

## MATERIALS AND METHODS

### Cell Culture and Enrichment of GSCs

The primary glioblastoma cell lines of T4105, T4302, and T4597 were generously provided by Dr. Jeremy Rich at Cleveland Clinic (Cleveland, OH). These tumor samples were originally derived from patient surgical specimens and serially passaged as subcutaneous xenograft tumors. Matched cultures enriched or depleted for the CD133+ glioma cells subpopulation was prepared following methods described in our previous publications (Wang et al., 2010b; Cheng et al., 2013; Ma et al., 2015; Gong et al., 2016; Ma et al., 2017). Briefly, cells were enzymatically dissociated from subcutaneous xenograft tumors and red blood cells were lysed in diluted phosphate-buffered saline solution (0.25×). The CD133+ and CD133 negative (CD133−) fractions were magnetically sorted using the CD133 Microbead kit (Miltenyi Biotec, Bergisch Gladbach, German) following the manufacturer's instructions. Dissociated CD133+ cells or unsorted neurospheres were then cultured overnight in stem cell media (neurobasal media supplemented with B27, epidermal growth factor, and basic fibroblast growth factor at 20 ng/ml) before cell sorting for recovery of cellular surface antigens. CD133− cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) but were cultured in stem cell media at least 24 h prior to experiments to control differences in cell media. All cells were cultured at 37 °C with 5% $CO_2$ and maintained for no more than five passages as these cells may spontaneously differentiate *in vitro*.

## Irradiation

Single cell suspension or monolayer culture for CD133+ cells were irradiated in medium with Varian 600 CD X-ray linear accelerator (Varian Medical Systems, Inc., Palo Alto, CA, USA) at a dose rate of 1, 2, or 3 Gy/min exposed at room temperature. Tumor-bearing mice receive whole brain X-ray radiation for 6 consecutive days at 3 Gy/day under anesthesia, and then tumors were measured by bioluminescence images.

## Reagent and Antibodies

NXD30001 was obtained from NexGenix Pharmaceuticals, 17AAG was generously provided by Kosan Bioscience, Inc. The antibodies used in this study including Phospho(p)-EGFR (1H12, #2236), EGFR (D6B6, #2085), pS473-Akt (D9E, #4060), Akt (40D4, #2920), Cleaved-Notch1 (D3B8, #4147), Notch1 (D1E11, #3608), c-Myc (D84C12, #5605), p-MEK (Thr286, #9127), p-ERK1/2 (D13.14.4E, #4370), ERK1/2 (L34F12, #4696), p-ATR (Ser428, #2853), p-CHK2 (Thr68, #2661), p-p53 (Ser15, #9286), p-H2AX (D7T2V, #80312), PERK (D11A8, #5683), IRE1α (14C10, #3294), BiP (C50B12, #3177), and CHOP (L63F7, #2895) were purchased from Cell signaling Technology. Mouse monoclonal antibody against actin (#MAB1501) was purchased from Millipore. Secondary antibodies were obtained from Santa Cruz Biotechnology, Inc.

## Cell Viability, Neurosphere Formation Assay, and Apoptosis Detection

GSC-enriched spheroid cultures (spheres) were used to test cell viability and neurospheres formation. For cell viability assay, cells were seeded into white, 96-Well White Flat Bottom (Corning, NY, USA) at 5,000 cells per well in a total volume of 100 μl media. Enriched cells were treated with 17AAG or NXD30001 at different concentrations, or then exposed to 3 Gy X-ray radiation for 1 min after 4 h. Proliferation assays *in vitro* testing were performed by counting the increase in viable cell numbers over 5 d using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). After 30 min incubation at room temperature, the signal from the viable cells was analyzed on a Molecular Devices Spectramax M5 (Molecular Devices, California, USA). Replicate measurements were analyzed with respect to dose and estimates of half maximal inhibitory concentrations (IC50) were calculated by logistic regression (GraphPad Prism 5.0). Averaged cell titer of the sham-treated control group on day 1 was assigned a value of 1. All other relative cell titers were normalized accordingly.

To measure neurospheres formation, CD133+ glioma cells were seeded in 24-well plates at 100 cells per well. Spheriod cultures were grown for 1–2 d prior to increasing dose of NXD30001 incubation. Cells were allocated into 3 groups and treated with NXD30001 for 24 h, then irradiated for 1 min at 1, 2, or 3 Gy, each group has four replicates. At 7 d after plating, neurospheres containing more than 50 cells were scored.

To detect apoptosis, the cell number was measured daily for 3 d was using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), the caspase 3/7 activities at 72 h after NXD30001 alone or with 3 Gy radiation treatment were measured using the Caspase-Glo3/7 assay (Promega, Madison, WI) according to the manufacturer's instructions. The caspase activities were normalized to the corresponding cell titers to generate the relative caspase 3/7 activities.

## Flow Cytometry

For apoptosis/necrosis detection, the CD133+ cells were grown in six-well plate (for suspension cells, Sarstedt) at a starting concentration of $5\times10^4$ cells per well. Then CD133+ cells ($10\times10^5$ cell/ml) were treated of NXD30001 (2.5, 5, 10 nM) with or without radiation or vehicle (DMSO 0.1%) for 72 h. At the desired time point, $2\times10^6$ cells were spun at 500 g for 5 min at 4°C and washed with PBS. Pellets were resuspended in 1 ml of cold PBS and added dropwise while gently vertexing to 9 ml 70% ethanol in a 15 ml polypropylene centrifuge tube. Fixed cells were then frozen at −20°C overnight. The next day, cells were centrifuged at 500 g for 10 min at 4°C and washed with 3 ml of cold PBS. Cells were resuspended in 500 μl of propidium iodide staining solution (0.2 mg/ml RNAse A, 0.02 mg/ml propidium iodide, 0.1 % Triton-X in PBS) and incubated for 20 min at 37°C. The cells were washed and resuspended in Annexin-V/propidium iodide buffer solution containing Annexin V-FITC (BD Pharmigen, 51-65874X) and Propidium Iodide (BD Pharmigen, 51-66211E). Samples were immediately analyzed on a five-laser BD LSRII. Visualizations and analyses of apoptotic fractions were generated using BD FACSDiva™ software.

## Immunoblotting Analysis

Lysates for blotting were prepared by seeding $5\times10^5$ of the GBM cells onto culture dish, cells were treated with 17-AAG or NXD30001 at different concentrations with or without radiation at 3 Gy for various time points. After collecting the cells the pellets were washed three times with ice-cold PBS and the pellet after the final wash was resuspended in lysis buffer containing NP40, 50 mM Tris HCl (pH=8.0), 120 mM NaCl, 5 mM EDTA, 1% protease inhibitors (Roche Diagnostics, Indianapolis, IN), and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined by using the Bio-Rad DC protein assay (Bio-rad, California, USA). Samples were loaded into a Novex Tris-Glycine Gel (Life Technologies, Grand Island, NY) and separated by electrophoreses at 125 V. The gels were then transferred onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA) by a wet Bio-Rad trans-blot system (Bio-rad, California, USA) and blocked by incubation with 5% dry milk in TBST (TBS with 0.1% Tween20). Primary antibodies were added to blocking solution and incubated overnight at 4°C on a shaker. Blots were washed several times with TBS-T-BSA. Chemiluminescent detection was performed with appropriate secondary antibodies. To quantify equal loading, the membranes were re-probed with a primary antibody targeting β-actin.

## *In Vivo* Assay With an Orthotopic Glioblastoma Model

All animal experiments were performed according to approved Institutional Animal Care and Use Committee (IACUC)

protocols at Vanderbilt University Medical Center. T4302 CD133+ cells were implanted into the right cerebrum of female athymic nude mice for intracranial glioblastoma induction, as described previously (Cheng et al., 2013; Ma et al., 2015; Gong et al., 2016; Ma et al., 2017). Briefly, mice were anesthetized and placed in a small animal stereotactic frame (ASI Instruments, Houston, TX). The injection location was 1.5 mm anterior to the coronal suture, 2.5 mm to the right of the sagittal suture, and 3–3.5 mm below the skull. Approximately $5\times10^3$ tumor cells suspended in 10 µl phosphate buffered saline were injected over 2 min using a 26–27 gauge syringe (approximately 3 mm deep), and the needle was left in position for 5 min and then withdrawn slowly. Upon withdrawal of the needle, the muscle and skin were closed with 5–0 silk sutures immediately. Before implantation, we first infected T4302 CD133+ cells with lentivirus directing expression of firefly luciferase and screened by 1 µg/ml puromycin for 3 d. After implantation, the mice were maintained for 10 d for tumor establishment and randomly divided into four treatment groups (n=10 per group) including vehicle, NXD30001, radiation, and NXD30001 combined with radiation. In NXD30001 and untreated groups, mice were treated intraperitoneally with either NXD30001 at 50 mg/kg every other day (q.o.d. IP) or a similar volume of vehicle control for 3 weeks. In radiotherapy alone or combined with NX30001 groups, animals received 3 Gy X-ray radiation in the whole brain once per day alone for 6 consecutive days or received same radiation plan one day after the second dose of NXD30001. Mice were weighted every time before drug administration. Tumor progression was monitored by bioluminescence imaging weekly from day 10 before NXD30001 or/and radiation treatment. Mice were imaged after injection of 150 mg/kg of D-luciferin (Promega, Madison, WI) using a Xenogen IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA). Quantification was performed using Living Image software Caliper Life Sciences, Hopkinton, MA) with standardized rectangular regions of interests covering the mouse trunk and extremities. Animals were sacrificed upon the development of apparent symptoms, such as lethargy or hunched posture. The median survival was determined by the Kaplan-Meier method using the GraphPad Prism 5.0 software.

## Statistical Analysis

Values are reported as the mean ± the standard error. GraphPad Software 5.0 (GraphPad Software, Inc.) was used to determine statistical significance with either two-tailed Student's t test or ANOVA as indicated. Significance testing of survival ratio was performed by log-rank analysis.

# RESULTS

## NXD30001 Preferentially Kills GSCs

We detected the efficacy of 17-AAG and NXD30001 against GSCs, a clinically relevant model of human GBM as described before (Lee et al., 2006; Guerrero-Cazares et al., 2012). The results indicated that both 17-AAG and NXD30001 were more effective to T4105 CD133+ GBM cells in comparison to that in T4105 CD133− cells (**Figure 1A**). Same effect was also found in T4302 and T4597 pair cells with NXD30001 treatment (**Figure 1B**). **Figure 1C** showed that NXD30001 markedly decreased cell viability and proliferation in T4105, T4302, and T4597 GSCs cell lines. The growth of above CD133+ cell lines was markedly reduced by NXD30001 administration with IC50 values that ranged from 7 nM to 15 nM, while the IC50 values were higher in the three corresponding CD133− cells. Taken together, these data indicated that GBM CD133+ cells were more sensitive to NXD30001 than GBM CD133− cells.

## NXD30001 Promotes GSCs Sensitivity to Radiation

In order to determine inhibitory potency of NXD30001 combination with radiation on GSCs, the neurosphere formation and Caspase-Glo3/7 assays were performed in T4302 CD133+ cells. The cell viability assay demonstrated that the radio-sensitivity of GSCs was more evident when treated with NXD30001 in comparison with 17-AAG (**Figure 2A**). More severely impaired cell growth with dose response was showed in treatment with NX30001 and 3 Gy radiation than NX30001 alone with days (**Figure 2B**). Neurosphere formation of GSCs was substantially reduced by NXD30001 with or without radiation in a concentration-dependent manner (**Figure 2C**), which indicated that NXD30001 might reduce the self-renewal potential of GSCs and lead to a reduction in tumor recurrence capacity. Caspase-Glo3/7 assay result revealed that the caspase activation of GSCs was significantly higher in 10 nM NXD30001 monotherapy group, and this effect was particularly prominent in treatment with NXD30001 + radiation (**Figure 2D**). Furthermore, the Annexin-V assay and flow cytometry confirmed that the number of Annexin-V and V/PI positive cells increased dose-dependently when treated with NXD30001 + radiation (**Figure 2E**), indicating that NXD30001 enhances apoptosis after radiation exposure. Above all, out study demonstrated that NXD30001 exhibited a radio-sensitization potential for GBM through inhibiting proliferation and inducing apoptosis of GSCs.

## NXD30001 Decreases Phosphorylation Levels of Multiple Hsp90 Client Proteins in Combination With Radiation in GSCs

To determine the mechanism by which NXD30001 achieves its antitumor and radio-sensitizing effects on GSCs, we assessed the levels of known Hsp90 client proteins involved in GBM pathology when treated with 17-AAG or NXD30001 in T4105 CD133+ cells and treated with NXD30001 ± 3 Gy radiation in T4302 CD133+ cells. In T4105 CD133+ cells, 17-AAG and NXD30001 were shown to induce the degradation of Hsp90 client proteins, such as p-EGFR, p-Akt, c-Myc, Cleaved-Notch1, and p-MEK in a dose-dependent manner (**Figure 3**). The expressions of most client proteins began to decrease after 20

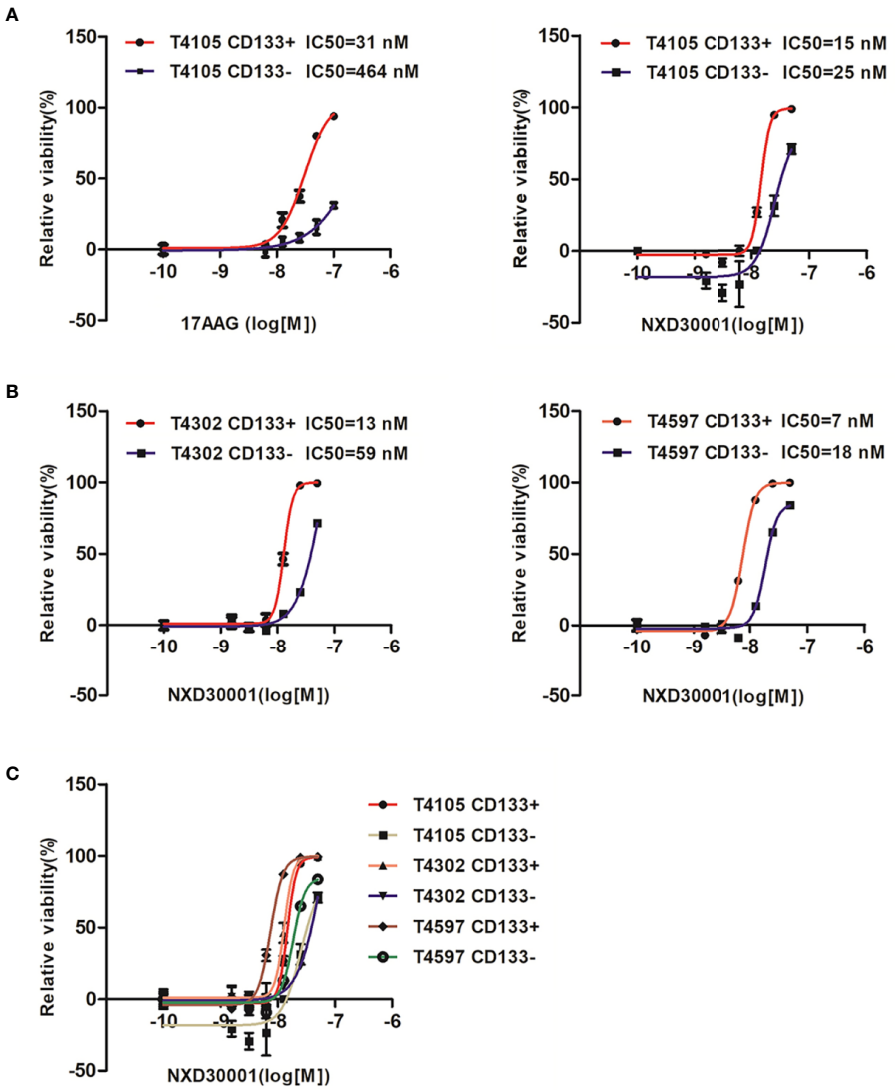


**FIGURE 1 |** NXD30001 preferentially kills GSCs. **(A)** T4105 cells were treated with 17-AAG or NXD30001 at indicated concentrations by two-fold dilution. **(B)** Two pair cells of T4302 and T4597 were also used to detect the IC50 values for NXD30001. **(C)** The cell viability in CD133+ and CD133– cells including T4105, T4302, and T4597 were compared to find the discrepancy between them. IC50 values were determined and calculated by GraphPad Prism5. The cell viability was determined after 5-d incubation by CellTiter-Glo kits (Promega). The results are the average of triplicate samples. Error bars represent the standard error.

nM NXD30001 or 50 nM 17-AAG treatment, which indicated that NXD30001 had a stronger cell growth inhibiting effect than 17-AAG (**Figure 3**). In T4302 CD133+ cells, significant downregulation of Hsp90 client proteins, including p-EGFR, p-Akt, p-ERK1/2, and Cleaved-Notch1 was found when treated with NXD30001 alone, and this effect was more markedly after combined NXD30001 and radiation therapy (**Figure 4**). Although no significant variation of c-Myc protein expression was found in NXD30001 monotherapy group, c-Myc level decreased significantly when treated with 20 nM NXD30001 + radiation (**Figure 4**). These preliminary results suggested that NXD30001 may regulate radio-resistance pathways in GSCs through, at least in part, stabilization of important pro-survival factors, such as EGFR, Akt, Notch1, and c-Myc.

## NXD30001 Reduces Activation of DNA Damage Response in GSCs After Radiation

GSCs confers radio-resistance through preferential activation of the DNA damage checkpoint response to increase DNA repair capacity, which initiate tumor recurrent after radiotherapy (Bao et al., 2006; Yamamori et al., 2013; Skvortsova et al., 2015). The present data showed that the phosphorylation levels of DNA damage related proteins including ATR, CHK2, p53, and H2AX

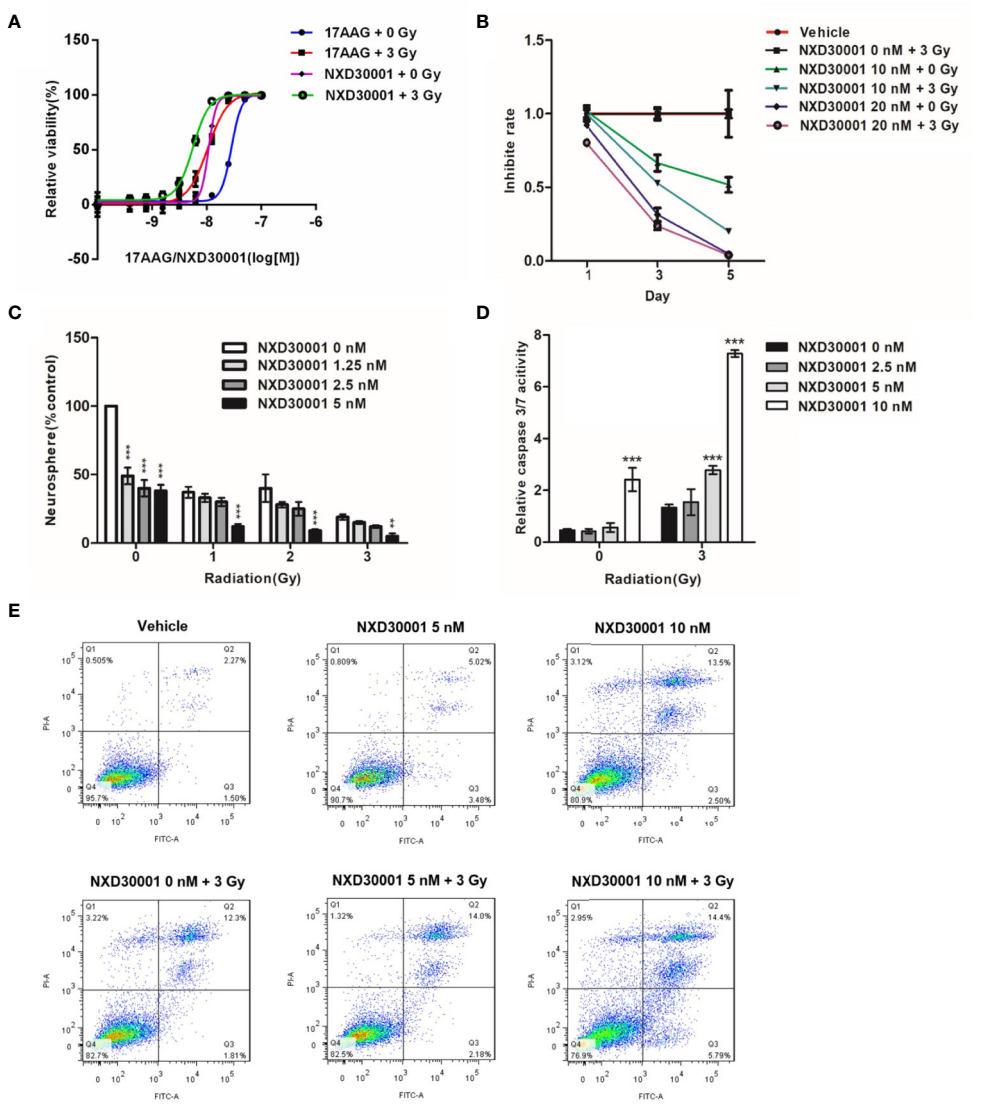


**FIGURE 2 |** NXD30001 Renders GSCs More Sensitive to Radiation. **(A)** Cell viability assay of T4302 CD133+ cells treated with various concentrations of 17-AAG or NXD30001 with or without 3 Gy dose after 5-d incubation. **(B)** T4302 CD133+ cells growth was detected in the treatment with NXD30001 (10 and 20 nM) alone or combined with radiation after 5 d. **(C)** T4302 CD133+ cells were seeded at 100 cells per well in 24-well plate. Neurospheres were scored after 7-d incubation of NXD30001 without or with 1, 2, and 3 Gy of radiation. $^{**}P < 0.01$, $^{***}P < 0.001$. **(D)** The caspase 3/7 activity was determined 72 h after various treatments and normalized to cell titer of the corresponding treatment groups. The results are the average of triplicate samples. Error bars represent the standard error. $^{**}P < 0.01$, $^{***}P < 0.001$. **(E)** The Annexin V-FITC and flow cytometric assay was performed to detect apoptosis/necrosis. The dual parameter dot plots cells in the upper right quadrant were represent as late apoptosis (AnnexinV$^+$-PI$^-$), whereas cells in lower right quadrant were represent as early apoptosis (AnnexinV$^+$-PI$^+$).

were not significantly altered in T4302 CD133+ cells following NXD30001 treatment with radiation, while significant down-regulation of p-CHK2 and p-H2AX levels was found in 10 nM NXD30001 monotherapy group (**Figure 5**). These results demonstrated that NXD30001 might reduce activation of postradiation DNA damage response in GSCs, and is speculated to impair survival signaling to induce GSCs death after radiotherapy.

## NXD30001 in Combination With Radiation Impairs ER Stress in GSCs

Unfolded protein response (UPR) that induced by ER stress response in cancer plays an important role in resistance to chemotherapy or radiation (Sui et al., 2013). In GSCs, the expression levels of UPR related proteins, such as PERK, IRE1α, GRP78/BiP, and CHOP were increased by activation of ER stress to keep cells survival (Yamamori et al., 2013;

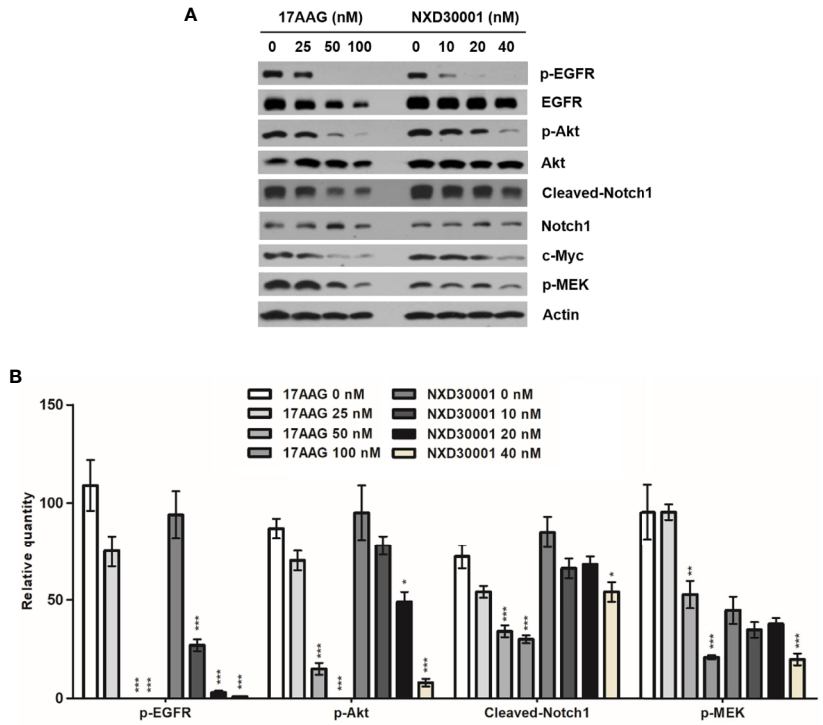


**FIGURE 3 |** NXD30001 or 17-AAG dose-dependently decreased phosphorylation levels of multiple Hsp90 client proteins in GSCs. **(A)** Representative western blotting bands showing the expression levels of p-EGFR, EGFR, p-Akt, Akt, Cleaved-Notch1, Notch1, c-Myc, p-MEK in T4105 CD133+ cell cultures treated with different concentrations of 17-AAG or NXD30001. The result was quantified from the average of three different samples. **(B)** Quantification of western blotting bands. Error bars represent the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Skvortsova et al., 2015; Le Reste et al., 2016). To determine whether NXD30001 and radiation treatment result in impairing ER stress response to induce GSCs apoptosis, the expressions of the above ER stress sensors were detected in T4102 CD133+ cells. The results showed that PERK, IRE1α, BiP, and CHOP expression levels were significantly downregulated with NXD30001 administration, and the effect was more conspicuous when treated in combination with radiation (**Figure 6**). These data provide evidence regarding the suppressive role of NXD30001 ± radiation on GSCs through impairing ER stress.

## NXD30001 in Combination With Radiation Represses Orthotopic GBM

We evaluated the therapeutic potential of combination of NXD30001 and radiotherapy by using orthotopic GBM model *in vivo*. The administration of NXD30001 and irradiation plan was showed in **Figure 7A**. Without any treatment, mice implanted with GSCs were expected to die around 30 d according to our previous studies (Cheng et al., 2013; Ma et al., 2015; Gong et al., 2016; Ma et al., 2017). Treatment with NXD30001 alone extended the median survival time of tumor-bearing mice by approximately 20% of the vehicle subjects (37 d vs. 31 d, $P<0.05$, **Figure 7B**). Radiation alone increased the median survival of tumor-bearing mice from 31 d to 38 d ($P<0.05$), while radiation in combination with NXD30001 extended median survival to 43 d ($P<0.05$, **Figure 7B**). The bioluminescence imaging results have shown that the growth rate and volume of tumors in combined NXD30001 and radiation group were significantly lower than those in vehicle group (**Figure 7C**). These *in vivo* results were well concordant with that of *in vitro* assays, suggesting that NXD30001 in combination with radiation would be an effective treatment for glioblastoma.

# DISCUSSION

Glioblastomas is one of the most lethal and frequent form of primary brain tumors, which undergoes continuous uncontrolled cell growth and proliferation. It is characterized by highly aggressive growth, invasiveness, and poor prognosis due to genetic and signaling abnormalities, which leads to an innate resistance to current therapies including radiation, alkylating chemotherapeutic agent temozolomide, and surgery (Gittleman et al., 2018). In this study, we assessed the antitumor effect of a brain-penetrating Hsp90 inhibitor, NXD30001 on GSCs *in vitro* and *in vivo* as a monotherapy or in combination with radiation. Our results showed that combined NXD30001 and radiotherapy may improve the antineoplastic activity in

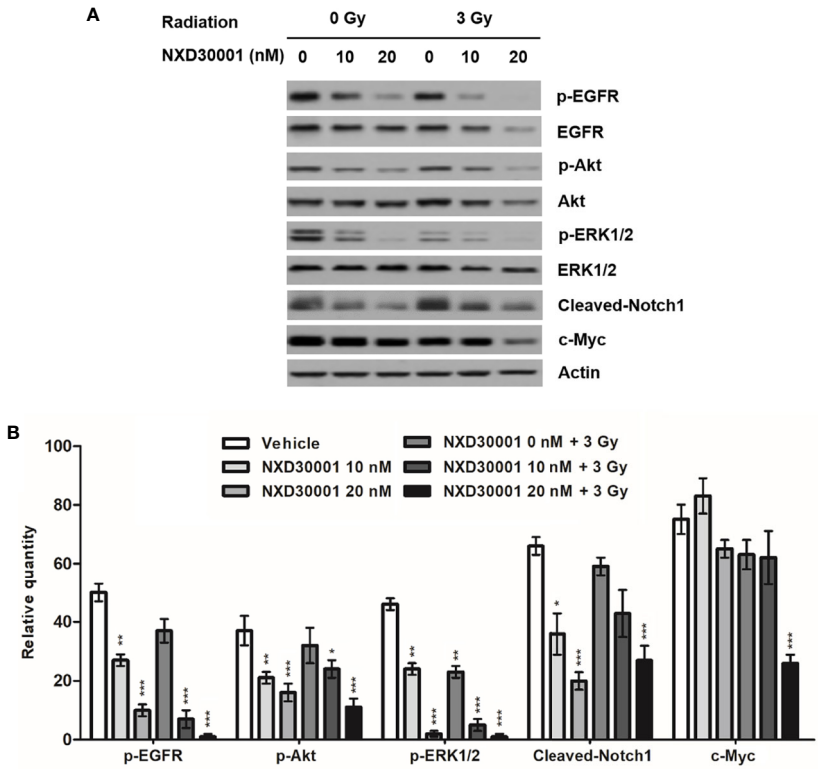


**FIGURE 4 |** NXD30001 dose-dependently decreased phosphorylation levels of multiple Hsp90 client proteins in combination with radiation in GSCs. **(A)** Representative western blotting bands showing the expression level of p-EGFR, EGFR, p-Akt, Akt, p-ERK1/2, ERK1/2, Cleaved-Notch1, c-Myc in T4302 CD133+ cell cultures treated with different concentrations of NXD30001 with or without radiation. The result was quantified from the average of three different samples. **(B)** Quantification of western blotting bands. Error bars represent the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

GBM through inhibiting GSCs proliferation and enhancing cell apoptosis by attenuating phosphorylation of multiple Hsp90 client proteins. NXD30001 also increased radio-sensitivity of GSCs through impairing DNA damage response and ER stress. Furthermore, NXD30001 in combination with radiation exerts better inhibitive effect on *in vivo* GBM growth, and provides a statistically significant prolonged survival of GBM-bearing mice.

CD133 accompanying with other neural and hematopoietic stem cell marker including Musashi-1, Nestin, Sox2, and Olig2 can identify glioma stem cells from different molecular subtypes of glioma. CD133+ GBM cells exhibit transcription profiles resembling the proneural subtype, whereas CD133− GBM cells may be associated with gliomas of the mesenchymal subtype (Joo et al., 2008; Lottaz et al., 2010). It has been reported that CD133+ glioma cells were sufficient to develop xenografted tumors *in vivo* that recapitulated the heterogeneity of the original tumor, and thus is considered as GSCs (Singh et al., 2004; Beier et al., 2007). GSCs has been shown more resistant to current therapeutic approaches than the matched non-stem glioma cells (Bao et al., 2006). Previous study has confirmed that GSCs was more sensitive to NXD30001 than non-GSCs (Zhu et al., 2010), which is inconsistent with our findings in this study.

Currently, the unsatisfactory therapeutic outcome in GBM are usually caused by the upregulation in expression of drug efflux transporters, reduced sensitivity to apoptotic signals, and increased expression of growth factors (Aldape et al., 2015). GBMs exhibit various signaling abnormalities, including EGFR amplifications, inactivating PTEN mutations, PDGF autocrine loops, and subsequent overactivation of their associated downstream signal generators. It is noteworthy that many of these proteins are Hsp90 client proteins and are responsible for initiation and maintenance of tumor (Pratt and Toft, 2003; Dutta Gupta et al., 2019). Thus, inhibiting Hsp90 activity will lead to downregulation of multiple oncogenic molecules and Hsp90 inhibitors may be effective chemotherapeutic agents for the treatment of GBM (Matts and Manjarrez, 2009). NXD30001 is a novel radicicol-based series of Hsp90 inhibitors and readily crosses the BBB and accumulates in the brain, made it be an attractive therapeutic candidate for GBM. Our study showed a significant inhibition of GSCs proliferation and induction of GSCs apoptosis after treatment with NXD30001 alone or combined with radiation, and these effects may be achieved through downregulating the phosphorylation levels of multiple Hsp90 client proteins including EGFR, EGFR, Akt, and c-Myc.

It is well known that radiotherapy could effectively repress GBM cell growth and induce cell death by leading to various types of DNA damage (Hathout and Pope, 2016). However, it is usually limited widespread used in curing GBM due to inherent tumor

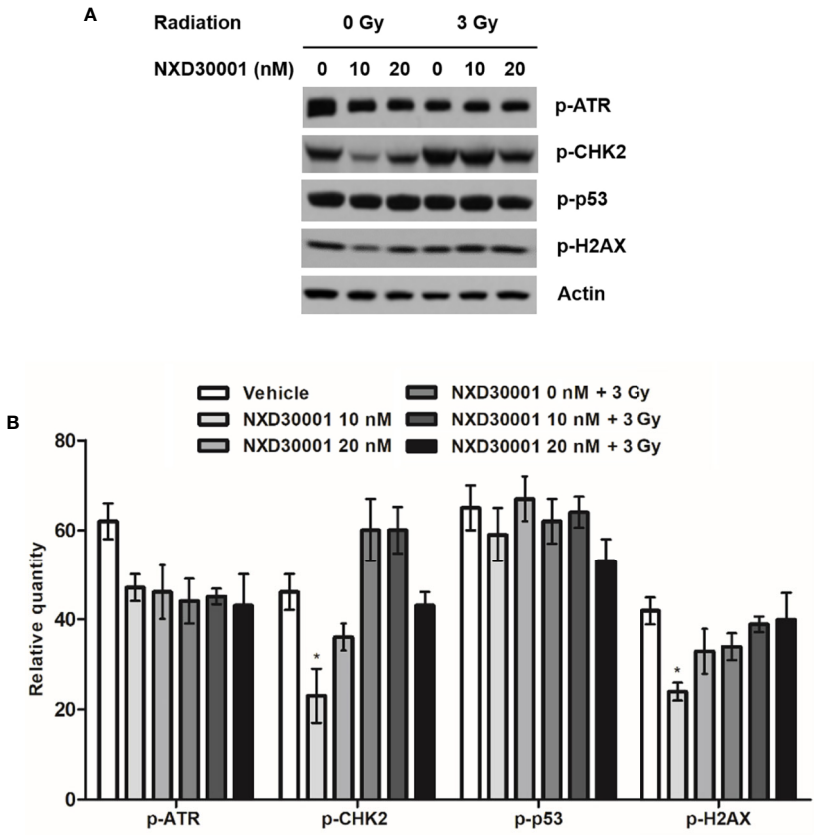


**FIGURE 5 |** NXD30001 reduces activation of DNA damage response in GSCs after radiation. **(A)** Representative western blotting bands showing the expression level of p-ATR, p-CHK2, p-p53, p-H2AX in T4302 CD133+ cell cultures treated with different concentrations of NXD30001 with or without radiation. The result was quantified from the average of three different samples. **(B)** Quantification of western blotting bands. Error bars represent the standard error. *$P < 0.05$.

radiation resistance that caused by a DNA damage response (DDR) cascade, which is able to repair DNA damage and injury in surrounding normal tissues (Frosina, 2010; Schmalz et al., 2011; Alexander et al., 2012). When DNA damage initiates in tumor cells, the DDR is also activated to induce cell cycle arrest and lesion repair, which lead to tumor growth and recurrence. However, when the damage exceeds capacity of DNA repair, apoptosis will be triggered, further leading to tumor cell death. Generally, the activation of DDR is implicated in radio-resistance of the GSCs and it involved a range of DNA damages related proteins such as ataxia telangiectasia Rad3-related (ATR), which initiates a transduction cascade activating downstream effectors, including H2AX histone, 53 binding protein 1 (53BP1), and the checkpoint kinases Checkpoint 1 (CHK1) and Checkpoint 2 (CHK2) (Kastan and Bartek, 2004; Norollahi et al., 2019). It has been reported that there was an aberrant constitutive activation of DDR by upregulation of related proteins under DNA replication stress produced by oncogenes in GBM (Bartkova et al., 2010). In our study, the expression levels of DNA damage related proteins like ATR, CHK2, p53, and H2AX were not significantly altered in GSCs following NXD30001 treatment combined with radiation, indicating that NXD30001 was able to enhance the radio-sensitivity of GBM by reduction of the DNA damage response after radiotherapy.

ER stress is another important response involved in tumor development and growth. It is induced when the ER failed in maintaining cellular homeostasis, which is caused by constantly exposure to both intrinsic stresses like genomic instability, increased metabolic burden, oncogene expression, and extrinsic stresses, such as hypoxia, oxidative stress, and nutrient deprivation (Wang et al., 2010a). Cancer cells usually have stronger ability in protein synthesis and folding by inducing UPR through the activation of ER transmembrane protein sensors (Roy and Kumar, 2019). The UPR related proteins, such as protein kinaseR–like ER kinase (PERK), inositol-requiring enzyme 1a (IRE1α), binding immunoglobulin protein (BiP/GRP78) and transcription factor C/EBP homologous protein (CHOP) could be suppressed to mitigate ER stress condition for cancer survival including GBM (Le Reste et al., 2016; Penaranda Fajardo et al., 2016; Madden et al., 2019). In addition, ER stress response in cancer is reported to act as a key driver in tumorigenesis and the development of resistance to chemotherapy or radiation via activation of UPR (Bahar et al., 2019). Therefore, we speculate that reduction of ER stress

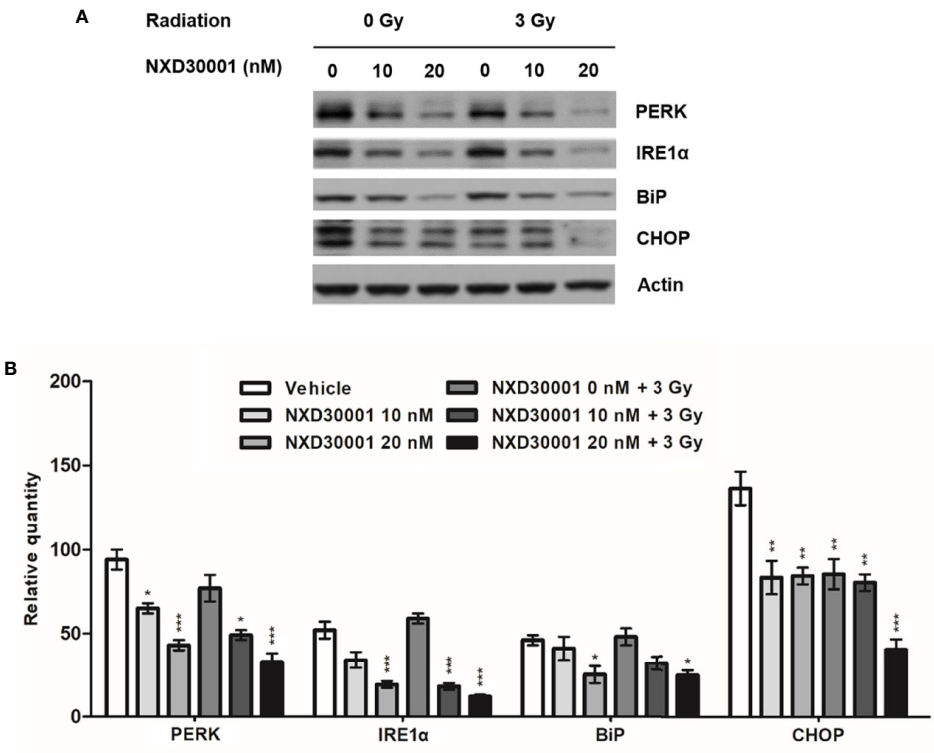


**FIGURE 6 |** NXD30001 in combination with radiation impairs ER stress in GSCs. **(A)** Representative western blotting bands showing the expression level of PERK, IRE1α, BiP, CHOP in T4302 CD133+ cell cultures treated with different concentrations of NXD30001 with or without radiation. The result was quantified from the average of three different samples. **(B)** Quantification of western blotting bands. Error bars represent the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

response may impair cellular homeostasis and lead to cell apoptosis, which might be an effective strategy for GBM therapy. Our results showed that the expression levels of PERK, IRE1α, BiP, and CHOP proteins were decreased dose-dependently in the treatment of NXD30001 alone or combined with radiation in GSCs, suggesting that NXD30001 could improve radiation sensitivity through impairing ER stress and cell survival response ultimately leading to GSCs death.

Notably, although Hsp90 inhibitor NXD30001 has shown effective effects in enhancing radiosensitivity of GBM by targeting multiple radio-resistant pathways in our study, it is premature to conclude that the combination of NXD30001 and radiotherapy is a promising candidate to enhance GBM treatment. An effective animal model that better mimics the radiosensitivity of GBM is needed to be used in the future studies in order to irradiate with a dose closer to those used to treat GBM patients.

## CONCLUSION

The present study demonstrated that the Hsp90 inhibitor, NXD30001 could inhibit GSCs growth and proliferation and induce GSCs apoptosis as a monotherapy. Moreover, NXD30001 markedly increased radio-sensitivity of GSCs through decreasing phosphorylation levels of multiple Hsp90 client proteins, and impairing DNA damage response and ER stress response. In an orthotopic GBM model, NXD30001 in combination with radiotherapy could significantly inhibit tumor growth and extend median survival of tumor-bearing mice, which provides a valuable basis for its use in the treatment of GBM.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The animal study was reviewed and approved by Vanderbilt Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

HC, ZC, and LX conceived and designed the experiments. HC and ZC performed the experiments and analyzed the data. YG

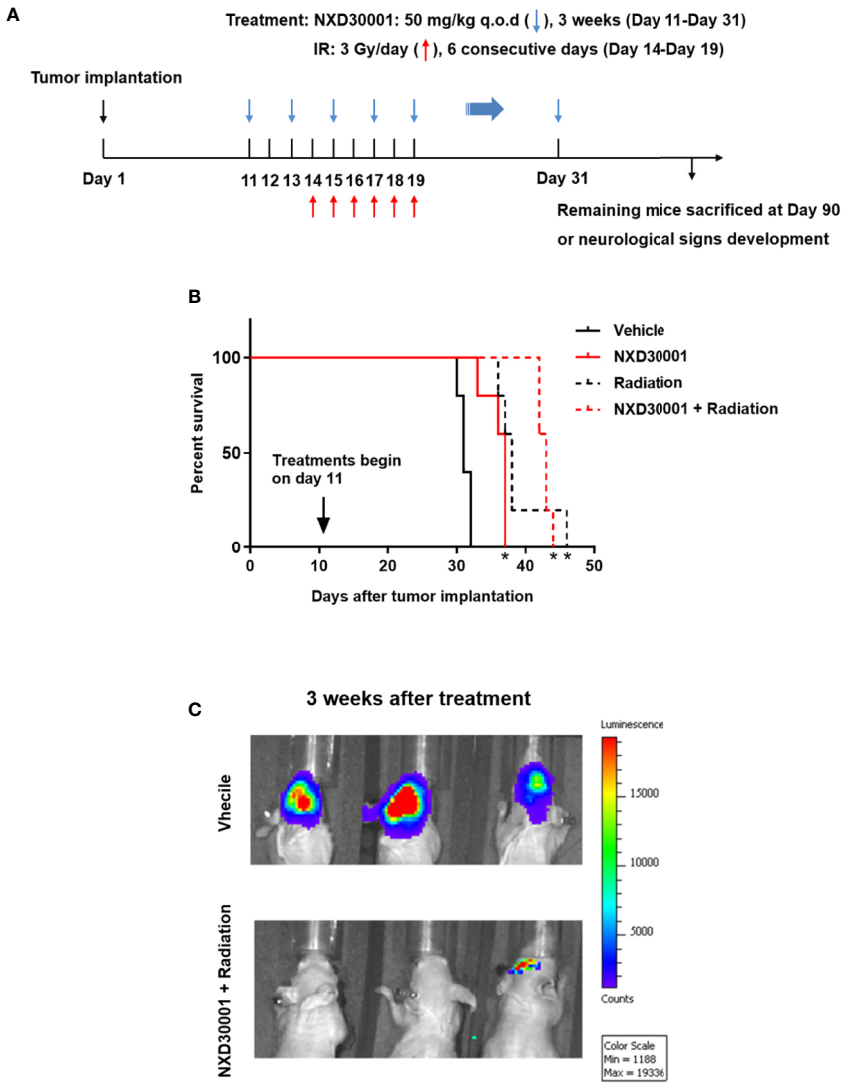


**FIGURE 7 |** NXD30001 in combination with radiation represses orthotopic GBM. **(A)** T4302 GSCs was implanted into the right cortex of 4–6 weeks old athymic nude mice. Mice were maintained for 10 d to allow tumor establishment and randomized prior to treatment. From day 11, NXD30001 was administrated intraperitoneally at 50 mg/kg q.o.d. for 3 weeks. One day after the second dose of NXD30001, mice received radiation in the whole brain for 6 consecutive days at 3 Gy/day. The rest mice in each group were maintained upon neurological signs developed. **(B)** The survival percentage of tumor-bearing mice analyzed using Kaplan-Meyer curves. *$P < 0.05$ vs vehicle treated group. **(C)** A representative bioluminescence images of tumor-bearing mice 3 weeks after NXD30001+radiation treatment.

and YM contributed reagents, materials, and analysis tools. RT and JW searched and reviewed the literatures. HC drafted the paper. ZC and LX critically revised the article. All authors contributed to the article and approved the submitted version.

## FUNDING

HC was partly supported by Shanghai Pujiang Program (18PJD034). LX was partly supported by National Natural Science Foundation of China (81801169). ZC was partly supported by grants from the National Natural Science Foundation of China (81372395). JW was supported by the American Brain Tumor Association and the Southeastern Brain Tumor Foundation NIH/NCI grant (1R01CA166492).

## ACKNOWLEDGMENTS

We are grateful for Dr. Jeremy Rich of Cleveland Clinic for providing primary glioblastoma xenograft lines. The abstract of this article is published as a conference paper (Chen et al., 2019).

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

BRIEF RESEARCH REPORT
published: 13 November 2020
doi: 10.3389/fnins.2020.589924

# Effects of Photons Irradiation on $^{18}$F-FET and $^{18}$F-DOPA Uptake by T98G Glioblastoma Cells

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The differential diagnosis between brain tumors recurrence and early neuroinflammation or late radionecrosis is still an unsolved problem. The new emerging magnetic resonance imaging, computed tomography, and positron emission tomography diagnostic modalities still lack sufficient accuracy. In the last years, a great effort has been made to develop radiotracers able to detect specific altered metabolic pathways or tumor receptor markers. Our research project aims to evaluate irradiation effects on radiopharmaceutical uptake and compare the kinetic of the fluorinate tracers. T98G glioblastoma cells were irradiated at doses of 2, 10, and 20 Gy with photons, and $^{18}$F-DOPA and $^{18}$F-FET tracer uptake was evaluated. Activity and cell viability at different incubation times were measured. $^{18}$F-FET and $^{18}$F-DOPA are accumulated via the LAT-1 transporter, but $^{18}$F-DOPA is further incorporated, whereas $^{18}$F-FET is not metabolized. Therefore, time-activity curves (TACs) tend to plateau with $^{18}$F-DOPA and to a rapid washout with $^{18}$F-FET. After irradiation, $^{18}$F-DOPA TAC resembles the $^{18}$F-FET pattern. $^{18}$F-DOPA activity peak we observed at 20 min might be fictitious, because earlier time points have not been evaluated, and a higher activity peak before 20 min cannot be excluded. In addition, the activity retained in the irradiated cells remains higher in comparison to the sham ones at all time points investigated. This aspect is similar in the $^{18}$F-FET TAC but less evident. Therefore, we can hypothesize the presence of a second intracellular compartment in addition to the amino acidic pool one governed by LAT-1, which could explain the progressive accumulation of $^{18}$F-DOPA in unirradiated cells.

**Keywords: photon irradiation, $^{18}$F-DOPA, glioblastoma, radionecrosis, T98G cells, $^{18}$F-FET**

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***Edited by:***
*Fabiana Novellino, National Research Council (CNR), Italy*

***Reviewed by:***
*Ravindra M. Samartha, Bhopal Memorial Hospital & Research Centre, India*
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***Specialty section:***
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

***Received:*** *31 July 2020*
***Accepted:*** *20 October 2020*
***Published:*** *13 November 2020*

***Citation:***
*Pasi F, Persico MG, Marenco M, Vigorito M, Facoetti A, Hodolic M, Nano R, Cavenaghi G, Lodola L and Aprile C (2020) Effects of Photons Irradiation on $^{18}$F-FET and $^{18}$F-DOPA Uptake by T98G Glioblastoma Cells. Front. Neurosci. 14:589924. doi: 10.3389/fnins.2020.589924*

## INTRODUCTION

Glioblastoma multiforme (GBM) represents the most common and aggressive primary malignancy form, with a poor prognosis (survival is about 12–18 months). Robust neovascularization, massive glioma cell invasiveness within the whole brain parenchyma, and resistance to conventional therapies characterize GBM evolution, flowing into frequent recurrence and relapse (Gilbert, 2011).

Diagnosis of GBM is based on magnetic resonance imaging, computed tomography, and positron emission tomography (PET) imaging. To date, the imaging protocol is still controversial and lacks of accurate tools, because of the abundant rate of peritumoral necrosis and inflammation that smooth the abilities to closely define the tumor boundaries and affect the efficiency of early diagnosis (Petrujkiæa et al., 2019).

Moreover, the GBM multimodal treatment provides the combination of surgical resection, radiotherapy, and chemotherapy that lead to a local inflammation process, the so-called radionecrosis phenomenon. Radionecrosis strongly affects the ability of rapid and unambiguous relapse discrimination, compromising the treatment strategies and options during the follow-up of the patient (Carlsson et al., 2014).

Recent advances in imaging techniques have opened endless opportunities for molecular diagnostic and therapeutic procedures. Molecular imaging could help in early detection, characterization, and "real-time" monitoring of various diseases. Regarding cancers, it could be an excellent tool to investigate treatment's efficacy, allowing areas of interest to be monitored during therapy and supporting the follow-up process. PET has proven to be a useful imaging modality in the distinction between benign and malignant lesions and in the assessment of peritumoral areas (Graves et al., 2007; Buroni et al., 2015; Frosina, 2016).

$^{18}$F-FDG is the most common PET radiopharmaceutical, able to detect the upper glucose uptake in the tumor region. Unfortunately, $^{18}$F-FDG is not applicable to detect brain tumors, such as GBM, due to the physiological high glucose metabolism rate in the brain (Nanni et al., 2010).

Hence, in the last years, a great effort has been made to develop alternative metabolic or receptorial radiotracers, able to detect specific altered metabolic pathways or tumor receptor markers.

$^{18}$F-choline ($^{18}$F-FCH), a fluorinated phosphatidylcholine precursor, is approved for diagnosis of prostate and hepatocellular carcinomas, and its use is currently extending to other cancers, including GBM. Our previous research demonstrated that $^{18}$F-FCH is able to trace tumor behavior in terms of higher uptake for increased doses of radiation treatment (Pasi et al., 2017).

Nevertheless, $^{18}$F-FCH has shown several limitations in the diagnosis of those tumors characterized by a high inflammatory component, therefore reducing the PET clinical impact (Spaeth et al., 2006). To overcome these crucial issues, amino acidic radiopharmaceuticals, such as $^{18}$F-ethyl-L-tyrosine ($^{18}$F-FET) and 6-$^{18}$F-L-3,4-dihydroxyphenylalanine ($^{18}$F-DOPA), have been developed. The overexpression of amino acidic transporters is a peculiar feature of cancer cells, because of the significant increase in protein synthesis and could be a reason of their future use in diagnostics (Stadlbauer et al., 2008).

$^{18}$F-FET is a tyrosine analog tracer that is internalized mostly by L-type-amino acid methionine Na + -independent transporter 1 (LAT-1), expressed in brain endothelial cells and in tumor cells, including GBM.

Another amino acidic tracer is the $^{18}$F-DOPA, an analog of L-DOPA, carried in the cytoplasm of cancer cells by the LAT system, as for $^{18}$F-FET. $^{18}$F-DOPA was initially employed to evaluate presynaptic dopaminergic neuronal function in patients with movement disorders as precursor of dopamine with accumulation in the basal ganglia and without significant accumulation in the brain parenchyma. The oncological interest of $^{18}$F-DOPA arises from the incidental discovery of a G-II oligoastrocytoma in a patient with suspicious Parkinson disease (Heiss et al., 1996).

In this article, we focused on comparing $^{18}$F-FET and $^{18}$F-DOPA, investigating their kinetic uptake in GBM cells in basal condition and after crescent doses of photon irradiation.

As discussed, the differential diagnosis between brain tumor recurrence and early neuroinflammation or late radionecrosis is still an unsolved problem. Our research project aims to evaluate the cellular response of T98G glioblastoma cells, chosen because of its radioresistant and aggressive features, by isolating it from the influence of the microenvironment. Although this approach is in part limiting of what occurs *in vivo*, it allows identifying the alterations within the tumor cell in basal conditions and after irradiation that could be useful in the clinic. In fact, it is of great importance for physicians to consider the different kinetics pathways of uptake concerning the two radiopharmaceuticals, in order to define the one of major interests, and the behavior of the different types of cells after irradiation. This is the first step of a larger project, which consists of the study of neoplastic, endothelial, microglia, and tumor cells incubated with medium harvested from irradiated ones, containing inflammatory and growth factors, cytokines, receptor ligands, and other factors that could contribute to the development of radiation necrosis. Although this system limits translatability due to the difference in tissue attenuation *in vivo* compared to cell cultures (Paget et al., 2019), our aim is to study the influence of ionizing radiation on the transport mechanism of labeled amino acids, namely, $^{18}$F-FET and $^{18}$F-DOPA, used in PET imaging and therefore on tumor cell uptake by discriminating early/late recurrence radionecrosis.

## MATERIALS AND METHODS

### Cell Culture

Human glioblastoma T98G cells were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, United Kingdom). T98G cells were cultured in eagle minimum essential medium (EMEM; Euroclone SpA, MI, Italy) supplemented with 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO, United States), 100 U/mL penicillin/streptomycin (Euroclone SpA, MI, Italy), 2 mM L-glutamine (Euroclone SpA, MI, Italy), and 0.01% sodium pyruvate (Sigma–Aldrich, St. Louis, MO, United States) at 37°C in an atmosphere of 5% $CO_2$. Stock cultures were maintained in exponential growth as monolayers in 75-cm$^2$ Corning plastic tissue-culture flasks (VWR International PBI Srl, MI, Italy).

### Irradiation Treatments

Cells were irradiated at doses of 2, 10, and 20 Gy with photons at room temperature using a LINAC at 6 MeV

(ELEKTA Synergy; Radiotherapy Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy) with a dose rate of 3 Gy/min. The flasks containing the cells were placed vertically at the isocenter, and a 5-mm-thick plastic sheet was placed below the flask surface to allow dose build-up (**Figure 1**). Sham-irradiated cells (0 Gy) were performed as control. An hour before irradiation, the medium was removed from the flasks, and fresh medium was added to the cells. Cells were replaced in incubator at 37°C after irradiation treatment.

## Radiopharmaceutical Incubation

$^{18}$F-DOPA (IASOdopa®) and $^{18}$F-FET (IASOglio®) were obtained from IASON GmbH (Graz, Austria). T98G cells grew adherent to the plastic surface at 37°C in 5% $CO_2$ in complete medium.

Samples with $2 \times 10^5$ cells per flask were irradiated at room temperature 20 h after seeding. A total of 36 h post-irradiation, tracer uptake was evaluated after addition of 100 kBq (100 μL) of to each flask with 2 mL of medium.

Activity in the adherent cells and the number of surviving cells and their viability at different incubation times (20, 40, 60, 80, and 120 min) were measured. Tracer incubation was done in complete medium. Control samples underwent the same procedure as other samples, but they were incubated with 100 μL of saline instead of a radiotracer.

## Cell Kinetic Studies and Uptake Evaluation

The cellular radiotracer uptake was determined with a 3 × 3" NaI(Tl) pinhole 16 × 40-mm gamma counter (Raytest,

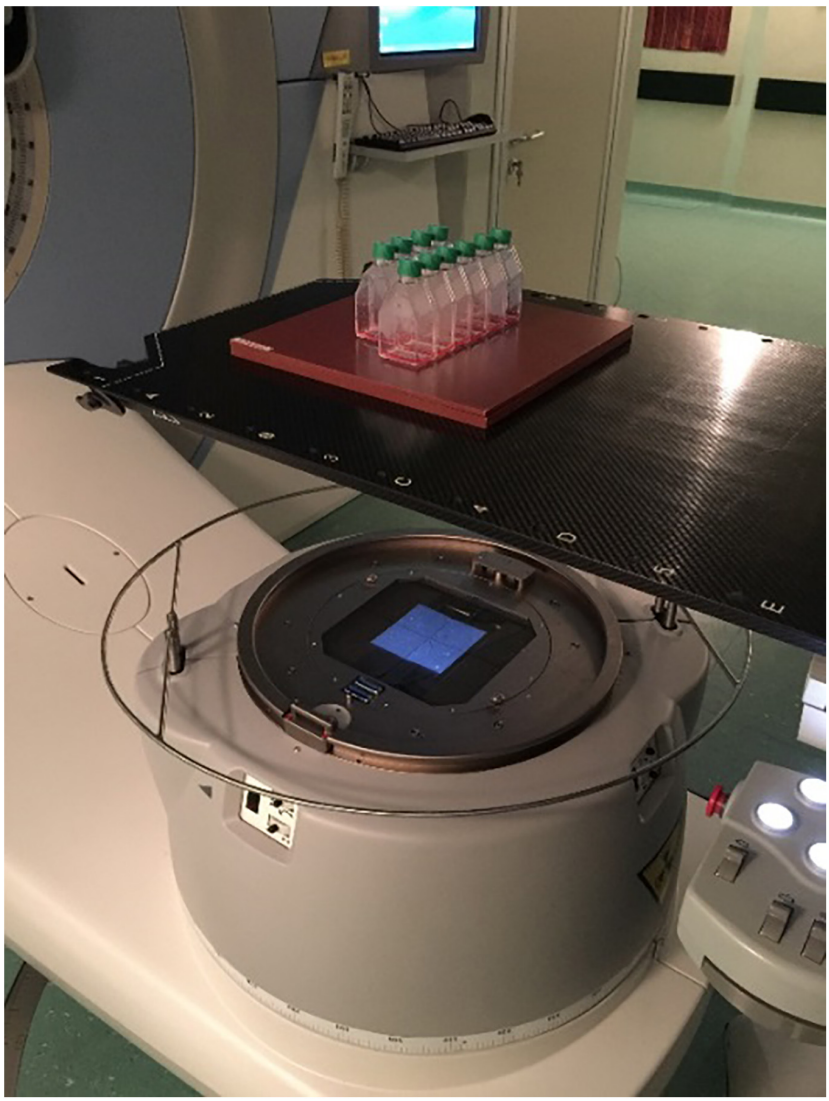

**FIGURE 1 |** Experimental setup used for T98G cell irradiations at radiotherapy unit.

Straubenhardt, Germany). All measurements were carried out under the same counting position, along with a standardized source to verify the counter's performance, and the data were corrected for background and decay. Total radioactivity was counted when the radiotracer was added to the medium in each flask (time 0). After 20, 40, 60, 90, and 120 min from time 0, the medium was harvested, the cells were rapidly washed three times with 1 mL of phosphate-buffered saline, and radiopharmaceutical uptake for each sample was assessed. The uptake measurements are expressed as the percentage of the administered dose of tracer per $2 \times 10^5$ cells after correction for negative control uptake (flasks containing no cells with complete medium and incubated with radiopharmaceutical). Damaged cells were evaluated with propidium iodide (PI) and annexin V fluorescent staining as previously described (Pasi et al., 2017).

## Statistical Analysis

*In vitro* experiments were conducted in duplicate for each experimental point and repeated twice the full experiment. All values are expressed as mean values with confidence interval (95% CI). The uptake of radiotracer is represented as a function of the incubation period; all values are shown in figures as a percentage of the administered dose per $2 \times 10^5$ cells (mean $\pm$ 95% CI). Therefore, if error bars on the $y$ axis do not overlap, the two points are considered significantly different ($p < 0.05$).

# RESULTS

Results were expressed as added dose (%) on $2 \times 10^5$ cells, corrected for decay and for non-specific binding. Sham-irradiated cells (0 Gy) were considered as control.

**Figure 2** shows the uptake of $^{18}$F-FET by T98G cells in basal conditions and after irradiation with photons at doses of 2, 10, and 20 Gy. In basal condition, the uptake increased up to 40 min and then decreased up to 80 min. Subsequently, it increased again to 100 min and then decreased.

After irradiation, the curves showed a dose-dependent uptake with a similar trend, showing a peak at 40 min. After 60 min, a plateau was reached for each irradiation dose.

The behavior of $^{18}$F-DOPA was different, as present on **Figure 3**; in basal conditions, T98G cells reached a maximal uptake 40 min after $^{18}$F-DOPA addition, afterward reaching a plateau. In photon-irradiated cells, the kinetic pattern changes dramatically; absolute uptake of $^{18}$F-DOPA increases more than two or three times after irradiation, and a peak activity was observable first at 20 min, and we cannot rule out earlier higher activity before 20 min, followed by a rapid washout and by a further reuptake at 80 min.

In **Figures 2**, **3**, statistical significance ($p < 0.05$) can be evaluated with the overlapping or not of error bars on $y$ axis. Indeed, they represent the 95% CI of the single experimental point; e.g., each value at 20 to 40 min about $^{18}$F-FET uptake (**Figure 2**) is significantly different from each other (0–2–10–20 Gy); in the same time series at 20 Gy, experimental point

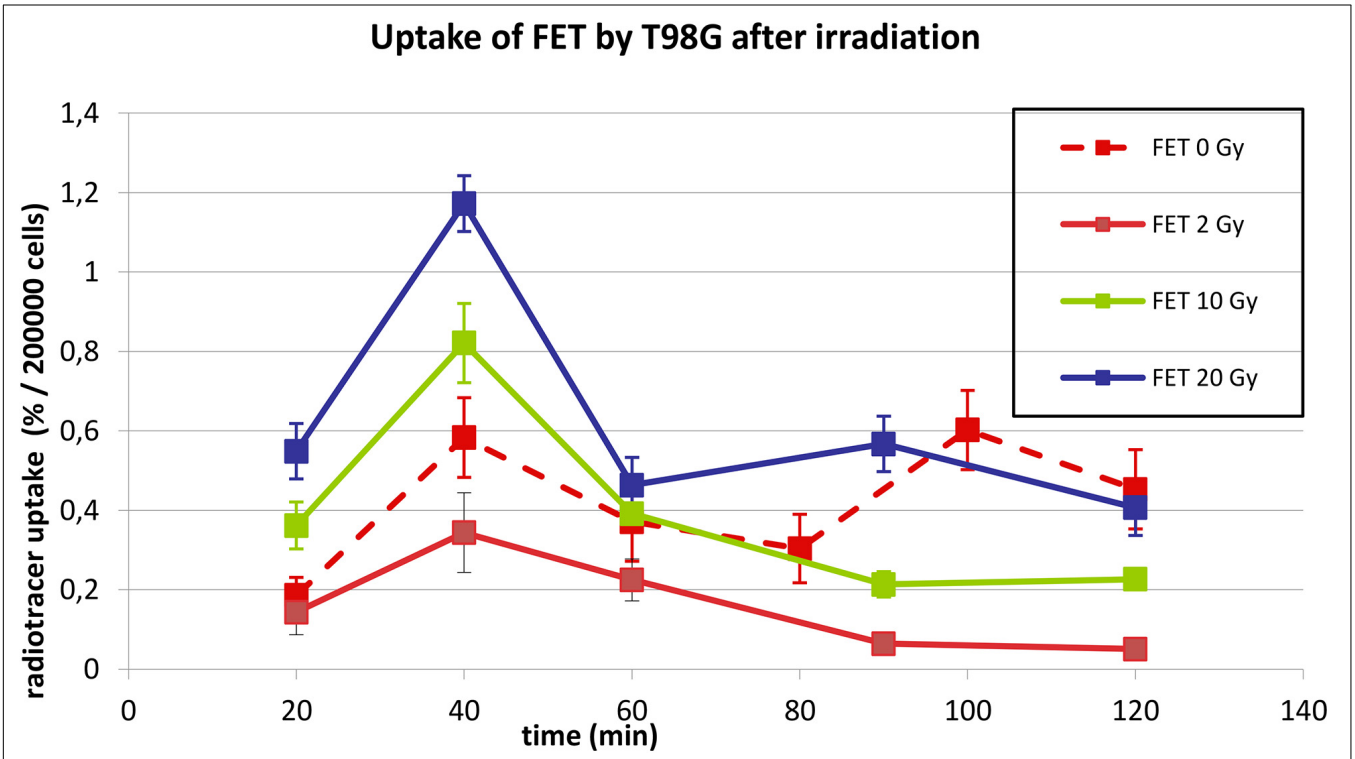


**FIGURE 2 |** Uptake of $^{18}$F-FET in T98G cells in basal condition and after photon irradiation with 2, 10, and 20 Gy.

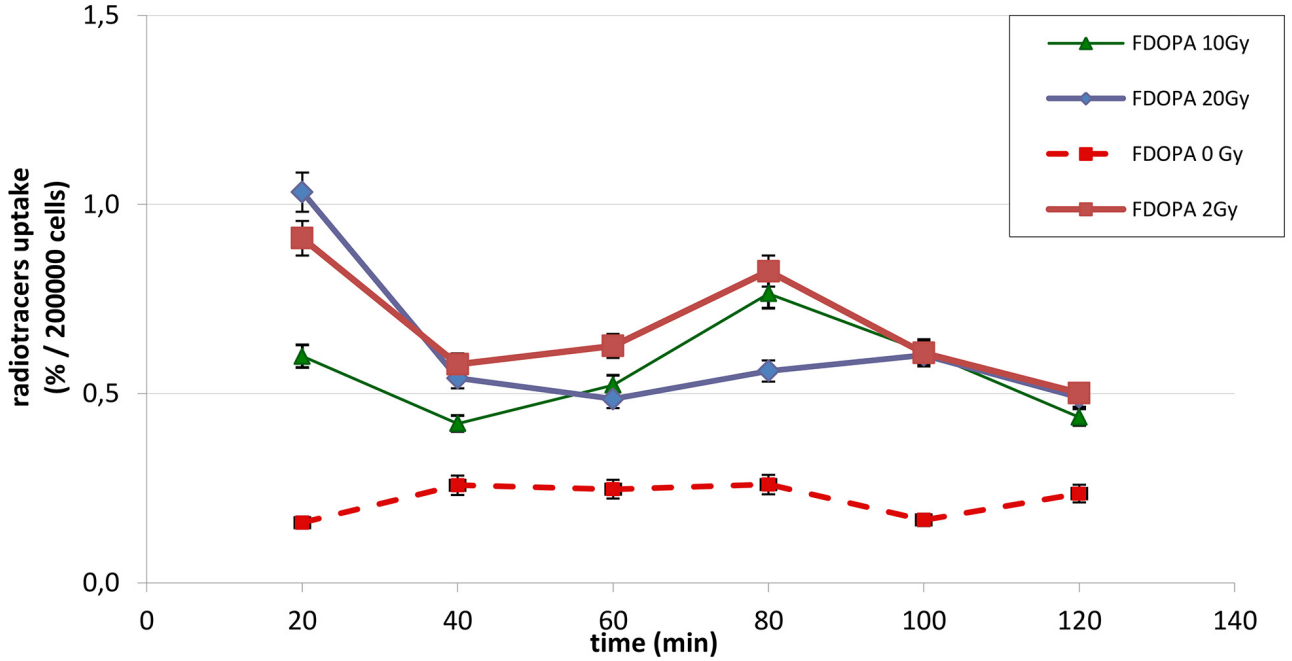


**FIGURE 3 |** Uptake of $^{18}$F-DOPA in T98G cells in basal condition and after photon irradiation with 2, 10, and 20 Gy.

at 40 min is statistically different from the others. Analogous evaluation can be drawn for all time series and in **Figure 3** as well.

After irradiation, as with other radiopharmaceuticals we previously reported (Pasi et al., 2017), the net uptake normalized to 2 × $10^5$ cells appears to be increased, but most noticeable is the striking change of the TAC pattern. We observed that the percentage of damaged cells 35 h after irradiation as shown by PI and annexin V fluorescent staining increases in an exponential pattern (4% at 2 Gy, 9% at 10 Gy and 21% at 20% vs. 3% of control), whereas the $^{18}$F-DOPA uptake at peak value increases in a linear one (**Figure 4**).

## DISCUSSION

Both FET and DOPA share the common characteristic to increase their uptake in T98G as response to radiation. We and other authors either for different labeled substrates and various cell lines have previously reported this apparent paradoxical behavior (Cheon et al., 2007; Pasi et al., 2017).

The main difference between the two tracers is related to the shape of TAC. In fact, $^{18}$F-FET TAC shows only a modification of the peak value, whereas with $^{18}$F-DOPA, major shape changes are observable. Sham cells show a maximal $^{18}$F-DOPA uptake at 40 min, plateaus during the observation time, whereas an early peak can be found after irradiation.

The irradiation system used in this work is LINAC, the medical linear accelerator used in clinic. It is used to mimic the cellular effect induced in cells by ionizing radiation at the same doses and energies used for therapy. It is the most used in clinic and in clinical research on cell cultures (Alexiou et al., 2019). The effect of treatment was evaluated after 36 h from irradiation. T98G cells seem not to respond to ionizing radiation after 24 h at a dose of 20 Gy (Murad et al., 2018). We considered more time for an effect to be appreciable compared to control.

Many factors have been postulated to explain modifications of tumor cells to ionizing radiation, which paradoxically can promote metastasis and invasion of cancer cells (Lee et al., 2017). $^{18}$F-FET does not participate in specific metabolic pathways such as catecholamine metabolism. The influence of catecholamine metabolism and melanin synthesis on $^{18}$F-FET uptake in tumors as well as in other tissues appears to be negligible.

Although $^{18}$F-FET is not incorporated into proteins, uptake by tumor cells is stereospecific and mediated by amino-acid transporters, whereas for methionine a 15% protein incorporation after 2-h incubation time was noted (Langen et al., 2017). On the other side, Habermeier and colleagues hypothesized an intracellular metabolism leading to another impermeable derivative trapped within glioma cell, suggesting an asymmetry of intracellular and extracellular recognition by LAT-1 (Habermeier et al., 2015).

$^{18}$F-FET uptake decreases after a peak of activity (Pasi et al., 2017). This pattern resembles that observed *in vivo* with PET (Galldiks et al., 2012) where the peak time is related to tumor aggressiveness followed by a steep decrease.

The increased uptake induced by irradiation has been previously described for $^{18}$F-FET in T98G (Pasi et al., 2017) and MCF7 cell lines (Cheon et al., 2007). Many factors could

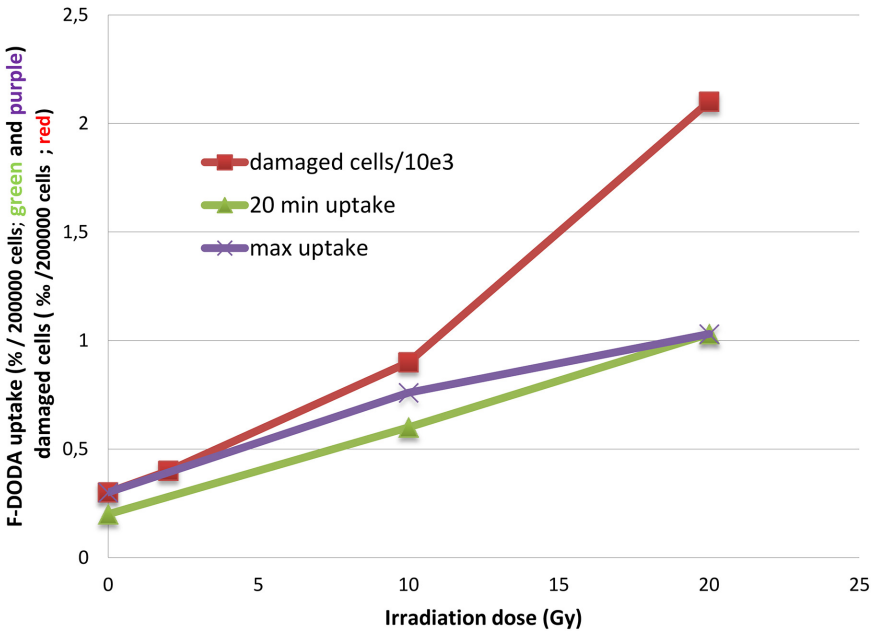


**FIGURE 4 |** Trend of $^{18}$F-DOPA uptake after 20 min and at the peak activity compared to the number of damaged cells.

be involved in this apparent paradox including up-regulation of the amino-acid transporter LAT-1 (Habermeier et al., 2015) and selection of more aggressive radioresistant clones (Pasi et al., 2017). The role of p53 seems less plausible in T98G p53mt (Van Meir et al., 1994); in fact, $^{18}$F-FET accumulation in MCF7 cell line increases independently of p53 status. Therefore, p53 does not influence the LAT-1 mechanism, whereas a clear influence of p53wt and p53mt on the $^{18}$F-FDG and $^{18}$F-FLT incorporation after beta and gamma radiation has been observed (Cheon et al., 2007).

As mentioned previously, in our *in vitro* model, $^{18}$F-DOPA reaches its maximum uptake after 40 min of incubation and plateaus up to 80 min before decreasing. This behavior does not support a simple transport uptake mechanism but suggests a further trapping mechanism.

The mechanism of $^{18}$F-DOPA accumulation in glioma has not been thoroughly elucidated, even if up-regulation of its transporter seems to play a key role. The LAT-1, a sodium-independent neutral amino-acid transporter, facilitates L-DOPA transport in endothelial cells of brain capillaries and in kidney epithelial cells. LAT-1 up-regulation correlates with tumor grade and a reduction of uptake with response to treatment and a similar consideration can be done for $^{18}$F-FET.

Nonetheless, the intensity of $^{18}$F-DOPA uptake is not directly correlated with the level of LAT-1 expression. A minimal expression of LAT1 is required for $^{18}$F-DOPA uptake, but this amount is not linearly related to the histochemical score of LAT-1 expression, suggesting that the balance between influx and efflux is not sufficient to understand the mechanism of uptake and retention (Dadone-Montaudié et al., 2017).

These results are partially contradictory with the Youland and colleagues' report (Youland et al., 2013), who found *in vivo* a linear relationship between standardized uptake value (SUV) and LAT-1 expression. GBM cell lines with low LAT-1 expression had significantly less $^{3}$H-L-DOPA uptake compared to cell lines with readily detectable LAT-1.

In addition, *in vitro* knockdown of LAT-1 reduces $^{3}$H-L-DOPA uptake in human glioma lines T98G and GBM28. Nevertheless, despite robust knockdown, the uptake is only reduced by approximately 50 to 70%, suggesting that other mechanisms account for tracer incorporation and differences related to brain lesions. Unfortunately, in Youland and colleagues' report, only one experimental time point is taken into account, i.e., 20 min after tracer addition, and the comparison of these data with our TAC is not possible.

Another difference between *in vivo* and *in vitro* model appears noticeable: $^{18}$F-DOPA competes with tyrosine for the LAT-mediated uptake. Tyrosine concentration in the medium used *in vitro* (EMEM) is 0.230 mmol/L, whereas in plasma it is 0.077 (Schmidt et al., 2016), indicating a competitor concentration twofold to threefold higher than *in vivo*.

The model works quite satisfactorily in brain gliomas (Schiepers et al., 2007; Wardak et al., 2014), where PET external detection is employed, but it does not take into account other signal sources as endothelial, infiltrating normal cells, vascularization, and metabolites such as L-3,4-dihydroxy-6-fluoro-3-O-methylphenylalanine (OMFD).

Retention after peak activity may be related to the presence of a second intracellular compartment, marked with a question point in the Schiepers and colleagues' study, in addition to the intracellular amino acidic pool (Schiepers et al., 2007; Youland et al., 2013). There are no evidences if this secondary compartment could be attributed to storage or a metabolic pool.

Differently from $^{18}$F-FET, the TAC shape we observed *in vitro* is different from the one reported *in vivo* with PET. The $^{18}$F-DOPA pattern we observed does not appear to mimic the PET *in vivo* TACs. Schiepers and colleagues' study (Schiepers et al., 2007) described an early steep ascent in tumor curve and a parallel cerebellum washout curve. On the contrary, striatum where metabolism is active leads to a plateau-shaped curve.

Ginet and colleagues, on the contrary, described different shapes of the PET TACs (Ginet et al., 2020). IDH mutation status and 1p/19q codeletion status seem to influence markedly the uptake rate and the residence time in the tumor of $^{18}$F-DOPA, whereas IDHwt GBM curve resembles $^{18}$F-FET curve with an early peak followed by a rapid washout. T98G, as many other glioma cell lines, does not display the IDH1$^{R132}$ variant or the 1p/19q total deletion (Bleeker et al., 2009; Ichimura et al., 2009).

*In vivo* $^{18}$F-DOPA is partially metabolized in blood and by peripheral tissues as liver to OMFD (L-3,4-dihydroxy-6-fluoro-3-*O*-methylphenylalanine), whereas such metabolic way is negligible in the brain and absent in the tumor. OMFD is transported bidirectionally in the brain, both in striatum and in tumor, showing a significant affinity for tumor cells (Chopra, 2007; Haase et al., 2007). It cannot be distinguished *in vivo* from parent $^{18}$F-DOPA by external detection because it retains the F-label. $^{18}$F-DOPA and OMFD are incorporated in tumor cell via the LAT-1. In an *in vitro* model, the OMFD contribution is absent and hampers a more precise comparison with the $^{18}$F-DOPA kinetics described by PET studies, where the combined activities of $^{18}$F-DOPA and OMFD appear to move in and out of tumor contributing in the same way as the signal detection.

The fluorinated amine shows a metabolic behavior similar to non-labeled ones, even if fluorinated compounds may have different biological properties in comparison to parent compounds. Isosteric F-substitution at ring position 6 is the best substrate for L-DOPA decarboxylase (DDC) (Daidone et al., 2012). The L-DDC is a pyridoxal-5-phosphate–dependent enzyme participating in the biosynthesis of catecholamines, catalyzing the decarboxylation of L-DOPA to dopamine. In addition to the central nervous system, DDC is present in many peripheral organs such as liver, kidneys, gastrointestinal tract, and pancreas, in which the biological function is yet to be determined (Patsis et al., 2012). Surprisingly, approximately 20% of non-neuroendocrine tumors express DDC, even if it has been found in three glioblastoma specimens (Gazdar et al., 1988). Novel DDC splice variants have been detected in 40 tumor cell lines from 11 types of cancer, including brain tumors U-87 MG, U-251 MG, D54, H4, and SH-SY5Y. These protein isoforms might participate in metabolic processes having alternative functions (Adamopoulos et al., 2019). For example, HeLa and HTB14 (human glioblastoma) coexpress the non-neural full-length DDC mRNA and the alternative neural transcript lacking exon 3, but no enzymatic activity is detectable in the cellular extracts (Chalatsa et al., 2011). Nevertheless, T98G cells do not seem to express the gene coding DDC (Functional Annotation of the Mammalian Genome, 2012).

Randomly, mis-incorporation of $^{18}$F-DOPA into newly synthetized proteins is another aspect that has been neglected until now, and it has been described in patients assuming L-DOPA for therapeutic purpose. However, this behavior depends on the ratio of L-DOPA to L-tyrosine in the cell (Rodgers et al., 2006; Chan et al., 2012). Therefore, this hypothesis is weak because in culture medium $^{18}$F-DOPA is present in femtomolar concentration while tyrosine in millimolar concentration.

Regarding T98G cell line used in our test, there is no further information available that can support a hypothetic metabolic pathway. On the other hand, based on TAC shape, which indicates retention with late egress, a second intracellular compartment can be hypothesized.

After irradiation, the TAC shape is more similar to the $^{18}$F-FET one with an early peak activity followed by a sharp decrease; nonetheless, the latter experimental points are higher than in basal condition. The $^{18}$F-DOPA activity peak we observed at 20 min might be fictitious, because earlier time points have not been evaluated, and a higher activity peak before 20 min cannot be excluded. Noticeably, the damaged cell percentage increased in an exponential manner, whereas $^{18}$F-DOPA uptake at peak value increases linearly (**Figure 4**).

The effects of irradiation on LAT-1 expression in tumors and on $^{18}$F-DOPA uptake are unknown. In addition, the comparison with PET activity in patients submitted to radiotherapy is not immediate and can furnish only limited information. Chiaravalloti and colleagues reported *in vivo* a significantly higher SUV related to a smaller interval from RT, whereas this relationship was not demonstrable after chemotherapy (Chiaravalloti et al., 2015). They attributed this finding to a blood–brain barrier disruption and a subacute or delayed inflammatory process. This explanation is appropriate for external imaging but not in our model where the microenvironment influence is absent. In addition, it is not possible to discriminate in this group patients who were free of disease versus recurrence in a short time. On the contrary, Dadone and colleagues did not find any significant correlation between the interval from radiotherapy to PET scan in terms of SUV (Dadone-Montaudié et al., 2017).

Therefore, taking into account the TAC in non-irradiated and in the 10- and 20-Gy irradiated cells, we can hypothesize the presence of a second intracellular compartment in addition to the amino acidic pool one governed by LAT-1, which could explain the progressive accumulation of $^{18}$F-DOPA in non-radiated cells. Indeed, if the LAT-1 system were the unique mechanism responsible for $^{18}$F-DOPA uptake, we should have observed an uptake pattern similar to the $^{18}$F-FET one. There are no indications if this second compartment has a metabolic or storage function and if there is an exchange between the two compartments. Protein incorporation is less likely, and some relationship with DDC is not demonstrable.

Another mechanism different from LAT-1 plays a significant role in the $^{18}$F-DOPA incorporation with a temporal shift in comparison with LAT-1, whose robust knockdown diminishes but does not abolish uptake. Therefore, in basal condition, the plateau phase could represent the additive effect of LAT-1 and the other mechanism of uptake and/or retention. After irradiation, this second mechanism is diminished or unchanged, whereas LAT-1 is up-regulated; therefore, the descending tail of the TAC

referable to LAT-1 activity can mask the contribution if any of this second hypothetical mechanism occurs.

The limitation of our experimental approach is related to the fact that an *in vitro* model cannot take into account many factors as the contribution of the microenvironment and labeled metabolites produced by non-tumor cells, which both provide signal detected by PET. In order to translate our results into preclinical application, physicians will have to consider the different kinetics pathways of uptake concerning the two radiopharmaceuticals. Further evaluation should be carried out to understand the underlying mechanism here hypothesized.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

FP designed and performed the experiments, and wrote the manuscript. MP designed and performed the experiments, and supervised the findings of this work. MM carried out the experiments and wrote the manuscript. MV carried out the experiments. AF discussed the results and contributed to the final manuscript. MH provided the radiopharmaceuticals and contributed to the final version of the manuscript. RN contributed to the implementation of the research and helped supervise the project. GC supervised the project. LL contributed to the implementation of the research and worked on the manuscript. CA conceived of the presented idea and contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

## FUNDING

The authors received funding for this work from Direzione Scientifica, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.

## ACKNOWLEDGMENTS

The authors thank the Department of Medical Physics, IRCCS Fondazione Policlinico San Matteo Foundation, Pavia, Italy for the collaboration in managing photon irradiation.

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**Conflict of Interest:** MH is Clinical Research Supervisor at IASON, Graz, Austria.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 25 November 2020
doi: 10.3389/fnins.2020.603647

# The Molecular and Microenvironmental Landscape of Glioblastomas: Implications for the Novel Treatment Choices

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Glioblastoma (GBM) is the most frequent and aggressive primary central nervous system tumor. Surgery followed by radiotherapy and chemotherapy with alkylating agents constitutes standard first-line treatment of GBM. Complete resection of the GBM tumors is generally not possible given its high invasive features. Although this combination therapy can prolong survival, the prognosis is still poor due to several factors including chemoresistance. In recent years, a comprehensive characterization of the GBM-associated molecular signature has been performed. This has allowed the possibility to introduce a more personalized therapeutic approach for GBM, in which novel targeted therapies, including those employing tyrosine kinase inhibitors (TKIs), could be employed. The GBM tumor microenvironment (TME) exerts a key role in GBM tumor progression, in particular by providing an immunosuppressive state with low numbers of tumor-infiltrating lymphocytes (TILs) and other immune effector cell types that contributes to tumor proliferation and growth. The use of immune checkpoint inhibitors (ICIs) has been successfully introduced in numerous advanced cancers as well as promising results have been shown for the use of these antibodies in untreated brain metastases from melanoma and from non-small cell lung carcinoma (NSCLC). Consequently, the use of PD-1/PD-L1 inhibitors has also been proposed in several clinical trials for the treatment of GBM. In the present review, we will outline the main GBM molecular and TME aspects providing also the grounds for novel targeted therapies and immunotherapies using ICIs for GBM.

**Keywords: GBM, tumor microenvironment, immune checkpoint inhibitors, tyrosine kinase inhibitors, CAR-T, treatment resistance**

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***Edited by:***
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***Specialty section:***
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

***Received:*** *07 September 2020*
***Accepted:*** *03 November 2020*
***Published:*** *25 November 2020*

***Citation:***
*Di Cintio F, Dal Bo M, Baboci L, De Mattia E, Polano M and Toffoli G (2020) The Molecular and Microenvironmental Landscape of Glioblastomas: Implications for the Novel Treatment Choices. Front. Neurosci. 14:603647. doi: 10.3389/fnins.2020.603647*

## INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive primary CNS tumor (Stupp et al., 2009; Louis et al., 2016; Mendes et al., 2018; Altmann et al., 2019; Ostrom et al., 2019) and it has been included in the group of diffuse astrocytic and oligodendroglial tumors by the 2016 CNS WHO (Louis et al., 2016). It is thought that genetic alterations affecting neuroglial stem or progenitors cells give origin to GBM. The incidence of this tumor seems to increase with age; in fact, 62 years is

the median age at diagnosis. Males are affected by GBM tumors 1.7 fold more often than females. According to the presence of mutations in the isocitrate dehydrogenase (*IDH*) 1 and *IDH2* genes GBM is subdivided by the WHO into two major types. More than 90% of GBM cases is constituted by GBM with wild type *IDH* (Louis et al., 2016). Clinically, grade IV lesions (namely primary GBM) are presented *de novo* by the majority of patients, while progression from a less aggressive form of WHO grade II diffuse astrocytomas and WHO grade III anaplastic astrocytomas (i.e., secondary GBM) is shown by a small fraction of patients (5–10%; Ohgaki and Kleihues, 2013; Louis et al., 2016). Primary GBM and secondary GBM differ in prognosis and age of onset. As for overall survival (OS) (Doetsch et al., 1999; Louis et al., 2016), primary GBM is typically diagnosed at older age and has a worse prognosis while secondary GBM are less common and affect people under the age of 45; also they develop into low-grade astrocytoma and are associated with better prognosis (Doetsch et al., 1999; Brennan et al., 2013; Ohgaki and Kleihues, 2013; Louis et al., 2016).

Standard of care first-line treatment is constituted by maximal surgical resection (complete resection is performed quite rarely because of the presence of diffuse infiltrations), followed by radiotherapy with concomitant and adjuvant chemotherapy such as the oral alkylating agent, temozolomide (TMZ). Upon this treatment combination GBM show a median OS of about 15 months (Canoll and Goldman, 2008; Stupp et al., 2009; Ohgaki and Kleihues, 2013; Levine et al., 2015).

The increase of patient survival is small and tumors invariably recur after TMZ (Canoll and Goldman, 2008; Stupp et al., 2009; Ohgaki and Kleihues, 2013; Levine et al., 2015). Following the first recurrence, treatment choices can be represented by further surgical resection when possible, or conventional chemotherapy, e.g., TMZ (with different dosing schedules) or nitrosoureas, or treatment with the anti-vascular endothelial growth factor (VEGF) agent, bevacizumab, or the use of the low-intensity alternating electric fields (TTFields). However, these treatments have not achieved significant improvements in survival (Canoll and Goldman, 2008; Stupp et al., 2009; Chamberlain and Johnston, 2010; Stupp et al., 2012; Ohgaki and Kleihues, 2013; Stupp and Hegi, 2013; Chamberlain, 2015). Moreover, the tyrosine kinase inhibitor (TKI) regorafenib has been introduced in the treatment of recurrent GBM (Lombardi et al., 2019).

A detailed characterization of the GBM-associated molecular signatures has made possible the development of novel therapies, including the use of TKIs (Friedman et al., 2009; Quant et al., 2009; Brennan et al., 2013; Wang et al., 2016; Lombardi et al., 2019). On the other hand, based on the results obtained in the context of other tumors (Brahmer et al., 2010; Eder and Kalman, 2014; Larkin et al., 2015; Weber et al., 2015; Kessler et al., 2018; Stathias et al., 2018), the use of programmed cell death protein (PD-1) receptor/programmed death ligand 1 (PD-L1) inhibitors has been suggested for gliomas, including GBM (Motzer et al., 2015; Goldberg et al., 2016; Reck et al., 2016; Schwartz et al., 2016; Reiss et al., 2017; Reardon et al., 2018; Cloughesy et al., 2019; Schalper et al., 2019).

In the present review, we will outline the principal GBM molecular and tumor microenvironment (TME) aspects providing also the grounds for novel targeted therapies and immunotherapy approaches using ICIs for the treatment of GBM affected patients.

## GENOMIC LANDSCAPE OF GBM

Specific molecular signatures of GBM have been identified through the introduction of next generation sequencing methods, in particular in untreated GBM tumors. It has been found mutations of several genes in GBM including phosphatase and tensin homolog (*PTEN*), tumor suppressor P53 (*TP53*), epidermal growth factor receptor (*EGFR*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), phosphatidylinositol 3-kinase regulatory subunit alpha (*PIK3R1*), platelet derived growth factor receptor alpha (*PDGFRA*), retinoblastoma 1 (*RB1*), neurofibromin 1 (*NF1*), gamma-aminobutyric acid receptor subunit alpha-6 (*GABRA6*), *IDH1*, mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6*), mutL homolog 1 (*MLH1*), and PMS1 homolog 2 (*PMS2*). Furthermore, several hotspot mutations have been found, like the *IDH1* R132H mutation, the B-Raf proto-oncogene (*BRAF*) V600E mutation (Zhao et al., 2009; Kloosterhof et al., 2011; Kannan et al., 2012; Schwartzentruber et al., 2012; Brennan et al., 2013; Eder and Kalman, 2014; Ceccarelli et al., 2016; Wang et al., 2016; Kessler et al., 2018; Stathias et al., 2018; D'Angelo et al., 2019).

Glioblastoma cases characterized by the presence of mutations in DNA mismatch repair (MMR) genes, e.g., *MSH2*, *MSH6*, *MLH1*, and *PMS2* have been suggested to be defined as having a hypermutated profile (Hunter et al., 2006; Cahill et al., 2007; Greenman et al., 2007; Tcga, 2008; Yip et al., 2009; Brennan et al., 2013; Daniel et al., 2019).

Frequent amplification events found in GBM concern chromosome 7 [*EGFR*/ MET proto-oncogene (*MET*)/ cyclin dependent kinase 6 (*CDK6*)], chromosome 12 [cyclin dependent kinase 4 (*CDK4*)/, mouse double minute 2 homolog (*MDM2*)], and chromosome 4 (*PDGFRA*). Gains of the genes SRY-box transcription factor 2 (*SOX2*), MYCN proto-oncogene (*MYCN*), cyclin D1 (*CCND1*), and cyclin E2 (*CCNE2*) have also been found (Hunter et al., 2006; Cahill et al., 2007; Kuttler and Mai, 2007; Parsons et al., 2008; Tcga, 2008; Yip et al., 2009; Brennan et al., 2013; Sanborn et al., 2013; Zheng et al., 2013; Furgason et al., 2015). Frequent deletions in GBM include deletions in cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*), deletions of 6q26 in which the minimal deleted region seems to include the QKI, KH domain containing RNA binding (*QKI*) gene, and single gene deletions of low-density lipoprotein receptor-related protein 1B (*LRP1B*), neuronal PAS domain protein 3 (*NPAS3*), limbic system associated membrane protein (*LSAMP*), SET and MYND domain-containing protein 3 (*SMYD3*) genes (Kamiryo et al., 2002; Hunter et al., 2006; Cahill et al., 2007; Tcga, 2008; Yip et al., 2009; Moreira et al., 2011; Chen et al., 2012; Mizoguchi et al., 2012; Brennan et al., 2013; Nobusawa et al., 2014; Tabouret et al., 2015; Yang et al., 2016).

Repeatedly, *EGFR* mutations have been found associated with regional gene amplification (Ekstrand et al., 1991; Jaros et al., 1992; Schlegel et al., 1994; Dunn et al., 2012; Brennan et al.,

2013; Cominelli et al., 2015). Remarkably, the aberrant exon 1–8 junction of epidermal growth factor receptor variant III (*EGFRvIII*) was found expressed in a relevant proportion of cases. Additional recurrent non-canonical *EGFR* transcript forms were also observed (Ekstrand et al., 1991; Jaros et al., 1992; Nishikawa et al., 1994; Tcga, 2008; Brennan et al., 2013; Cominelli et al., 2015). The O-6-methylguanine-DNA methyltransferase (*MGMT*) locus has been found methylated in about 50% of GBM cases (Esteller et al., 2000; Paz et al., 2004; Hegi et al., 2005; Tcga, 2008; Zawlik et al., 2009; Malmstrom et al., 2012; Reifenberger et al., 2012; Armstrong et al., 2013; Brennan et al., 2013; Wiestler et al., 2013; Wick et al., 2014, 2018; Cominelli et al., 2015).

Recent studies have defined the evolution of tumor cells in GBM cases undergoing therapy as a process of clonal replacement where a fraction of tumor cells is eliminated by the treatment while clones of resistant cells are positively selected (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016). Specifically, intratumoral heterogeneity, with the presence of resistant subclones, both in low grade and high grade glioma is frequently associated with treatment failure. Although a clearly defined pattern of tumor evolution has not yet been described in GBM, *TP53* gene mutations have been recently proposed as a marker of subclonal heterogeneity (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016).

Glioblastoma evolution is highly branched, specific alterations and evolutionary patterns frequently occurring depending on the treatment. There is no linear link between the dominant clone at diagnosis and the dominant clone at relapse (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016). Remarkably, genes such as *TP53, EGFR, PDGFRA* are frequently subjected to a process of mutational switching where a mutated version of a gene, found at diagnosis, is replaced by another mutated version of the same gene at relapse (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016). Hypermutated tumors, which are highly enriched for mutations at CpG dinucleotides, generally harbor mutations in *MMR* pathway genes, most commonly in *MSH6*. These *MMR* alterations have been thought to be associated with putative mutagenic mechanisms of TMZ treatment (Hunter et al., 2006; Cahill et al., 2007; Tcga, 2008; Yip et al., 2009; Brennan et al., 2013; Wang et al., 2016).

A gene expression-based molecular classification has been proposed to integrate somatic mutation and DNA copy number data (Verhaak et al., 2010; Behnan et al., 2019). According to this classification, GBM cases were subdivided in proneural, neural, classical and mesenchymal subtypes. These different subtypes have been associated with gene signatures of normal brain cell types of different neural lineages. Moreover, GBM cases included in the different subtypes have also been associated with a different pathogenesis with GBM clones developing as the result of different causes and/or from different cell type of origin. However, further studies, also investigating glioma stem cells, have been able to identify three subtypes: proneural, mesenchymal and classical subtypes (Verhaak et al., 2010; Behnan et al., 2019).

According to the first proposed classification, GBM cases belonging to the classical subtype show in about the 100% of cases the chromosome 7 amplification paired with chromosome 10 loss. This event is also very frequent in the totality of GBM cases. High-level of *EGFR* amplification has been observed in 97% of cases belonging to the classical subtype, whereas this alteration has been infrequently found in the other GBM subtypes. Moreover, in association with frequent EGFR alteration, a lack of *TP53* mutations has been found in a subset of the classical subtype (Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016). The focal 9p21.3 homozygous deletion, targeting the *CDKN2A* gene, has been also frequently found in the classical subtype, in the 94% of cases belonging to this subtype found associated with *EGFR* amplification. The homozygous 9p21.3 deletion has been also found mutually exclusive with aberrations in genes belonging to the RB1 pathway such as *RB1*, *CDK4* and cyclin-D2 (*CCDN2)*, thus suggesting that in the cases with focal *EGFR* amplification the *CDKN2A* deletion is the sole alteration affecting the RB1 pathway. GBM cases belonging to the classical subtype are also characterized by the high expression of genes belonging to the notch homolog 1, translocation-associated (NOTCH) pathway such as neurogenic locus notch homolog-3 (*NOTCH3*), jagged-1 (*JAG1*) and lunatic fringe (*LFNG*), sonic hedgehog pathway such as smoothened (*SMO*), growth arrest-specific protein 1 (*GAS1*) and zinc finger protein GLI1 (*GLI1*) and the neural precursor and stem cell marker nestin (NES) pathway (Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016).

Glioblastoma cases belonging to the proneural subtype were found to be mainly characterized by alterations of *PDGFRA* and point mutations of *IDH1*. The focal amplification at the locus 4q12 harboring *PDGFRA* was associated with high levels of *PDGFRA* gene expression and the frequent presence of mutations in the *PDGFRA* gene. The great majority of *IDH1* mutations has been found in GBM cases belonging to the proneural subtype. Of note, they have been found to be generally mutually exclusive to *PDGFRA* alterations. Loss of heterozygosity and mutations of the *TP53* gene have been found to be frequent events in the proneural subtype. *PIK3CA/PIK3R1* mutations have also been found in the proneural subtypes in cases without *PDGFRA* abnormalities. The proneural group has been found to be characterized also by the high expression of genes other than *PDGFRA* that characterize the oligodendrocytic development such as oligodendrocyte transcription factor (*OLIG2*) and homeobox protein nkx-2.2 (*NKX2-2*). This group has been found also characterized by the expression of proneural development genes such as *SOX* family genes and achaete-scute homolog 1 (*ASCL1*), doublecortin (*DCX*), delta-like 3 (*DLL3*), transcription factor 4 (*TCF4*; Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016).

Glioblastoma cases belonging to the neural subtype were characterized by the expression of genes well-known as neuron markers such as *GABRA1*, neurofilament light chain (*NEFL*), synaptotagmin-1 (*SYT1*) and solute carrier family 12 member 5 (*SLC12A5*). GBM cases belonging to the neural subtype show an enrichment in genes involved in neuron protection and in axon and synaptic transmission (Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016).

Glioblastoma cases belonging to the mesenchymal subtype are frequently characterized by the presence of focal hemizygous deletions at 17q11.2 region encompassing the *NF1* gene. This

has been frequently associated with low *NF1* expression levels. Moreover, mutations at the *NF1* gene have been found in GBM cases belonging to the mesenchymal subgroup. Concomitant *PTEN* mutations have also been found in mesenchymal subgroup cases carrying *NF1* mutations (Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016). GBM cases belonging to the mesenchymal subtype are also characterized by the expression of mesenchymal markers such as chitinase-3-like protein 1 (*CHI3L1*) and *MET*. It has been thought that the higher activity of mesenchymal and astrocytic markers such as *CD44* and *MERKT* is linked to an epithelial-to-mesenchymal transition proper of dedifferentiated and transdifferentiated tumors. Finally, GBM cases belonging to the mesenchymal subtype are also characterized by the high expression of genes belonging to the TNF superfamily pathway and NF-kB pathway such as tumor necrosis factor receptor type 1-associated death domain (*TRADD*), *RELB* and TNF receptor superfamily member 1A (*TNFRSF1A*) (Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016).

Several clinical features have been associated with the four subtypes. In particular, an association between proneural subtype and age as well as between this subtype and a trend for a longer survival. However, GBM belonging to the proneural subtype have not shown a survival advantage from aggressive treatment protocols. On the other hand, a clear treatment effect has been observed among GBM cases belonging to the classical and mesenchymal subtypes (Tcga, 2008; Verhaak et al., 2010; Brennan et al., 2013; Wang et al., 2016).

The proneural-to-mesenchymal transition upon tumor recurrence has been proposed as a mechanism of treatment resistance for GBM to radiotherapy and/or chemotherapy. GBM patients belonging to the mesenchymal subtype have been associated with survival shorter than the other subtypes, particularly when cases with low transcriptional heterogeneity are considered. Although in the context of poor prognosis patients, GBM cases belonging to the mesenchymal subtype have been found to show favorable response to immunotherapy and intensive radiotherapy and chemotherapy (Verhaak et al., 2010; Behnan et al., 2019).

Long non-coding RNAs (LncRNAs) are RNA transcripts longer than 200 base pairs which do not code for proteins. Although the human genome harbors more than 50,000 LncRNA genes, they are still poor characterized. However, LncRNAs have been found to play key roles in various cell activities related to regulation of gene expression, protein synthesis, stemness, immunity (Schlackow et al., 2017). Moreover, LncRNAs have been found to exert relevant roles in pathogenesis and progression of various cancers including GBM. In particular, a large number of LncRNAs has been found associated with deregulated gene expression and imbalanced biological processes in GBM (Zeng et al., 2018). In this context, the expression of the LncRNA P73 antisense RNA 1T (*TP73-AS1*) has been associated with poor outcome in GBM patients. GBM patients belonging to less aggressive subgroups have been found to be characterized by hypermethylation and low expression of *TP73-AS1*. Moreover, it has been found that *TP73-AS1* downregulation is associated with the loss of aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*) expression and the re-sensitivity of the GBM stem cell (GSC) population to TMZ treatment (Mazor et al., 2019). Expression of the LncRNA HOX transcript antisense intergenic RNA (*HOTAIR*) in GBM has been demonstrated to be significantly higher than in normal tissues and low grade gliomas. Moreover, *HOTAIR* has been demonstrated to be an independent prognostic factor in GBM associated with proliferation and tumorigenic potential of GBM cells (Zhang et al., 2015; Zhang et al., 2018). LncRNA colorectal neoplasia differentially expressed (*CRNDE*) has been found highly expressed in GBM and other brain cancers such as astrocytomas. It has also been explained that its overexpression is associated with promotion of tumor cell growth and migration (Ellis et al., 2014; Wang et al., 2016). LncRNA nuclear enriched abundant transcript 1 (*NEAT1*) has been shown to be a key regulator of nuclear domains implicated in mRNA nuclear retention and splicing. *NEAT1* has been found upregulated in human GBM tissues and GBM cell line models and a high *NEAT1* expression has been associated with larger tumor size, higher WHO grade, higher recurrence rate and unfavorable overall survival (He et al., 2016). The LncRNA X-inactive specific transcript (*XIST*) has been found highly expressed in glioma tissues and GSCc. The knockdown of *XIST* has been shown to suppress proliferation, migration, invasion and tumorigenic potential of GSCs by upregulating *miR152* (Yao et al., 2015). The LncRNA SOX2 overlapping transcript (*SOX2OT*) is characterized by the fact that its transcription genomic region includes the *SOX2* gene; a *SOX2OT* involvement in the transcriptional regulation of *SOX2* has also been observed. *SOX2OT* has been shown to be involved in the proliferation, migration, invasion of GSCs (Su et al., 2017). The LncRNA *H19* has been shown to be upregulated in glioma tissues and associated with poor outcome. Moreover, invasion, angiogenesis, stemness and tumorigenicity of GBM cells have been found enhanced when *H19* is overexpressed (Jiang et al., 2016). The LncRNA *LOC441204* has been found highly expressed in glioma tumor specimens and cell lines. Tumor cell proliferation has been found suppressed by knockdown of *LOC441204* in glioma. On the other hand, *LOC441204*-induced tumor cell growth has been shown to be modulated by the stabilization of the β-catenin pathway (Lin et al., 2017). Regarding the role of other LncRNAs, evidence has also been reported about the fact that the high expression of other LncRNAs such as maternally expressed gene 3 (*MEG3*), metastasis associated lung adenocarcinoma (*MALAT1*), cancer susceptibility candidate 2 (*CASC2*), taurine-upregulated gene 1 (*TUG1*), DBH antisense RNA 1 (*DBH-AS1), AC005035.1*, *AC010336.2*, *AC108134.2*, *AC116351.2*, *Clorf132*, *C10orf91*, *LINC00475*, *MIR210HG* could be associated with poor outcome in GBM cases (Zeng et al., 2018).

## ROLE OF THE GBM TUMOR MICROENVIRONMENT

The brain is distinguished from the other organs by the presence of the blood-brain-barrier (BBB). The BBB provides a selective barrier between the systemic circulation and the brain, thus representing a limit for the delivery of many therapeutic agents

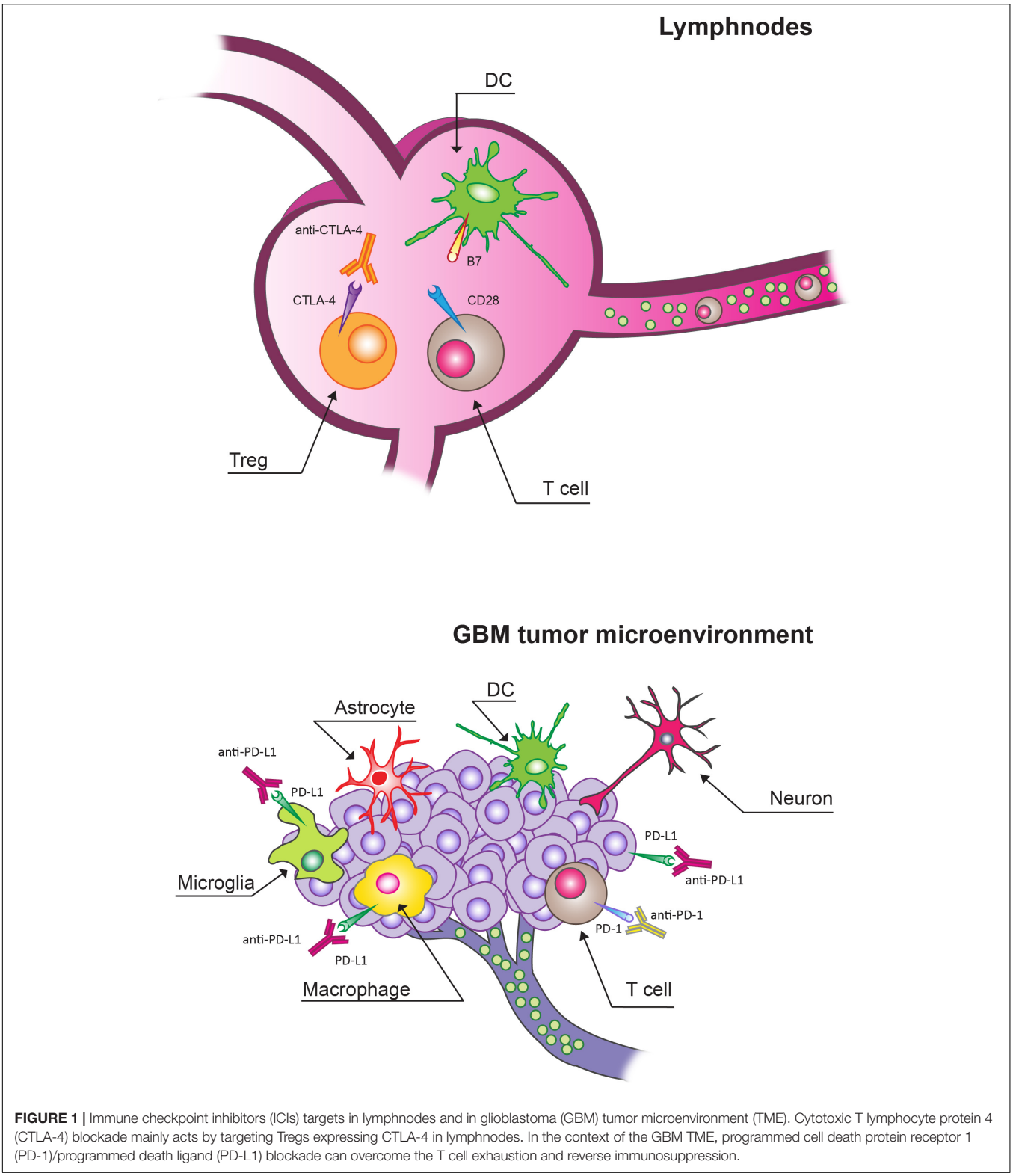


**FIGURE 1 |** Immune checkpoint inhibitors (ICIs) targets in lymphnodes and in glioblastoma (GBM) tumor microenvironment (TME). Cytotoxic T lymphocyte protein 4 (CTLA-4) blockade mainly acts by targeting Tregs expressing CTLA-4 in lymphnodes. In the context of the GBM TME, programmed cell death protein receptor 1 (PD-1)/programmed death ligand (PD-L1) blockade can overcome the T cell exhaustion and reverse immunosuppression.

(Chen et al., 2012; Miura et al., 2013). However, a loss of BBB integrity could be displayed in the presence of cancer, in particular during the cancer progression. This seems to be the reason why several agents, including ICIs, that are known to be not capable of penetrating the BBB, have however shown in some extent a clinical efficacy (de Vries et al., 2006; van Tellingen et al., 2015). Specialized endothelial cells, pericytes, and astrocytic foot processes, dictating junctional integrity, are the elements that

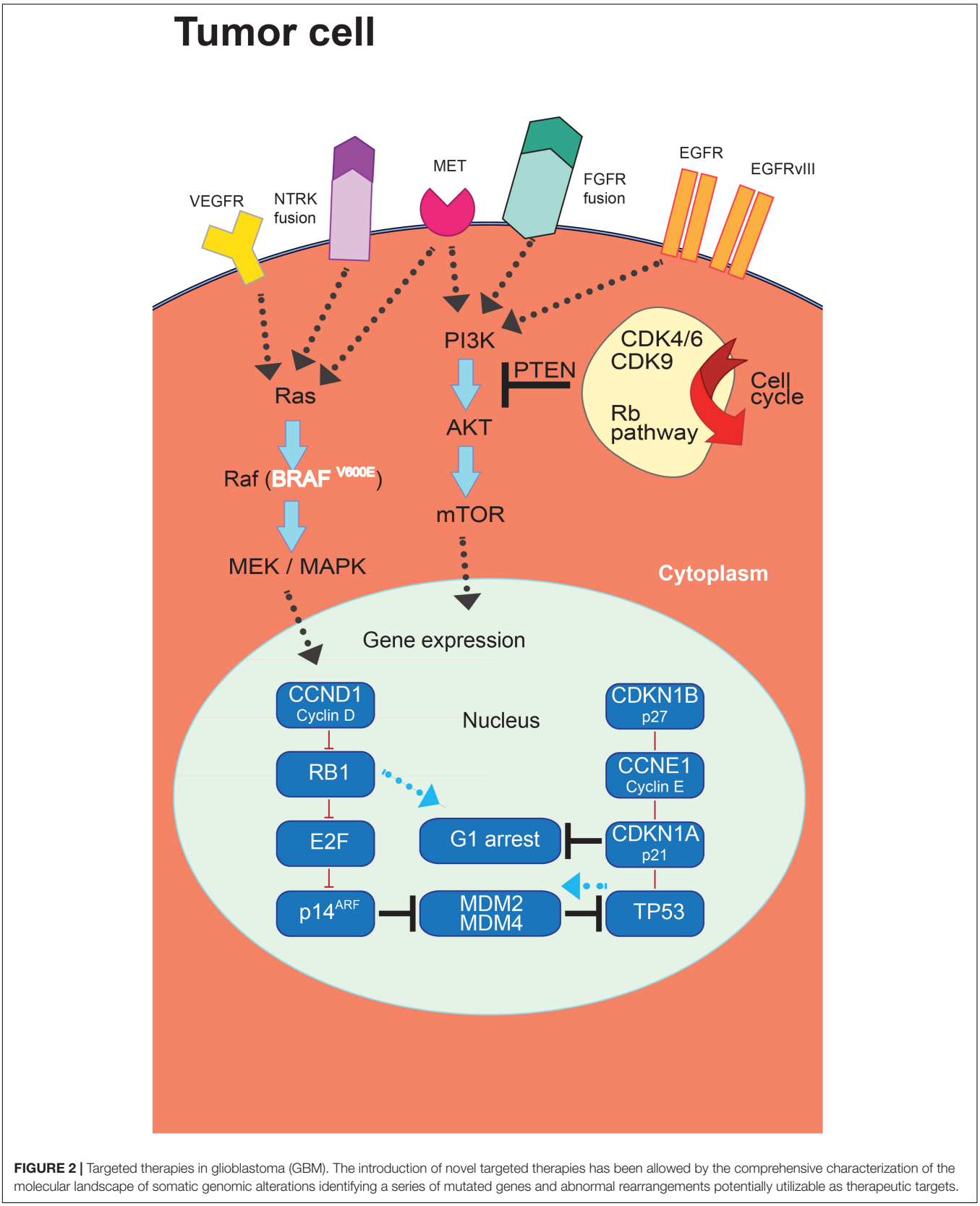


**FIGURE 2 |** Targeted therapies in glioblastoma (GBM). The introduction of novel targeted therapies has been allowed by the comprehensive characterization of the molecular landscape of somatic genomic alterations identifying a series of mutated genes and abnormal rearrangements potentially utilizable as therapeutic targets.

constitute the BBB. Moreover, BBB integrity can be also regulated by microglia, being these cells capable of repairing the BBB in a purinergic receptor P2RY12-dependent manner in case of injury (de Vries et al., 2006; van Tellingen et al., 2015).

The complex crosstalk of TME components is involved in the regulation of tumor progression (Quail and Joyce, 2013, 2017; Gritsenko et al., 2012; Nakasone et al., 2012; Quail and Joyce, 2013, 2017). The composition of ECM of normal brain is distinctive, with specific tissue-resident cell types such as neurons, astrocytes and microglia. Moreover, the BBB physically protects the ECM from inflammation (Novak and Kaye, 2000; Mahesparan et al., 2003). The most common component of the brain ECM is hyaluronic acid which is localized in the intraparenchymal region (Kim et al., 2018). The haptotactic cues from the vascular basement membrane, the enrichment of vascular derived chemoctatic cues, as well as interconnected axon tracts can determine the therapeutic resistance of GBM cells in the perivascular space further providing haptotactic cues for cellular invasion (Giese and Westphal, 1996; Nimsky et al., 2005; Gritsenko et al., 2012).

A diffuse invasion pattern characterizes GBM (Young et al., 2015). Healthy tissue beyond the tumor margin is infiltrated by the tumor cells, generally enriched in the GSC stem cell fraction, that either migrate individually or collectively practically impeding complete surgical resection (Eyler and Rich, 2008; Sherriff et al., 2013). On the other hand, GBM tumors rarely intravasate and metastasize from the brain to distant organs (Quail and Joyce, 2013, 2017).

Glioblastoma frequently develop in a hypoxic microenvironment which can modify the metabolic pathways of GBM cells. The brain has a high metabolism level in which the glucose is the major energy substrate and lactate, ketone bodies, fatty acids and aminoacids can also be employed. The metabolic homeostasis of the brain is maintained by the interaction among its various constituent cells such as astrocytes, neurons and microglia (Gritsenko et al., 2012; Nakasone et al., 2012; Quail and Joyce, 2013, 2017). This equilibrium can be altered by genomic aberrations and biochemical variations in GBM cells that often metabolize glucose into lactate even when oxygen is present in a process called Warburg effect. GBM cells can also increase intracellular lipid, aminoacid and nucleotide levels. These metabolic adaptations can favor GBM tumor growth (Gritsenko et al., 2012; Nakasone et al., 2012; Quail and Joyce, 2013, 2017).

Hypervascularity is a characteristic of GBM tumors with an increment in angiogenesis compared to healthy brain tissue. This tumor-associated vasculature is not completely formed, with leaky vessels, and associated with an increase in interstitial fluid pressure. A necrotic core softer than the surrounding tissue characterizes the TME of GBM (Brat and Van Meir, 2004; Brat et al., 2004; Persano et al., 2011; Hambardzumyan and Bergers, 2015; Chen and Hambardzumyan, 2018). High density regions called pseudopalisades are formed when cells migrate away from the hypoxic regions. Increased matrix production with respect to both necrotic regions and healthy tissues characterizes these regions (Brat and Van Meir, 2004; Brat et al., 2004; Persano et al., 2011; Hambardzumyan and Bergers, 2015; Chen and Hambardzumyan, 2018). GBM cells are capable of rapidly invading vasculature (Akiyama et al., 2001; Ponta et al., 2003; Zimmermann and Dours-Zimmermann, 2008; Dicker et al., 2014; Schiffer et al., 2018).

Circulatory and immune systems are connected by the lymphatic system that is involved, together with blood vessels, in the exchange of various elements including fluid, waste, debris as well as immune cells (Engelhardt et al., 2017). Together with the absence of a classic lymphatic drainage system, the CNS exhibits several other peculiar features, such as the presence of tight junctions in the BBB, as well as the limited rejection of foreign tissues within the CNS (Louveau et al., 2015; Schiffer et al., 2018).

There are functional lymphatic vessels in the CNS with the presence of different types of antigen-presenting cells (APCs), including microglia, macrophages, astrocytes and canonical APC such as dendritic cells (DCs; **Figure 1**; Louveau et al., 2015; Schiffer et al., 2018). In the brain, microglia are the predominant APCs whereas DCs carry out a less relevant role (Lowe et al., 1989; Ulvestad et al., 1994; Weiss et al., 2009; Goldmann et al., 2016).

Activated T cells can invade the CNS. These activated T cells can cover these compartments in an unrestricted manner. On the other hand, antigens can be presented locally or in the draining cervical lymph nodes. Moreover, the BBB can be compromised, thus allowing a relevant infiltration of multiple immune cell types from the peripheral circulation (Weiss et al., 2009). However, GBM tumors present low numbers of tumor-infiltrating lymphocytes (TILs) and other immune effector cell types compared to other tumor types (Schiffer et al., 2018). The interaction of TILs with the TME can cause their re-education. In particular, the local TME can alter T cell effector function in the process related to anti-tumor immunity even in the CNS, where T cell-mediated inflammatory responses are considered poor under normal physiological contexts. The number of antigen-specific TILs can remain relatively low besides frequently displaying an exhausted phenotype. The peculiar immune environment of the brain can be responsible for this reduced quantity and limited activity of T cells in GBM. In particular, there is a specific need of avoiding unrestrained inflammation in the brain given its solid enclosure and the potential for damage from increased intracranial pressure (Quail and Joyce, 2013, 2017; Gajewski et al., 2017; Keskin et al., 2019). This need is not present with the same extent in peripheral organs. In fact, this environment in which both inflammatory and adaptive immune responses are tightly regulated is specific of the brain; besides there is a variety of immunosuppressive mechanisms at both the molecular and cellular levels (Perng and Lim, 2015). In particular, stromal cells of the brain produce high levels of the classic immunosuppressive cytokines transforming growth factor β (TGFβ), interleukin-10 (IL-10) in response to inflammatory stimuli, including those derived from GBM tumors, in order to maintain homeostasis (Vitkovic et al., 2001; Gong et al., 2012). Furthermore, the accumulation of regulatory T cells (Tregs) is stimulated by IDO which can suppress T cell activity by depleting tryptophan from the microenvironment. Microglia and tumor- infiltrating myeloid cells can also inhibit T cell proliferation and function through the production of high levels of arginase that causes the

**TABLE 1 |** Clinical trials in glioblastoma (GBM) using tyrosine kinase inhibitors (TKIs).

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| mTORC1/mTORC2 kinase inhibitor AZD2014 in previously treated glioblastoma multiforme | Glioblastoma multiforme | Drug: AZD2014 | NCT02619864 | Completed | Phase I |
| Gefitinib and radiation therapy in treating patients with glioblastoma multiforme | Adult giant cell Glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma | Drug: Gefitinib | NCT00052208 | Completed | Phase I, II |
| Study of AEE788 in patients with recurrent/relapse glioblastoma multiforme (GBM) | Glioblastoma multiforme | Drug: AEE788 | NCT00116376 | Completed | Phase I, II |
| Clinical trial on the combination of avelumab and axitinib for the treatment of patients with recurrent glioblastoma | Recurrent glioblastoma (WHO-grade IV glioma) | Drug: Axitinib Drug: Avelumab | NCT03291314 | Completed | Phase II |
| AZD8055 for adults with recurrent gliomas | Glioblastoma Multiforme<br>Anaplastic astrocytoma<br>Anaplastic oligodendroglioma<br>Malignant glioma<br>Brainstem glioma | Drug: AZD8055 | NCT01316809 | Completed | Phase I |
| Sunitinib in treating patients with recurrent malignant gliomas | Astrocytoma<br>Adult diffuse astrocytoma<br>Adult giant cell Glioblastoma<br>Adult glioblastoma and other 5 | Drug: Sunitinib malate | NCT00499473 | Completed | Phase II |
| Study to assess safety, pharmacokinetics, and efficacy of oral CC-223 for patients with advanced solid tumors, non-hodgkin lymphoma or multiple myeloma | Multiple myeloma<br>Diffuse large B cell Lymphoma<br>Glioblastoma multiforme<br>Hepatocellular carcinoma and other 4 | Drug: CC-223 | NCT01177397 | Completed | Phase I,II |
| Trial of ponatinib in patients with bevacizumab-refractory glioblastoma | Glioblastoma | Drug: Ponatinib | NCT02478164 | completed | Phase II |
| A phase II exploratory, multicentre, open-label, non-comparative study of ZD1839 (iressa) and radiotherapy in the treatment of patients with glioblastoma multiforme | Glioblastoma | Drug: Gefitinib | NCT00238797 | Completed | Phase II |
| A study of the safety and efficacy of tarceva in patients with first relapse of grade IV glioma (glioblastoma multiforme) | Glioblastoma | Drug: Erlotinib HCl (OSI-774) | NCT00337883 | Completed | Phase II |
| Study of tesevatinib monotherapy in patients with recurrent glioblastoma | Glioblastoma<br>Recurrent glioblastoma<br>Brain tumor | Drug: Tesevatinib | NCT02844439 | Completed | Phase II |
| Oral tarceva study for recurrent/residual glioblastoma multiforme and anaplastic astrocytoma | Glioblastoma multiforme<br>Anaplastic astrocytoma | Drug: Erlotinib | NCT00301418 | Completed | Phase I,II |
| Gefitinib in treating patients with newly diagnosed glioblastoma multiforme | Adult giant cell glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma | Drug: Gefitinib | NCT00014170 | Completed | Phase II |
| Erlotinib and sorafenib in treating patients with progressive or recurrent glioblastoma multiforme | Adult giant cell glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma<br>Recurrent adult brain tumor | Drug: erlotinib hydrochloride<br>Drug: sorafenib tosylate | NCT00445588 | Completed | Phase II |
| AZD2171 in treating patients with recurrent glioblastoma multiforme | Adult giant cell Glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma<br>Recurrent adult brain tumor | Drug: cediranib maleate | NCT00305656 | Completed | Phase II |
| Dasatinib in treating patients with recurrent glioblastoma multiforme or gliosarcoma | Adult giant cell glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma<br>Recurrent adult brain neoplasm | Drug: Dasatinib | NCT00423735 | Completed | Phase II |

*(Continued)*

**TABLE 1 |** Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Cediranib maleate and cilengitide in treating patients with progressive or recurrent glioblastoma | Adult giant cell Glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma<br>Recurrent adult brain neoplasm | Drug: Cediranib maleate Drug: Cilengitide | NCT00979862 | Completed | Phase I |
| Sorafenib in newly diagnosed high grade glioma | Glioblastoma<br>Gliosarcoma<br>Anaplastic astrocytoma<br>Anaplastic oligoastrocytoma<br>Anaplastic oligodendroglioma | Drug: Sorafenib dose escalation | NCT00884416 | Completed | Phase I |
| E7050 in combination with E7080 in subjects with advanced solid tumors (dose escalation) and in subjects with recurrent glioblastoma or unresectable stage III or stage IV melanoma after prior systemic therapy (expansion cohort and phase 2) | Advanced solid tumors | Drug: E7050 Drug: Lenvatinib | NCT01433991 | Completed | Phase I,II |
| ZD 1839 in treating patients with glioblastoma multiforme in first relapse | Brain and central nervous system tumors | Drug: Gefitinib | NCT00016991 | Completed | Phase II |
| Ph I dasatinib + erlotinib in recurrent MG | Glioblastoma, Gliosarcoma | Drug: Erlotinib and dasatinib | NCT00609999 | Completed | Phase I |
| Bafetinib in treating patients with recurrent high-grade glioma or brain metastases | Adult anaplastic astrocytoma<br>Adult anaplastic Ependymoma<br>Adult anaplastic Oligodendroglioma<br>Adult giant cell glioblastoma<br>Adult glioblastoma and other 5 | Drug: Bafetinib | NCT01234740 | Completed | Phase I |
| A randomized phase II clinical trial on the efficacy of axitinib as a monotherapy or in combination with lomustine for the treatment of patients with recurrent glioblastoma | Glioblastoma multiforme | Drug: Axitinib Drug: Axitinib plus lomustine | NCT01562197 | Completed | Phase II |
| Cediranib in combination with lomustine chemotherapy in recurrent glioblastoma | Recurrent glioblastoma | Drug: Cediranib Drug: Lomustine chemotherapy Drug: Placebo Cediranib | NCT00777153 | Completed | Phase III |
| Radiation therapy and temozolomide followed by temozolomide plus sorafenib for glioblastoma multiforme | Glioblastoma multiforme | Drug: Temozolomide Drug: Sorafenib | NCT00544817 | Completed | Phase II |
| PTK787/ZK 222584 in combination with temozolomide and radiation in patients with glioblastoma taking enzyme-inducing anti-epileptic drugs | Glioblastoma | Drug: PTK787/ZK 222584 Drug: Temozolomide | NCT00385853 | Completed | Phase I |
| Study of imatinib mesylate in combination with hydroxyurea versus hydroxyurea alone as an oral therapy in patients with temozolomide resistant progressive glioblastoma | Glioblastoma multiforme Astrocytoma | Drug: Imatinib mesylate Drug: Hydroxyurea | NCT00154375 | Completed | Phase III |
| Open label trial to explore safety of combining afatinib (BIBW 2992) and radiotherapy with or without temozolomide in newly diagnosed glioblastoma multiform | Glioblastoma | Drug: Temozolomide Drug: BIBW2992 | NCT00977431 | Completed | Phase I |
| Ph. 2 sorafenib + protracted temozolomide in recurrent GBM | Recurrent glioblastoma Multiforme | Drug: Sorafenib and temozolomide | NCT00597493 | Completed | Phase II |
| Erlotinib and temozolomide with radiation therapy in treating patients with glioblastoma multiforme or other brain tumors | Adult giant cell glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma | Drug: Erlotinib hydrochloride Drug: Temozolomide | NCT00039494 | Completed | Phase II |
| Erlotinib and radiation therapy in treating young patients with newly diagnosed glioma | Brain and central nervous system tumors | Drug: Erlotinib hydrochloride | NCT00124657 | Completed | Phase I,II |
| Safety and efficacy study of tarceva, temodar, and radiation therapy in patients with newly diagnosed brain tumors | Glioblastoma multiforme<br>Gliosarcoma | Drug: Tarceva Drug: Temodar | NCT00187486 | Completed | Phase II |

*(Continued)*

**TABLE 1 |** Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Study of sunitinib before and during radiotherapy in newly diagnosed biopsy-only glioblastoma patients | Glioblastoma | Drug: Sunitinib | NCT01100177 | Completed | Phase II |
| Dasatinib and bevacizumab in treating patients with recurrent or progressive high-grade glioma or glioblastoma multiforme | Glioblastoma multiforme | Biological: Bevacizumab<br>Drug: Basatinib | NCT00892177 | Completed | Phase II |
| Lapatinib in treating patients with recurrent glioblastoma multiforme | Brain and central nervous system tumors | Drug: Lapatinib ditosylate | NCT00099060 | Completed | Phase I,II |
| Gefitinib in treating patients with recurrent or progressive CNS tumors | Brain and central nervous system tumors | Drug: Gefitinib | NCT00025675 | Completed | Phase II |
| Radiation therapy, temozolomide, and erlotinib in treating patients with newly diagnosed glioblastoma multiforme | CNS tumor<br>Adult | Drug: Erlotinib hydrochloride<br>Drug: Temozolomide | NCT00274833 | Completed | Phase II |
| Sunitinib tumor levels in patients not on enzyme-inducing anti-epileptic drugs undergoing debulking surgery for recurrent glioblastoma | Glioblastoma<br>Brain tumor | Drug: Sunitinib | NCT00864864 | Completed | Early Phase I |
| Study of bevacizumab plus temodar and tarceva in patients with glioblastoma or gliosarcoma | Glioblastoma<br>Gliosarcoma | Drug: Bevacizumab<br>Drug: Tarceva<br>Drug: Temozolomide | NCT00525525 | Completed | Phase II |
| A Phase II trial of sutent (sunitinib; SU011248) for recurrent anaplastic astrocytoma and glioblastoma | Anaplastic astrocytoma<br>Glioblastoma | Drug: Sunitinib malate | NCT00606008 | Completed | Phase II |
| Cediranib, temozolomide, and radiation therapy in treating patients with newly diagnosed glioblastoma | Adult giant cell<br>Glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma | Drug: Cediranib maleate<br>Drug: Temozolomide | NCT00662506 | Completed | Phase I,II |
| Dasatinib or placebo, radiation therapy, and temozolomide in treating patients with newly diagnosed glioblastoma multiforme | Brain and central nervous system tumors | Drug: Dasatinib<br>Drug: Temozolomide | NCT00869401 | Completed | Phase I,II |
| Erlotinib compared with temozolomide or carmustine in treating patients with recurrent glioblastoma multiforme | Brain and central nervous system tumors | Drug: Carmustine<br>Drug: Erlotinib hydrochloride<br>Drug: Temozolomide | NCT00086879 | Completed | Phase II |
| Sorafenib combined with erlotinib, tipifarnib, or temsirolimus in treating patients with recurrent glioblastoma multiforme or gliosarcoma | Adult giant cell glioblastoma<br>Adult glioblastoma<br>Adult gliosarcoma<br>Recurrent adult brain tumor | Drug: Sorafenib tosylate<br>Drug: Erlotinib hydrochloride<br>Drug: Tipifarnib<br>Drug: Temsirolimus | NCT00335764 | Completed | Phase I,II |
| Phase II Imatinib + hydroxyurea in treatment of patients with recurrent/progressive grade II low-grade glioma (LGG) | Glioblastoma<br>Gliosarcoma | Drug: Imatinib mesylate and hydroxyurea | NCT00615927 | Completed | Phase II |
| Sorafenib tosylate and temsirolimus in treating patients with recurrent glioblastoma | Adult glioblastoma<br>Adult gliosarcoma<br>Recurrent adult brain neoplasm | Drug: Sorafenib tosylate<br>Drug: Temsirolimus | NCT00329719 | Completed | Phase I,II |
| Phase I : cediranib in combination with lomustine chemotherapy in recurrent malignant brain tumor | Recurrent glioblastoma<br>Brain tumor | Drug: Cediranib<br>Drug: Lomustine | NCT00503204 | Completed | Phase I |
| Ph I SU011248 + irinotecan in treatment of Pts w MG | Glioblastoma | Drug: SU011248 and irinotecan | NCT00611728 | Completed | Phase I |
| Ph I zactima + imatinib mesylate and hydroxyurea for pts w recurrent malignant glioma | Glioblastoma<br>Gliosarcoma | Drug: Zactima, gleevec, hydroxyurea | NCT00613054 | Completed | Phase I |
| Imatinib mesylate and hydroxyurea in treating patients with recurrent or progressive meningioma | Glioblastoma<br>Gliosarcoma | Drug: Hydroxyurea<br>Drug: Imatinib mesylate | NCT00354913 | Completed | Phase II |
| Bevacizumab and sorafenib in treating patients with recurrent glioblastoma multiforme | Brain and central nervous system tumors | Biological: Bevacizumab<br>Drug: Sorafenib tosylate | NCT00621686 | Completed | Phase II |
| BIBW 2992 (afatinib) with or without daily temozolomide in the treatment of patients with recurrent malignant glioma | Glioma | Drug: BIBW 2992<br>Drug: TMZ<br>Drug: BIBW 2992 plus TMZ | NCT00727506 | Completed | Phase II |
| Bevacizumab and erlotinib after radiation therapy and temozolomide in treating patients with newly diagnosed glioblastoma multiforme or gliosarcoma | Brain and central nervous system tumors | Drug: Bevacizumab<br>Drug: Erlotinib hydrochloride | NCT00720356 | Completed | Phase II |
| Ph I gleevec in combo w RAD001 + hydroxyurea for Pts w recurrent MG | Glioblastoma<br>Gliosarcoma | Drug: Gleevec, RAD001, and hydroxyurea | NCT00613132 | Completed | Phase I |

*(Continued)*

**TABLE 1 |** Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| GW572016 to treat recurrent malignant brain tumors | Glioma<br>Brain tumor<br>Glioblastoma multiforme<br>GBM<br>Gliosarcoma<br>GS | Drug: Lapatinib ditosylate | NCT00107003 | Completed | Phase II |
| Temozolomide and radiation therapy with or without vatalanib in treating patients with newly diagnosed glioblastoma multiforme | Brain and central nervous system tumors | Drug: Temozolomide<br>Drug: Vatalanib | NCT00128700 | Completed | Phase I,II |
| Ph II erlotinib + sirolimus for pts w recurrent malignant glioma multiforme | Glioblastoma<br>Gliosarcoma | Drug: Erlotinib + Sirolimus | NCT00672243 | Completed | Phase II |
| Afatinib (BIBW 2992) QTcF trial in patients with relapsed or refractory solid tumors | Neoplasms | Drug: BIBW 2992 | NCT00875433 | Completed | Phase II |
| Phase (Ph) II bevacizumab + erlotinib for patients (Pts) with recurrent malignant glioma (MG) | Glioblastoma<br>Gliosarcoma | Drug: Bevacizumab and erlotinib | NCT00671970 | Completed | Phase II |
| Everolimus and gefitinib in treating patients with progressive glioblastoma multiforme or progressive metastatic prostate cancer | Brain and central nervous system tumors<br>Prostate cancer | Drug: Everolimus<br>Drug: Gfitinib | NCT00085566 | Completed | Phase I,II |
| Sorafenib in treating patients with recurrent or progressive malignant glioma | Adult anaplastic astrocytoma<br>Adult anaplastic Oligodendroglioma<br>Adult giant cell Glioblastoma and other 2 | Drug: Sorafenib tosylate | NCT00093613 | Completed | Phase I |
| AZD7451 for recurrent gliomas | Glioblastoma multiforme | Drug: AZD7451 | NCT01468324 | Completed | Phase I |
| Gefitinib and radiation therapy in treating children with newly diagnosed gliomas | Untreated childhood anaplastic astrocytoma<br>Untreated childhood anaplastic oligodendroglioma<br>Untreated childhood brain stem glioma<br>Untreated childhood giant cell glioblastoma and other 4 | Drug: Gefitinib | NCT00042991 | Completed | Phase I,II |
| Erlotinib in treating patients with recurrent malignant glioma or recurrent or progressive meningioma | Adult anaplastic astrocytoma<br>Adult anaplastic oligodendroglioma<br>Adult giant cell glioblastoma<br>Adult glioblastoma and other 5 | Drug: Erlotinib hydrochloride | NCT00045110 | Completed | Phase I,II |
| Gamma-secretase inhibitor RO4929097 and cediranib maleate in treating patients with advanced solid tumors | Adult anaplastic astrocytoma<br>Adult anaplastic ependymoma<br>Adult anaplastic oligodendroglioma<br>Adult brain stem glioma<br>Adult giant cell glioblastoma<br>Adult glioblastoma and other 41 | Secretase inhibitor RO4929097<br>Drug: Cediranib maleate | NCT01131234 | Completed | Phase I |
| EGFR inhibition using weekly erlotinib for recurrent malignant gliomas | Brain cancer | Drug: Erlotinib | NCT01257594 | Completed | Phase I |
| Lapatinib in treating young patients with recurrent or refractory central nervous system tumors | Recurrent childhood anaplastic Astrocytoma<br>Recurrent childhood brain stem gliom<br>Recurrent childhood ependymoma<br>Recurrent childhood giant cell glioblastoma<br>Recurrent childhood glioblastoma and other 3 | Drug: Lapatinib ditosylate | NCT00095940 | Completed | Phase I,II |
| Erlotinib in treating patients with solid tumors and liver or kidney dysfunction | Astrocytoma<br>Adult anaplastic ependymoma<br>Adult anaplastic oligodendroglioma | | | | |

*(Continued)*

**TABLE 1 |** Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Adult brain stem glioma<br>Adult diffuse astrocytoma<br>Adult ependymoblastoma<br>Adult giant cell glioblastoma and 79 more | Drug: Erlotinib hydrochloride | NCT00030498 | Completed | Phase I | |
| ZD1839 and oral irinotecan in treating young patients with refractory solid tumors | Glioblastoma<br>Rhabdomyosarcomas<br>Neuroblastoma<br>Osteosarcoma | Drug: Irinotecan, Gefitinib | NCT00132158 | Completed | Phase I |
| Apatinib in recurrent or refractory intracranial central nervous system malignant tumors | Efficacy and safety | Drug: Apatinib<br>Drug: Temodar | NCT03660761 | Completed | Phase II |
| Bevacizumab and cediranib maleate in treating patients with metastatic or unresectable solid tumor, lymphoma, intracranial glioblastoma, gliosarcoma, or anaplastic astrocytoma | Adult grade III lymphomatoid granulomatosis<br>Adult nasal type extranodal NK/T-cell lymphoma<br>Anaplastic large cell lymphoma<br>Angioimmunoblastic T-cell lymphoma<br><br>Childhood burkitt lymphoma and other 56 | Biological: Bevacizumab<br>Drug: Cediranib maleate | NCT00458731 | Completed | Phase I |
| Erlotinib and Temsirolimus in Treating Patients With Recurrent Malignant Glioma | Adult anaplastic astrocytoma<br>Adult anaplastic oligodendroglioma Adult diffuse astrocytoma<br>Adult giant cell glioblastoma<br>Adult glioblastoma and other 6 | Drug: Erlotinib<br>Drug: Temsirolimus | NCT00112736 | Completed | Phase I,II |
| Pazopanib in combination with lapatinib in adult patients with relapsed malignant glioma (VEG102857) | Glioma | Drug: Pazopanib<br>Drug: Lapatinib | NCT00350727 | Completed | Phase I |
| BIBF 1120 for recurrent high-grade gliomas | Glioblastoma Gliosarcoma<br>Anaplastic astrocytoma<br>Anaplastic oligodendroglioma<br>Anaplastic oligoastrocytoma | Drug: BIBF 1120 | NCT01380782 | Completed | Phase II |
| Imetelstat sodium in treating young patients with refractory or recurrent solid tumors or lymphoma | Brain and central nervous system tumors<br>Lymphoma Lymphoproliferative disorder<br>Small intestine cancer<br>Unspecified childhood solid tumor, protocol specific | Drug: Imetelstat sodium | NCT01273090 | Completed | Phase I |
| Imatinib mesylate in treating patients with gliomas | Brain and central nervous system tumors | Drug: Imatinib mesylate | NCT00039364 | Completed | Phase II |
| Imatinib mesylate in treating patients with recurrent malignant glioma or meningioma | Brain and central nervous system tumors | Drug: Imatinib mesylate | NCT00010049 | Completed | Phase I,II |
| Tumor tissue analysis in patients receiving imatinib mesylate for malignant glioma | Brain and central nervous system tumors | Drug: Imatinib mesylate | NCT00401024 | Completed | Phase I |
| Imatinib mesylate, vatalanib, and hydroxyurea in treating patients with recurrent or relapsed malignant glioma | Brain and central nervous system tumors | Drug: Hydroxyurea<br>Drug: Imatinib mesylate Drug: Vatalanib | NCT00387933 | Completed | Phase I |
| Gefitinib plus temozolomide in treating patients with malignant primary glioma | Brain and central nervous system tumors | Drug: Gefitinib<br>Drug: Temozolomide | NCT00027625 | Completed | Phase I |
| Imatinib mesylate and temozolomide in treating patients with malignant glioma | Brain and central nervous system tumors | Drug: Imatinib mesylate<br>Drug: Temozolomide | NCT00354068 | Completed | Phase I |
| Erlotinib and sirolimus in treating patients with Recurrent malignant glioma | Brain and central nervous system tumors | Drug: Erlotinib + Sirolimus | NCT00509431 | Completed | Phase I |
| SU5416 in treating patients with recurrent astrocytoma or mixed glioma that has not responded to radiation therapy | Brain and central nervous system tumors | Drug: Semaxanib | NCT00004868 | Completed | Phase I,II |

*(Continued)*

**TABLE 1** | Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Lenalidomide in combination with bevacizumab, sorafenib, temsirolimus, or 5-fluorouracil, leucovorin, oxaliplatin (FOLFOX) | Advanced cancers | Drug: Lenalidomide<br>Drug: Bevacizumab<br>Drug: Sorafenib Drug: Temsirolimus<br>Drug: Oxaliplatin<br>Drug: Leucovorin<br>Drug: 5-fluorouracil | NCT01183663 | Completed | Phase I |

*Summarized in the Table 1 are the ongoing clinical trials present on ClinicalTrials.gov searching the keywords "glioblastoma multiforme" and "kinase inhibitor" The research has been done adding the following filters: "Completed"; "The research has been performed on October 21st, 2020."*

depletion of tissue arginine levels (Uyttenhove et al., 2003; Fecci et al., 2006a,b; Wainwright et al., 2012).

Immune checkpoints exert a key role in central and peripheral tolerance by counteracting activating signaling (Xu et al., 2018). Under physiological conditions, immune checkpoint molecules represent a negative feedback to regulate inflammatory responses following T cell activation (Krummel and Allison, 1996; Chambers et al., 2001; Collins et al., 2002; Stone et al., 2009; Inarrairaegui et al., 2018). A mechanism used by tumors, including GBM, to inhibit and escape the anti-tumor immune response is represented by the expression of checkpoint molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD1 (Stone et al., 2009; Francisco et al., 2010; Cheng et al., 2013; Bhandaru and Rotte, 2017; Hui et al., 2017; Wei et al., 2017, 2018, 2019a,b; Rotte et al., 2018; Kalbasi and Ribas, 2020; Sharma and Allison, 2020).

## SMALL MOLECULES FOR TARGETED THERAPIES IN GBM

The progresses in the molecular classification of GBM have allowed the identification of dysregulated pathways that could represent potential targets for new treatment strategies (**Figure 2**).

Glioblastoma is a vascularized tumor which is histologically characterized by the expression of VEGF and other proangiogenic cytokines involved in the stimulation of endothelial cell proliferation, migration and survival (Schiffer et al., 2018). In patients with a relapsed GBM, the TKI regorafenib has received approval in the GBM treatment (Lombardi et al., 2019).

Other TKIs targeting VEGF family components have been proposed for the treatment of GBM besides regorafenib. Of note, vascular normalization has been proposed as an alternative strategy for the employment of antiangiogenic therapies in which the objective is to modulate the tumor vasculature in order to reduce hypoxia, and to support physiological angiogenesis. This process could ultimately improve perfusion and drug delivery. In this context, promising results in reducing angiogenesis and normalizing vascularization have been shown by cediranib and sutinib (Batchelor et al., 2013; Grisanti et al., 2019).

The PI3K/mammalian target of rapamycin (mTOR) pathway is a targetable pathway in GBM. In this context, the mTOR inhibitor temsirolimus did not show a treatment efficacy as single agent in recurrent GBM (Chang et al., 2005). Similarly, the pan-PI3K inhibitor buparlisib did not demonstrate a treatment efficacy (Wen et al., 2019). Also treatment combinations of mTOR pathway inhibitors with radiotherapy and TMZ or in combination with radiotherapy only did not show efficacy (Ma et al., 2015; Wick et al., 2016).

Targeting MDM2 and mouse double minute 4 homolog (MDM4) activity has been suggested for GBM cases carrying *MDM2* or *MDM4* gene amplification (Wick et al., 2019).

Moreover, the *CDK4/6* inhibitor palbociclib failed to demonstrate the efficacy of this treatment in GBM (Taylor et al., 2018). CDK9 is an alternative targetable CDK (Taylor et al., 2018).

The use of TKIs targeting EGFR as single agents did not demonstrate significant activity for GBM treatment (Lassman et al., 2005; Hegi et al., 2011). It has not yet been agreed on the potential use of MET as target for GBM treatment. The use of the TKIs crizotinib and cabozantinib in recurrent GBM has achieved modest efficacy after several attempts (International Cancer Genome Consortium PedBrain Tumor Project, 2016; Wen et al., 2018). Tests have been carried out for larotrectinib and entrectinib in neutrophic tyrosine receptor kinase (NTRK) fusion-positive GBM without any confirmation on their efficacy (Ferguson et al., 2018). Notwithstanding the frequent expression in GBM of fibroblast growth factor receptors (FGFRs), a relevance as potential therapy target seems to be restricted to GBM exhibiting FGFR-transforming acidic coiled-coil containing protein TACC fusions (Singh et al., 2012), as shown by using the pan-FGFR kinase inhibitor erdafitinib (Di Stefano et al., 2015). A modest treatment efficacy has been obtained for the possible targeting of *BRAF*V600E mutation in GBM (Kaley et al., 2018). Finally, eribulin has been proposed to inhibit TERT activity in GBM (Takahashi et al., 2019). A list of the current clinical trials employing TKIs for GBM treatment is reported in **Table 1**.

## USE OF ICIs FOR GBM TREATMENT

Following the results of ICIs use in other cancers, the use of PD-1/PD-L1 inhibitors has been proposed for GBM (**Table 2**). Clinical trial results have shown that GBM patients with unresectable tumors do not benefit from monotherapy with nivolumab in terms of survival improvement when compared

**TABLE 2 |** Clinical trials in glioblastoma (GBM) using immune checkpoint inhibitors (ICIs).

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Neoantigen-based personalized vaccine combined with immune checkpoint blockade therapy in patients with newly diagnosed, unmethylated glioblastoma | Glioblastoma | Biological: NeoVax<br>Biological: Nivolumab<br>Biological: Ipilimumab | NCT03422094 | Suspended | Phase I |
| Autologous dendritic cells, metronomic cyclophosphamide and checkpoint blockade in children with relapsed HGG | Childhood glioblastoma | Drug: depletion of regulatory T cells<br>Biological: cancer vaccine<br>Biological: checkpoint blockade | NCT03879512 | Recluting | Phase I,II |
| Cytokine microdialysis for real-time immune monitoring in glioblastoma patients undergoing checkpoint blockade | Glioblastoma | Drug: Nivolumab<br>Drug: BMS-986016 | NCT03493932 | Recluting | Phase I |
| Laser interstitial thermotherapy (LITT) combined with checkpoint inhibitor for recurrent GBM (RGBM) | Glioblastoma Adult | Drug: Pembrolizumab at 7 days prior<br>Drug: Pembrolizumab at 14 days post<br>Drug: Pembrolizumab at 35 days post | NCT03277638 | Recluting | Phase I,II |
| Pilot surgical trial to evaluate early immunologic pharmacodynamic parameters for the PD-1 checkpoint inhibitor, pembrolizumab (MK-3475), In patients with surgically accessible recurrent/progressive glioblastoma | Brain cancer | Drug: MK-3475 | NCT02852655 | Active, not recruiting | Phase I |
| A study testing the effect of immunotherapy (ipilimumab and nivolumab) in patients with recurrent glioblastoma with elevated mutational burden | Recurrent glioblastoma<br>Secondary glioblastoma | Biological: Ipilimumab<br>Biological: Nivolumab | NCT04145115 | Not yet recruiting | Phase II |
| First-in-human, phase 1b/2a trial of a multipeptide therapeutic vaccine in patients with progressive glioblastoma | Glioblastoma<br>Adult | Biological: Multiple dose of EO2401 | NCT04116658 | Not yet recruiting | Phase I,II |
| A phase 1 study of PVSRIPO and pembrolizumab in patients with recurrent glioblastoma | Glioblastoma Recurrent glioblastoma<br>Supratentorial glioblastoma<br>Brain tumor | Biological: PVSRIPO<br>Biological: Pembrolizumab | NCT04479241 | Not yet recruiting | Phase I |
| Nivolumab, BMS-986205, and radiation therapy with or without temozolomide in treating patients with newly diagnosed glioblastoma | Glioblastoma | Biological: IDO1 Inhibitor BMS-986205<br>Biological: Nivolumab<br>Drug: Temozolomide | NCT04047706 | Recluting | Phase I |
| Immunogene-modified T (IgT) cells against glioblastoma multiforme | Glioblastoma multiforme of brain<br>Glioblastoma multiforme | Biological: Antigenspecific IgT cells | NCT03170141 | Enrolling by invitation | Phase I |
| An investigational immunotherapy study of nivolumab compared to temozolomide, each given with radiation therapy, for newly diagnosed patients with glioblastoma (GBM, a malignant brain cancer) | Brain Cancer | Drug: Nivolumab Drug: Temozolomide | NCT02617589 | Active, not recruiting | Phase III |
| Translational study of nivolumab in combination with bevacizumab for recurrent glioblastoma | Recurrent adult brain tumor | Drug: Nivolumab<br>Drug: Bevacizumab | NCT03890952 | Recluting | Phase II |
| Immunological and functional characterization of cellular population CD45+ infiltrating human glioblastoma | Glioblastoma | | NCT03687099 | Recluting | Observational |

*(Continued)*

**TABLE 2 |** Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Avelumab in patients with newly diagnosed glioblastoma multiforme | Glioblastoma Multiforme of brain | Biological: Avelumab | NCT03047473 | Active, not recruiting | Phase II |
| Capecitabine + bevacizumab in patients with recurrent glioblastoma | Glioblastoma | Drug: Capecitabine Drug: Bevacizumab | NCT02669173 | Recluting | Phase I |
| VXM01 plus avelumab combination study in progressive glioblastoma | Recurrent glioblastoma | Biological: VXM01 Biological: Avelumab | NCT03750071 | Recluting | Phase I,II |
| Immunotherapy (nivolumab and ipilimumab) before and after surgery for the treatment of recurrent or progressive high grade glioma in children and young adults | Glioblastoma Malignant glioma Recurrent glioblastoma Recurrent malignant glioma Recurrent grade III Glioma Grade III GLioma | Biological: Ipilimumab Biological: Nivolumab Drug: Placebo Administratio | NCT04323046 | Not yet recruiting | Phase I |
| CART-EGFRvIII + Pembrolizumab in GBM | Glioblastoma | Biological: CARTEGFRvIII T cells Biological: Pembrolizumab | NCT03726515 | Active, not recruiting | Phase I |
| INO-5401 and INO-9012 delivered by electroporation (EP) in combination with cemiplimab (REGN2810) in newly diagnosed glioblastoma (GBM) | Glioblastoma | Biological: INO-5401 Biological: INO-9012 Biological: Cemiplimab Drug: Temozolomide | NCT03491683 | Active, not recruiting | Phase I,II |
| Combination adenovirus + pembrolizumab to trigger immune virus effects | Brain cancer Brain neoplasm Glioma Glioblastoma Gliosarcoma and other 3 | Biological: DNX-2401 Biological: Pembrolizumab | NCT02798406 | Active, not recruiting | Phase II |
| GMCI, nivolumab, and radiation therapy in treating patients with newly diagnosed high-grade gliomas | Glioma Malignant | Biological: AdV-tk Drug: Valacyclovir Drug: Temozolomide Biological: Nivolumab | NCT03576612 | Recluting | Phase I |
| Nivolumab, BMS-986205, and radiation therapy with or without temozolomide in treating patients with newly diagnosed glioblastoma | Glioblastoma | Biological: IDO1 Inhibitor BMS-986205 Biological: Nivolumab Drug: Temozolomide | NCT04047706 | Recluting | Phase I |
| Study of the IDO pathway inhibitor, indoximod, and temozolomide for pediatric patients with progressive primary malignant brain tumors | Glioblastoma Multiforme Glioma Gliosarcoma Malignant brain tumor Ependymoma and other 3 | Drug: Indoximod Drug: Temozolomide Drug: Cyclophosphamide Drug: Etoposide | NCT02502708 | Active, not recruiting | Phase I |
| A phase 0 study of AZD1775 in recurrent GBM patients | Glioblastoma | Biological: AZD1775 | NCT02207010 | | Early phase I |
| Nivolumab in people with IDH-mutant gliomas with and without hypermutator phenotype | Glioma Glioblastoma High grage glioma Low grade glioma Malignant glioma | Drug: Nivolumab | NCT03718767 | Recluting | Phase II |
| A pilot study to evaluate PBR PET in brain tumor patients treated with chemoradiation or immunotherapy | Intracranial tumors Glioblastoma Melanoma | Biological: Cancer immunotherapy Radiation: Radiation and chemotherapy | NCT02431572 | Completed | |

*(Continued)*

**TABLE 2** | Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| HSV G207 with a single radiation dose in children with recurrent high-grade glioma | Neoplasms<br>High grade glioma<br>Glioblastoma multiforme<br>Malignant glioma of brain<br>Anaplastic astrocytoma of brain and other 3 | Drug: Biological G207 | NCT04482933 | Not yet recruiting | Phase II |

*Summarized in the Table are the ongoing clinical trials present on ClinicalTrials.gov searching the keywords "glioblastoma multiforme" and "Checkpoint." The research has been done adding the following filters: "Not yet recruiting"; "Recruiting"; "Enrolling by invitation"; "Active, not recruiting"; "Suspended"; "Terminate"; "Completed"; "Withdrawn"; "Unknown status"; "The research has been performed on October 21st, 2020."*

to bevacizumab (Reiss et al., 2017). Moreover, pembrolizumab showed limited activity for GBM (Reardon et al., 2014, 2016; Schwartz et al., 2016; Reardon et al., 2017; Reiss et al., 2017; Wen et al., 2018).

Recent tests have been carried out involving patients with newly diagnosed or relapsed GBMs for the use of ICIs (e.g., nivolumab or pembrolizumab) in neoadjuvant and/or adjuvant administration, although no straightforward results have been obtained (Cloughesy et al., 2019; Schalper et al., 2019).

Glioblastoma tumors of cases non-responsive to ICIs have shown an enrichment in mutations of the *PTEN* gene (Zhao et al., 2019) that has been associated with an immunosuppressive TME characterized by the presence of GBM cells expressing CD44. *PTEN* mutant tumors were characterized by highly clustering tumor cells with a lack of T cell infiltration (Peng et al., 2016; George et al., 2017). Furthermore, the poor responsiveness to ICIs of GBM cases carrying *PTEN* mutations has been related to a low PD-L1 expression for the involvement of the PI3K-mTOR pathway that is downstream to PTEN (Lastwika et al., 2016).

Responsiveness to ICI was associated with the presence of mutations of *BRAF*/protein tyrosine phosphatase non-receptor type 11 (*PTPN11*). In this subset of *BRAF*/*PTPN11* GBM patients, treatment combinations of ICIs and MAPK inhibitors could be introduced (Toso et al., 2014; Toso et al., 2014; Ebert et al., 2016; Wang et al., 2016).

The heterogeneous response rate to ICIs highlights the need of identifying the subgroups of patients who could benefit the most from the use of this immunotherapy treatment. PD-L1 expression was the first marker evaluated as predictor of a clinical response to ICIs (Ansell et al., 2015). PD-L1 expression in gliomas was associated with *IDH* status (Berghoff and Preusser, 2016; Garber et al., 2016; Berghoff et al., 2017). Importantly, mesenchymal GBM has been found having high levels of PD-L1 expression that may suggest that the expression of immune checkpoint proteins and aggressiveness of GBM tumors may be correlated (Garber et al., 2016). More recently, the tumor mutational burden has been proposed as a predictive marker of responsiveness to ICIs. However, it has not generally been demonstrated that the tumor mutational burden is capable of sufficiently predicting long term clinical benefits (Champiat et al., 2014; Rizvi et al., 2015; Schumacher et al., 2015; Le et al., 2017). Moreover, recent studies have shown that higher somatic mutation and neoepitope loads have not been found in GBM cases responsive to ICIs (Zhao et al., 2019). The infiltration of mutation-reactive class I and class II T cells into the tumor seems not to be precluded by a low mutational load in GBM (Cloughesy et al., 2019; Schalper et al., 2019; Zhao et al., 2019). The presence of alterations in the *MMR* genes is another proposed biomarker (Cloughesy et al., 2019; Schalper et al., 2019). The expression of MHC class I molecules has been associated to responsiveness to ICIs since it is involved in the presentation of antigens and characterized by highly heterogeneous expression levels in GBM (Indraccolo et al., 2019).

## DISCUSSION

Surgery followed by radiotherapy and chemotherapy with alkylating agents constitutes the standard first-line treatment of GBM (Stupp et al., 2005; Canoll and Goldman, 2008; Levine et al., 2015). Complete resection of the GBM tumors is generally not possible given its high invasive features. Although this combination therapy can prolong survival, the prognosis is still poor due to several factors including chemoresistance. Multiple mechanisms appear to be involved in the development of drug resistance in GBM including overexpression of drug efflux transporter pumps such as p-glycoprotein, the presence of a GSC population, a relevant activity of DNA repair mechanisms and dysregulated apotosis processes such as MGMT, the MMR pathway, the base excision repair (BER) pathway and the TP53 pathway (Walker et al., 1992; Bobola et al., 1996; Qian and Brent, 1997; Jaeckle et al., 1998; Chen et al., 1999; Esteller et al., 2000; Middlemas et al., 2000; Paz et al., 2004; Hegi et al., 2005; Helleday et al., 2005; Bryant and Helleday, 2006; Zawlik et al., 2009; van Nifterik et al., 2010; Malmstrom et al., 2012; Reifenberger et al., 2012; Armstrong et al., 2013; Brennan et al., 2013; Wiestler et al., 2013; Wick et al., 2014, 2018; Erasimus et al., 2016; Peng et al., 2016; Sun et al., 2018; Gupta et al., 2018; Zhang et al., 2018; Christmann and Kaina, 2019; Hafner et al., 2019; Mantovani et al., 2019). Tumor/TME interactions also contribute to the development of drug resistance in GBM tumor cells (Hanahan and Weinberg, 2011; Ab and Jn, 2012; Rodriguez-Hernandez et al., 2014; Munoz et al., 2015).

Systemic delivery uses existing vessels to deliver anti-tumor drugs to the tumor. To overcome the impediment of the

**TABLE 3 |** Clinical trials in glioblastoma (GBM) using chimeric antigen receptor-T (CAR-T).

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Pilot study of autologous anti-EGFRvIII CAR-T cells in recurrent glioblastoma multiforme | Glioblastoma multiforme | Biological: anti-EGFRvIII CAR-T cells drug: cyclophosphamide Drug: Fludarabine | NCT02844062 | | Phase I |
| Pilot study of B7-H3 CAR-T in treating patients with recurrent and refractory glioblastoma | Recurrent glioblastoma Refractory glioblastoma | Drug: B7-H3 CAR-T Drug: Temozolomide | NCT04385173 | Recruiting | Phase I |
| B7-H3 CAR-T for recurrent or refractory glioblastoma | Recurrent glioblastoma Refractory glioblastoma | Drug: Temozolomide Biological: B7-H3 CAR-T | NCT04077866 | Not yet recruiting | Phase I, II |
| CD147-CAR-T cells in patients with recurrent malignant glioma | Recurrent glioblastoma CD147 positive | Biological: CD147-CAR-T | NCT04045847 | Active, not recruiting | Early phase I |
| CART-EGFRvIII + pembrolizumab in GBM | Glioblastoma | Biological: CART-EGFRvIII T cells Biological: Pembrolizumab | NCT03726515 | Active, not recruiting | Phase I |
| EGFRvIII CAR-T cells for newly diagnosed WHO grade IV malignant glioma | Glioblastoma Gliosarcoma | Biological: EGFRvIII CAR-T cells | NCT02664363 | Terminated | Phase I |
| Chimeric antigen receptor (CAR- T) cells with a chlorotoxin tumor-targeting domain for the treatment of recurrent or progressive glioblastoma | Recurrent glioblastoma Recurrent malignant glioma recurrent WHO grade II glioma recurrent WHO grade III glioma | Biological: Chlorotoxin (EQ)-CD28-CD3zeta-CD19t-expressing CAR-TTlymphocytes NCI SYs | NCT04214392 | Recluting | Phase I |
| IL13Ralpha2-targeted chimeric antigen receptor (CAR-T) T cells with or without nivolumab and ipilimumab in treating patients with recurrent or refractory glioblastoma | Recurrent glioblastoma Refractory glioblastoma | Biological: IL13Ralpha2-specific Hinge-optimized 4-1BB-co-stimulatory CAR/Truncated CD19-expressing autologous TN/MEM Cells Biological: Ipilimumab Biological: Nivolumab | NCT04003649 | Recluting | Phase I |
| Autologous T cells redirected to EGFRVIII-with a chimeric antigen receptor in patients with EGFRVIII+ glioblastoma | Patients with residual or reccurent EGFRvIII+ glioma | Biological: CART-EGFRvIII T cells | NCT02209376 | Terminated Result | Phase I |
| NKG2D-based CAR-T-cells Immunotherapy for patient with r/r NKG2DL+ solid tumors | Hepatocellular carcinoma Glioblastoma Medulloblastoma Colon cancer | Biological: NKG2D-based CAR-T-cells | NCT04270461 | Not yet recruiting | Phase I |
| Pilot study of autologous chimeric switch receptor modified T Cells in recurrent glioblastoma multiforme | Glioblastoma multiforme | Biological: Anti-PD-L1 CSR T cells Drug: Cyclophosphamide Drug: Fludarabine | NCT02937844 | | Phase I |
| Intracerebral EGFR-vIII CAR-T cells for recurrent GBM | Recurrent glioblastoma Recurrent gliosarcoma | Biological: EGFRvIII-CARs | NCT03283631 | Recluting | Phase I |
| Combination of immunization and radiotherapy for malignant gliomas (*In situ* Vac1) | High grade glioma Glioblastoma Glioma of brainstem Glioma Malignant | Combination Product: Combined immune adjuvants and radiation | NCT03392545 | Recluting | Phase I |
| CAR-T cell receptor immunotherapy targeting EGFRvIII for patients with malignant gliomas expressing EGFRvIII | Malignant glioma Glioblastoma Brain cancer Gliosarcoma | Biological: Epidermal growth factor receptor (EGFRv)III Chimeric antigen receptor (CAR) transduced PBL Drug: Aldesleukin Drug: Fludarabine Drug: Cyclophosphamide | NCT01454596 | Completed | Phase I, II |
| Immunogene-modified T (IgT) cells against glioblastoma multiforme | Glioblastoma multiforme of brain glioblastoma multiforme | Biological: Antigen-specific IgT cells | NCT03170141 | Enrolling by invitation | Phase I |
| CMV-specific cytotoxic T lymphocytes expressing CAR-T targeting HER2 in patients with GBM (HERT-GBM) | Glioblastoma multiforme | Biological: HER.CAR-TCMV-specific CTLs | NCT01109095 | Completed | Phase I |
| Genetically modified T-cells in treating patients with recurrent or refractory malignant glioma | Malignant glioma Refractory brain neoplasm Recurrent brain neoplasm Glioblastoma | Biological: IL13Rα2-specific, hinge-optimized, 41BB-costimulatory CAR/truncated CD19-expressing Autologous T lymphocytes Biological: Vaccine Therapy | NCT02208362 | Recluting | Phase I |

*(Continued)*

**TABLE 3 |** Continued

| Title | Condition or Disease | Intervention/Treatment | NCT Number | Status | Status Phase |
|---|---|---|---|---|---|
| Memory-enriched T cells in treating patients with recurrent or refractory grade III-IV glioma | Glioblastoma HER2/Neu positive Malignant glioma Recurrent glioma Refractory glioma WHO grade III glioma | Biological: CD19CAR-CD28-CD3zeta-EGFRt-expressing Tcm-enriched T-lymphocytes Biological: CD19CAR-CD28-CD3zeta-EGFRt-expressing Tn/mem-enriched T-lymphocytes | NCT03389230 | Recluting | Phase I |

*Summarized in the Table are the ongoing clinical trials present on ClinicalTrials.gov searching the keywords "glioblastoma multiforme" and "CAR-T." The research has been done adding the following filters: "Not yet recruiting"; "Recruiting"; "Enrolling by invitation"; "Active, not recruiting"; "Suspended"; "Terminate"; "Completed"; "Withdrawn"; "Unknown status"; "The research has been performed on October 21st, 2020."*

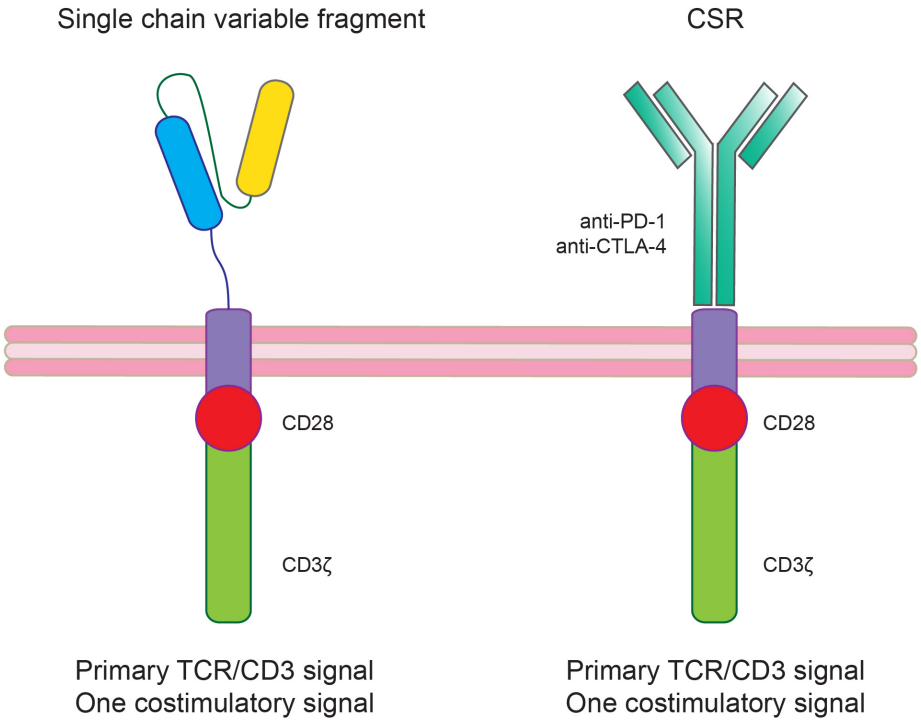


**FIGURE 3 |** Modified chimeric antigen receptor-T (CAR-T) cells to ameliorate treatment efficacy by counteracting the immunosuppressive glioblastoma (GBM) tumor microenvironment (TME). The co-expression of an activating chimeric switch receptor (CSR), that combines the extracellular ligand-binding domain of an inhibitory receptor (PD-1 or CTLA-4) fused through a transmembrane domain with the cytoplasmic co-stimulatory signaling domain of CD28, could improve CAR-T cell efficacy in GBM.

BBB several strategies have been proposed including chemical modification of the drugs, high dose chemotherapy capable of inducing a transient BBB disruption, nanoparticle-based drug delivery and peptide-based drug delivery. Nevertheless, no straightforward results have still been reached (Siegal, 2013).

Glioblastoma stem cell cell population has been shown to induce a certain degree of radio- chemoresistance given their high expression of anti-apoptotic proteins, ATP-binding cassette pumps, their increased capability of DNA damage repair, as well as their high capacity of migration and invasion (Bao et al., 2006; Calabrese et al., 2007; Eyler and Rich, 2008; Diehn et al., 2009; Pietras et al., 2014). GSCs have been found capable of secreting angiogenic factors which in turn are responsible for an enhancement in the formation of tumor blood vessels, this has been frequently associated with high tumor aggressiveness. Moreover, the TME cell components can promote GSC survival by VEGF secretion (Wada et al., 2006). The interaction of TME with GSCs can facilitate tumor progression and consequently therapeutic resistance (Chen and Liu, 2012; Miura et al., 2013).

Over the past 10 years, the knowledge regarding genomic features of GBM has been greatly increased by comprehensive multiplatform genome-wide analyses. As a result of these analyses, it has emerged that GBM comprises a group of highly heterogeneous tumor types, each with peculiar molecular/genetic features (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016).

In GBM the phase I/II and III trials investigating the use of therapies molecularly targeting oncogenic alterations did not generally show straightforward results and, consequently, their

clinical utilization is still limited. However, although limited activity or no therapeutic efficacy has so far been produced by the use of TKIs, improvement in understanding the mechanisms of action of these compounds could help to determine how to better incorporate their use in the existing treatment modalities. Redundancies are frequently present in the molecular pathways that can be targeted which makes the inhibition of any pathway largely ineffective (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016). The failure of targeted therapies can also be ascribed to another possible reason such as the fact that several genomic alterations are important only for the initial stages of tumor progression whereas other molecular mechanisms outweigh their role in the later stages. On the other hand, several genomic alterations in GBM can interfere with GBM cell metabolism. In particular, alterations in the growth factor signaling pathways that can control metabolic flux have been found in high frequency as well as recurrent mutations in *IDH1* and *IDH2* genes, whose encoded proteins are part of the tricarboxylic acid (TCA) cycle. Alterations of the cellular metabolism, which is controlled also by the biochemical microenvironment, could contribute to the failure of the proposed targeted therapies (Tcga, 2008; Brennan et al., 2013; Wang et al., 2016). A better understanding of the interactions constituting this interplay between altered genome and biochemical microenvironment could contribute to finding more effective treatment strategies in the reverting of altered cellular metabolism of GBM cells.

The TME of GBM is largely immunosuppressive, therefore efficiency of ICI treatments can be strongly affected by this condition (Akiyama et al., 2001; Brat and Van Meir, 2004; Brat et al., 2004; Nimsky et al., 2005; Zimmermann and Dours-Zimmermann, 2008; Persano et al., 2011; Sherriff et al., 2013; Dicker et al., 2014; Hambardzumyan and Bergers, 2015; Young et al., 2015; Chen and Hambardzumyan, 2018). GBM patients frequently present reduced levels of circulating $CD4^+$ and $CD8^+$ lymphocytes as a consequence of chemotherapy treatments (Gustafson et al., 2010; Mirzaei et al., 2017). A clear molecular/immunological signature that can be predictive of response to ICI treatments has not yet been identified (Motzer et al., 2015; Goldberg et al., 2016; Reck et al., 2016; Schwartz et al., 2016; Reiss et al., 2017; Reardon et al., 2018; Cloughesy et al., 2019; Schalper et al., 2019).

The treatment of different cancers has markedly been revolutionized by immunotherapy. Nevertheless, the data obtained so far concerning the use of ICIs for the treatment of GBM patients seem to be not sufficient to propose this type of immunotherapy as a standard treatment for GBM (Reardon et al., 2014, 2016; Motzer et al., 2015; Goldberg et al., 2016; Reck et al., 2016; Schwartz et al., 2016; Reardon et al., 2017; Reiss et al., 2017; Wen et al., 2018; Cloughesy et al., 2019; Schalper et al., 2019).

Another immunotherapy approach that can be used also in combinations with ICIs is the chimeric antigen receptor-T (CAR-T) cell therapy targeting specific tumor associated antigens. The introduction of CAR-T cell therapy approaches also in solid tumors including GBM has been favored by the success of this therapy in hematological malignancies (Neelapu et al., 2017; Maude et al., 2018). Concerning GBM treatment, several clinical trials have been proposed showing that there are still substantial obstacles including TME immune suppression (**Table 3**; Morgan et al., 2010; Brown et al., 2015, 2016; Zah et al., 2016; Ahmed et al., 2017; Walseng et al., 2017; Richman et al., 2018). To increase CAR-T treatment efficacy several CAR-T modifications have been proposed such as the knocking out of genes encoding T cell inhibitory receptors or signaling molecules (e.g., PD-1 or CTLA-4) or the co-expression of activating chimeric switch receptor (CSR; **Figure 3**; Prosser et al., 2012; Shin et al., 2012; Ankri et al., 2013; Kobold et al., 2015; Liu et al., 2016).

Understanding the molecular and immunological complexity of GBM more and more could provide the grounds for the introduction of other immunotherapeutic approaches such as the use of CAR-T cell therapy, in combination with ICIs or TKIs, in the treatment paradigm of GBM.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

Supported by Progetto Giovani Ricercatori n. GR-2016-02364678, Ministero della Salute, Rome, Italy.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 30 November 2020
doi: 10.3389/fnins.2020.598266

# Perampanel Add-on to Standard Radiochemotherapy *in vivo* Promotes Neuroprotection in a Rodent F98 Glioma Model

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**Specialty section:**
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

**Received:** 24 August 2020
**Accepted:** 10 November 2020
**Published:** 30 November 2020

**Citation:**
Lange F, Hartung J, Liebelt C, Boisserée J, Resch T, Porath K, Hörnschemeyer J, Reichart G, Sellmann T, Neubert V, Kriesen S, Hildebrandt G, Schültke E, Köhling R and Kirschstein T (2020) Perampanel Add-on to Standard Radiochemotherapy in vivo Promotes Neuroprotection in a Rodent F98 Glioma Model. Front. Neurosci. 14:598266. doi: 10.3389/fnins.2020.598266

An abnormal glutamate signaling of glioblastoma may contribute to both tumor progression and the generation of glioma-associated epileptic seizures. We hypothesized that the AMPA receptor antagonist perampanel (PER) could attenuate tumor growth and epileptic events. F98 glioma cells, grown orthotopically in Fischer rats, were employed as a model of glioma to investigate the therapeutic efficiency of PER (15 mg/kg) as adjuvant to standard radiochemotherapy (RCT). The epileptiform phenotype was investigated by video-EEG analysis and field potential recordings. Effects on glioma progression were estimated by tumor size quantification, survival analysis and immunohistological staining. Our data revealed that orthotopically-growing F98 glioma promote an epileptiform phenotype in rats. RCT reduced the tumor size and prolonged the survival of the animals. The adjuvant administration of PER had no effect on tumor progression. The tumor-associated epileptic events were abolished by PER application or RCT respectively, to initial baseline levels. Remarkably, PER preserved the glutamatergic network activity on healthy peritumoral tissue in RCT-treated animals. F98 tumors are not only a robust model to investigate glioma progression, but also a viable model to simulate a glioma-associated epileptiform phenotype. Furthermore, our data indicate that PER acts as a potent anticonvulsant and may protect the tumor-surrounding tissue as adjuvant to RCT, but failed to attenuate tumor growth or promote animal survival.

**Keywords: epilepsy, glioblastoma, glioma, perampanel, radiochemotherapy, glutamate, glutamate receptors**

## INTRODUCTION

Glioblastoma (WHO grade IV) is a devastating disease with a median survival of 15 months (Delgado-López and Corrales-García, 2016) and severely reduced quality of life (QOL) due to cognitive decline and neurological deficits. Moreover, symptomatic epilepsy is frequently reported in patients with primary brain tumors, and in many cases an epileptic seizure is the initial symptom

of malignant brain tumors (van Breemen et al., 2007). Low-grade gliomas (WHO grade I-II) exhibit a seizure prevalence ranging from 70 to 90%. In high-grade gliomas (WHO grade III-IV), up to 62% of the patients suffer from tumor-associated epilepsy (Kerkhof et al., 2013). Different mechanisms have been proposed to play a role in the generation of glioma-induced seizures (Huberfeld and Vecht, 2016). One major pathological mechanism is an altered glutamate signaling of glioma cells and their microenvironment. Our current understanding involves the glioma-expressed branched-chain amino acid transaminase 1 (BCAT1) that transfers α-amino groups from branched-chain amino acids to α-ketoglutarate, thereby producing glutamate and the respective branched-chain α-ketoacid (Tönjes et al., 2013). Intracellular glutamate, in turn, is exchanged for cystine via the xCT antiporter, which is often over-expressed in glioma tissue (Ye et al., 1999; Chung et al., 2005; Savaskan et al., 2008). This situation may be exacerbated by the downregulation or mislocalization of EAAT2 (excitatory amino acid transporter 2) which clears the neurotransmitter from the extracellular space (Ye et al., 1999; de Groot et al., 2005). It is assumed that these mechanisms contribute substantially to an elevated peritumoral glutamate level up to 100-times compared to unaffected brain tissue (Roslin et al., 2003; Marcus et al., 2010). High glutamate levels may promote proliferation and migration of glioma cells in an autocrine manner (Ishiuchi et al., 2007; Lyons et al., 2007) and also cause hyperexcitation of the surrounding neuronal tissue that eventually results in $Ca^{2+}$-induced excitotoxicity of neurons (Noch and Khalili, 2009). Recently, this glioma model of glutamate interaction was expanded by the description of neurogliomal synapses (Venkataramani et al., 2019; Venkatesh et al., 2019). Synaptic transmission is based on glutamate and postsynaptic α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptor expression, with the subunit GluA2 (GluR2) crucially involved. The authors demonstrated that glutamatergic signaling via integration of glioma cells into neuronal circuits promotes tumor cell growth and invasiveness.

Based on those pathological findings, anticonvulsants addressing tumor-associated seizures and, at the same time, interfering with glioma progression could be promising therapeutic candidates. Previous data suggested that the non-competitive AMPA receptor antagonist perampanel (PER) (Hanada et al., 2011) had antitumoral effects *in vitro* – unlike levetiracetam, valproate or carbamazepine (Lange et al., 2019), and attenuated patient-derived xenograft tumor growth in mice (Venkataramani et al., 2019). Furthermore, we have shown that systemic monotherapy of low-dose PER inhibited epileptiform discharges in organotypic brain slices of glioma (Mayer et al., 2019). Intriguingly, PER has been ascribed a neuroprotective effect in neurodegeneration occurring after pilocarpine-induced status epilepticus (Wu et al., 2017) and ischemia (Nakajima et al., 2018; Mazzocchetti et al., 2020).

Therefore, we established a rodent glioma model in order to evaluate the neuroprotective potential of PER as adjuvant treatment to standard radiochemotherapy (RCT). We demonstrated that orthotopic implantation of F98 glioma cells into Fischer 344 rats produced a valuable model to mimic orthotopic glioma progression in the rat neocortex with reduced survival and glioma-associated epilepsy. Our most important finding was that combined RCT and PER preserved physiological synaptic activity in the peritumoral tissue, while both RCT and PER alone were ineffective in this respect. These data suggest that PER promotes neuroprotection by reducing putatively detrimental glutamatergic effects within the peritumoral microenvironment.

# MATERIALS AND METHODS

## Cell Culture

The rat F98 glioma cell line was obtained from the American Type Culture Collection (ATCC). F98 cells were cultured in Dulbecco's modified eagle medium (DMEM)/F-12 (Merck, Darmstadt, Germany), supplemented with 10% fetal calf serum (FCS, Merck), and grown at 37°C in a 5% $CO_2$ humidified atmosphere. In regular intervals, cell culture supernatants were tested for mycoplasma contamination employing MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

## Animal Tumor Model and Stereotactic Glioma Implantations

F98 cells were implanted unilaterally into the sensomotor neocortex of Fischer 344 rats (Charles River, Sulzfeld, Germany) via stereotactic surgery to imitate human brain cancer (Mathieu et al., 2007). The experimental protocol of Mathieu et al. (2007) was modified with respect to the number of injected glioma cells, implantation volume, and position to fit with own studies in Wistar rats (Mayer et al., 2019). All procedures were conducted according to national and international guidelines on the ethical use of animals (European Council Directive 86/609/EEC, approval of local authority LALLF M-V/TSD/7221.3-1-020/20). All efforts were made to minimize animal suffering and to reduce the number of animals used. The animals were housed under environmentally controlled conditions (12 h light/dark cycles, lights switched on from 6 a.m. to 6 p.m., and 40–60% relative humidity). For the exploratory study a total of 105 Fischer 344 rats were included.

For the stereotactic glioma implantation, Fischer 344 rats (9–12 weeks old) were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and the head was fixed in a stereotactic frame (Narishige, Tokyo, Japan). Following a scalp incision, the skull was freed from extracranial muscles and a hole of 0.7 mm diameter was manually drilled into the skull in left parasagittal position (relative to bregma: 1.8 mm posterior, 2.5 mm left, 2 mm deep; positions are illustrated in **Supplementary Figure 1A**). Trypsinized F98 cells from a subconfluently growing culture were prepared for injection with a concentration of $10^4$ cells/μl phosphate-buffered saline (PBS). The glioma cell suspension was injected at a rate of 1 μl every 2 min (total of 10 μl, equivalent to $10^5$ cells) using a Hamilton syringe (Model 701 N SYR; Hamilton, Reno, NV, United States). After completing the injection, the drill hole was covered with Heliobond®, and the scalp was sutured. Sham-operated animals

underwent the same procedure with 10 µl PBS instead of the cell suspension.

One week after glioma cell injection, Fischer 344 rats were randomly divided into four groups and treatment with PER only, RCT only or combined RCT/PER was started (see **Supplementary Figure 1B** for overview of experimental treatment protocol). Regardless of RCT or not, PER (15 mg/kg bw/day; Eisai Inc., Tokyo, Japan; formulated 1:1 in DMSO: PEG300) was delivered at a rate of 10 µl/h via a subcutaneously implanted, pre-loaded mini-osmotic pumps (Model 2ML1, Alzet, Cupertino, CA, United States). Sham-operated and F98-bearing cohorts w/o therapy received mini-osmotic pumps loaded with the vehicle only. Temozolomide (30 mg/kg bw/day; Selleck Chemicals, Houston, TX, United States) was administrated via intraperitoneal injections in a daily routine for five consecutive days (days 7–11 post-surgery, **Figure 4A**). On the same days, irradiation (5 × 4 Gy, a total of 20 Gy) of the whole brain (**Supplementary Figures 1C–E**) was performed in low-dose anesthesia with ketamine (20 mg/kg i.p.) and xylazine (2 mg/kg i.p.).

## Analysis of Video-EEG Recordings

Ten F98 glioma-bearing Fischer 344 rats were monitored by video-EEG (4 untreated, 2 RCT-treated, 2 Per-treated, and 2 RCT/PER-treated). To this end, these animals additionally received single-channel bipolar EEG recording during glioma implantation as previously described (Bajorat et al., 2011). Both electrodes were placed epidurally above the cortex with respect to the manufacturer's instructions (electrode 1: 7.0 mm posterior to bregma, 1.5 mm left to sagittal suture; electrode 2: 2.0 mm anterior to bregma, 1.5 mm right to sagittal suture; **Supplementary Figure 1A**). Continuous 24/7 video-EEG data (sample rate 500 Hz, low-pass filter 30 Hz) were recorded employing a telemetric system (ETA-F20; Dataquest A.R.T.4.2., Data Sciences International, St. Paul, MN, United States) in combination with a light/dark network camera equipped with an infrared filter (Axis 223M; Axis Communications, Lund, Sweden). At nighttime, a small lamp over each cage improved video quality. Epileptiform potentials and seizures were analyzed manually by screening the video-EEG.

The video-EEG registration was maintained throughout the remaining lifetime of the animals. According to pre-defined humane endpoints, animals were sacrificed prior to reaching a moribund stage. While we aimed to investigate epileptiform potentials and epileptic seizures during the course of the tumor disease with or without therapy, we observed some variation in disease progression leading to variable survival. In order to compare similar clinical states, we defined a pre-final period as the time prior to reaching the humane endpoints from day 9 to 2 (relative to sacrifice). To rule out epileptiform activity due to the surgical procedure and/or anesthesia, we analyzed the EEG 12 h post-surgery for 12 consecutive hours.

## Neocortical Slice Preparations

For electrophysiological recordings, Fischer 344 rats were deeply anesthetized by diethyl ether inhalation (Mallinckrodt Baker, Deventer, Netherlands) and decapitated. The brain was quickly removed and transferred into chilled and oxygenated (95% $O_2$/5% $CO_2$) dissection solution containing (in mmol/l) 87 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 $NaH_2PO_4$, 0.5 $CaCl_2$, 7 $MgCl_2$, 10 D-glucose and 75 sucrose adjusted to pH 7.4 with an osmolarity of 326–328 mosmol/l $H_2O$. Next, the cerebellum was removed and the brain was sectioned (400 µm coronal slices) using a vibratome (Integraslice 7550 MM, Campden Instruments Ltd., United Kingdom) in chilled and oxygenated artificial cerebrospinal fluid (aCSF), comprised of (in mmol/l) 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 $NaH_2PO_4$, 2.5 $CaCl_2$, 1.5 $MgCl_2$, and 10 D-glucose adjusted to pH 7.4 with an osmolarity of 304–312 mosmol/l $H_2O$. After preparation, slices were transferred into a submerged-type storage chamber for maintenance with oxygenated aCSF and kept for 1.5 h of equilibrium, before starting recordings.

## Field Potential Recordings

For electrophysiological recordings, slices were transferred into an interface chamber (BSC-HT, Harvard Apparatus, Holliston, MA, United States) maintained at 32°C (TC-10, npi electronic GmbH, Tamm, Germany) and superfused with aCSF (perfusion rate of 2–3 ml/min). Under visual control electrodes were placed 500–1,000 µm from glioma above neocortical layers II/III and field potentials were recorded from with conventional aCSF-filled glass micropipette electrodes (Ag/AgCl with a resistance of approx. 2–5 MΩ). The analog recording data were amplified, filtered at 1 kHz by an EXT-10-2F (npi electronic GmbH), and digitized using a Micro1401 analog-to-digital converter (Cambridge Electronic Design, Cambridge, United Kingdom) run by the Signal 2.16 software (Cambridge Electronic Design). To evoke spontaneous physiological network activity in the brain tissue, the slices were exposed to three different aCSF solutions: (i) aCSF with 0 mM $MgCl_2$ and addition of 5 µM gabazine (Tocris, Bristol, United Kingdom), (ii) aCSF with 8 mM KCl, 0 mM $MgCl_2$ and 5 µM gabazine, (iii) aCSF with 0 mM $MgCl_2$, 5 µM gabazine, and 50 µM 4-aminopyridine (4-AP; Tocris). Spontaneous physiological activity was defined as deflections with an amplitude at least twice the background potential and was observed under all conditions [(i): 20.3 ± 3.1 $min^{-1}$, (ii): 37.9 ± 5.3 $min^{-1}$, and (iii): 11.8 ± 1.2 $min^{-1}$]. We chose protocol (i), since this evoked a medium incidence of spontaneous physiological activity; results of the other two protocols are shown in **Supplementary Figure 2**. To analyze *ex vivo* effects of PER on glutamatergic network activity, the anticonvulsant alone or in combination with D-AP5 [D-(-)-2-Amino-5-phosphonopentanoic acid; Tocris] were added to the aCSF solution.

## Tumor Size Quantification and Immunohistological Analysis

For histological analysis, F98 glioma-bearing brains of Fischer 344 rats were fixed in 3.7% paraformaldehyde phosphate buffer overnight, then cryo-protected with 30% sucrose in PBS overnight and frozen. For the quantification of tumor volume, brains were cut into 30-µm slices. The high expression of NeuN was used to distinguish between tumor cells from

the surrounding tissue (Wolf et al., 1996). To this purpose, NeuN expression was detected employing an anti-NeuN primary antibody (Abcam, Ab104225, Cambridge, United Kingdom) and an anti-Rabbit IgG (H + L) Cross-Adsorbed, Cyanine5 (Thermo Fisher Scientific, Karlsruhe, Germany) as the secondary antibody. Afterward, the slices were counterstained and mounted with ProLong Gold Antifade Reagent containing 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Darmstadt, Germany). Fluorescence analysis was performed by using a laser-scanning microscope (Leica DMI 6000, Wetzlar, Germany) and Leica Application Suite (v. 2.0.0.13332) software. After detection of F98 cells in frontal slices, the tumor area of every 500 μm was estimated and tumor volume was quantified as described before (Mayer et al., 2019). Routine hematoxylin and eosin staining for determination of tumor establishment was performed on 30-μm sections using standard procedures.

Additionally, the expression of the AMPA receptor subunit GluA2 in the tumor area, the peritumoral tissue and the contralateral hemisphere was determined using anti-GluA2 antibody (Alomone labs; AGC-005; Jerusalem, Israel) as the primary antibody and anti-rabbit Cyanine5 as the secondary antibody. For each animal in 1–3 slices (depending on the size of the tumor) GluA2 expression was determined. GluA2 data are presented as relative immunofluorescence (IF): $IF_{ROI}/IF_{contralateral}$. This ratio was normalized to $IF_{peritumoral}/IF_{contralateral}$ of the mean of untreated animals.

## Statistical Analysis

Statistical analysis was performed with SigmaPlot 13.0. Experimental results are illustrated in box plots or given as mean ± standard error of the mean (SEM) for the indicated number of experiments. Mean group differences were tested using non-parametric analysis of variance employing the Kruskal–Wallis test followed by *post hoc* Dunn's test for multiple comparisons and Mann–Whitney *U* test for single comparison of two groups. For the analysis of spike load a two-way ANOVA followed by Bonferroni *t*-test was used. A significance level of $p < 0.05$ was considered to be statistically significant.

# RESULTS

## Orthotopic F98 Glioma Is a Valuable Model for the Human Disease

First, we established a rat glioma model that shows both orthotopic glioma progression and tumor-associated symptomatic epilepsy. While *in vivo* implantation of F98 cells is a well-known glioma model (Kirschstein and Köhling, 2016), the epileptic phenotype has not yet been addressed. In prolonged video-EEG recordings, we could show that both epileptiform EEG potentials and motor seizures occurred during the pre-final clinical stage [from day 9 to 2 (relative to sacrifice); **Figure 1A** and **Supplementary Figure 3**]. Seizure frequency varied from 1–10 per day (**Figure 1B**), and the interictal spike load was also variable among the animals tested (**Figure 1C**). Obviously, spike load and seizure rates were poorly related in untreated animals. Intriguingly, there was a heterogeneous distribution of spikes between the light phase and the dark phase during the day. All analyzed animals showed a significantly higher spike load during the dark phase than during the light phase ($p < 0.05$, *U*-test; **Figure 1C**). Hence, F98 glioma-bearing animals develop symptomatic epilepsy.

A further prerequisite for a brain tumor model is reduced lifespan and limited response to standard tumor therapy. We tested the effect of standard RCT consisting of temozolomide (TMZ, 30 mg/kg) and radiotherapy (RT, 5 Gy) on five consecutive days (cumulative doses 150 mg/kg TMZ and 20 Gy, for treatment protocol see **Supplementary Figure 1**). As shown in **Figure 2A**, animals reached pre-defined humane endpoints 16.2 ± 0.8 days after glioma implantation, hence showed a drastically reduced survival. This finding is in line with previous published survival data (Mathieu et al., 2007). Although of little clinical significance, we treated three rats exclusively with PER (mean survival 20.3 ± 3.2 days), with no significant effects on survival in comparison to untreated animals. In marked contrast, combined whole-brain RT together with TMZ for five days significantly prolonged the survival to 30.8 ± 5.0 days ($p < 0.05$, one-way ANOVA on ranks followed by Dunn's test). Adding PER (15 mg/kg) to this standard RCT could not prolong the survival (28.5 ± 2.4 days), but significantly reduced the inter-individual variance in our cohort ($p < 0.05$, *F*-test, **Figure 2A**). These findings demonstrate that PER for up to 50 days was well tolerated in F98 glioma-bearing animals showing a limited, but significant response to standard tumor therapy such as RCT.

Next, we asked whether tumor therapy affects the epileptic phenotype in our glioma model. To this end, we included a further glioma-bearing, but untreated animal in our EEG analysis to validate our previous results. Two animals received RCT alone or PER respectively, and two rats were given a combined therapy of RCT and PER. Since seizure rates differed substantially among the animals, we analyzed the spike load in the F98 model expressed as the ratio of spikes/h during the pre-final period relative to baseline values – as defined 12 h after tumor implantation (**Figure 2B**). These analyses revealed a reduction from 14.1 ± 4.1 in untreated animals to 0.8 ± 0.1 spikes/h in PER-treated animals which represents an attenuation down to 5.7% ($p < 0.05$, one-way ANOVA on ranks followed by Dunn's test; **Figure 2B**). This anticonvulsive effect is in line with experiences from pilot studies of human glioma-associated epilepsy.

In RCT-treated rats spike load was reduced to 0.2 ± 0.1 spikes/h (1.4% of untreated animals; $p < 0.05$, one-way ANOVA on ranks followed by Dunn's test; **Figure 2B**). The number of spikes during the pre-final stage was drastically reduced compared to post-surgery baseline (indicated by gray lines; **Figure 2C**). These findings indicate that RCT given in the second week after tumor implantation persistently suppressed the epileptic phenotype in later stages. It is important to note that this is in agreement with retrospective studies in humans also showing an anticonvulsive effect by TMZ and RT (Rudà et al., 2013; Koekkoek et al., 2015, 2016). Combined RCT-PER treatment also prevented the increase of spike load (1.2 ± 0.3; $p < 0.05$ versus untreated animals, one-way ANOVA on ranks followed by Dunn's test; **Figures 2B,C**). The time courses of all four groups for the last 12-h interval are

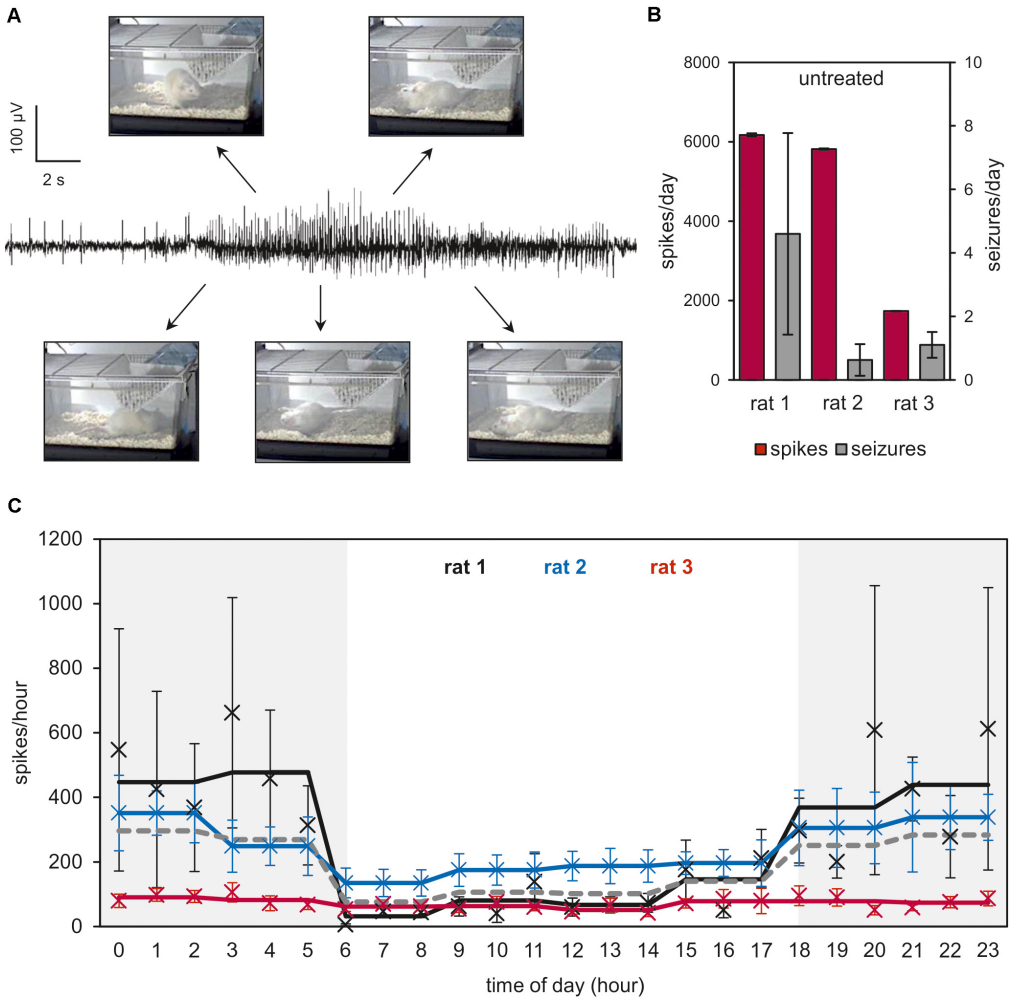


**FIGURE 1 |** Video-EEG analysis of F98 glioma-bearing Fischer 344 rats. Single-channel electrodes were implanted epidurally to record an EEG trace of F98 glioma-bearing Fischer 344 rats for the whole time of the animal experiments. Rats were housed in individual cages with 12-h light/dark cycles (illumination: 6:00–18:00 h). **(A)** Sample trace of a representative seizure. Photos illustrate the association of field records with motor expressions during seizure. More details of ictal potentials are shown in **Supplementary Figure 3**. **(B)** The last 9–2 days (relative to sacrifice) in the life of the animals were analyzed and seizures were counted for each animal individually. **(C)** Number of spikes and distribution over time of day are shown as means ± SEM (5–9 data points per time of day). Means of 3-h-intervals are presented as lines. Gray-shaded areas indicate time of day without illumination in the animal housing facility. The dark gray dotted line represents mean of 3-h-intervals of all three animals.

illustrated in **Supplementary Figure 4**. A two-way ANOVA followed by Bonferroni *t*-test revealed that no significant difference between time of day and treatment regime was found (**Supplementary Figure 4**). In line with this, seizures responded well to PER, regardless whether RCT was co-administered (**Supplementary Figure 5**).

Prolonged survival following tumor therapy may predict a treatment-related reduction of tumor size. To test this directly, we analyzed the tumor size in histological sections 2 weeks after glioma implantation. The presence of the F98 tumor cells was verified by routine hematoxylin-eosin staining (**Figure 3A**), and the tumor size was three-dimensionally reconstructed using NeuN-based immunofluorescence micrographs (**Figures 3B,C**). While systemic administration of 15 mg/kg PER had only limited effects on F98 glioma tumor size (83.7 ± 14.7% of untreated animals), RCT significantly reduced the tumor volume to 17.1 ± 2.5% of untreated animals ($p < 0.05$, one-way ANOVA on ranks followed by Dunn's test; **Figure 3B**). Adding PER, however, to this standard RCT had no further significant effect on glioma size (29.8 ± 5.3% of untreated animals). In summary, by implantation of F98 tumor cells into Fischer 344 rats we have established a valuable model of glioma-related mortality and morbidity including an epileptiform phenotype with response to standard RCT.

## Neuroprotective Effects of *in vivo* PER Add-on in the RCT-Treated Glioma Model

In order to evaluate PER effects on the physiological network activity within the peritumoral tissue, we induced spontaneous

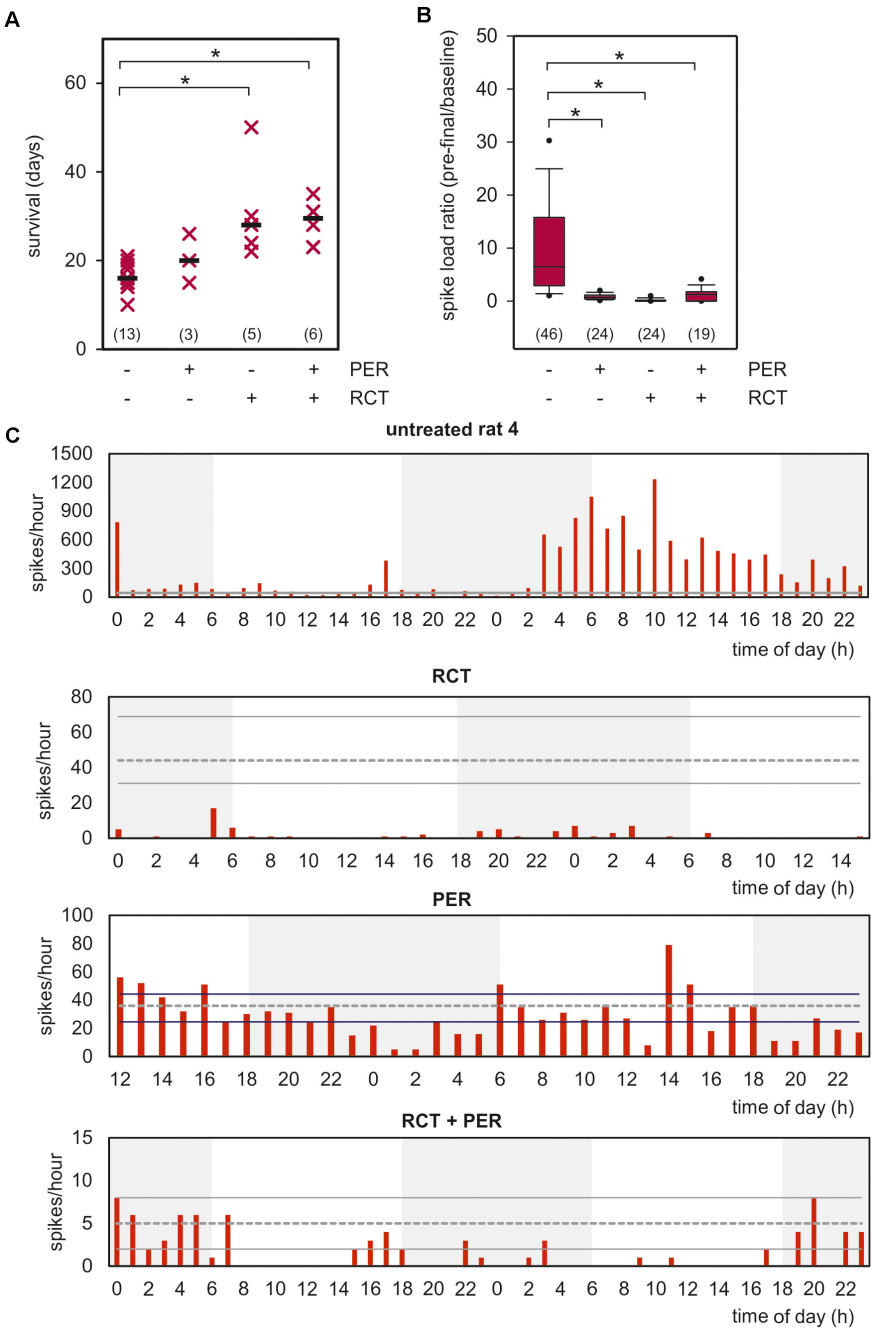


**FIGURE 2 |** Impact of PER, RCT, and adjuvant PER treatment to RCT on clinical progression. **(A)** Rats with orthotopically-growing F98 glioma were sacrificed when endpoint criteria of the experiment design were reached and survival was estimated. Survival of each animals is illustrated as single point, median is shown as black bar ($n$ = number of animals per group); $^*p < 0.05$ [Kruskal–Wallis test followed by *post hoc* analysis (Dunn's test)]. **(B)** Box plot shows video-EEG analysis of untreated animals versus RCT, PER, or RCT + PER cohorts. Data represent the number of analyzed hours in each cohort (data were obtained from 4 untreated, 2 RCT-treated, 2 PER-treated, and 2 RCT + PER-treated animals), $^*p < 0.05$ [Kruskal–Wallis test followed by *post hoc* analysis (Dunn's test)]. **(C)** Spike distribution over time of one animal from each investigated group is shown. Data range from 40–48 h of the last 3–4 days of the rats. Gray dotted line presents median of spikes of 12h-baseline as described in section "Materials and Methods," whereas solid lines show upper and under quartiles.

potential deflections in slices using specific recording solutions in pilot experiments (see section "Materials and Methods" and **Supplementary Figure 2**). Since we aimed to yield a medium incidence of spontaneous deflections, we chose a bath with 0 mM $MgCl_2$ and 5 μM gabazine. Under these conditions, we obtained 20.3 ± 3.1 spontaneous deflections per minute in the peritumoral tissue from tumor-bearing, but otherwise untreated animals (**Figures 4B,C**). This network activity was significantly

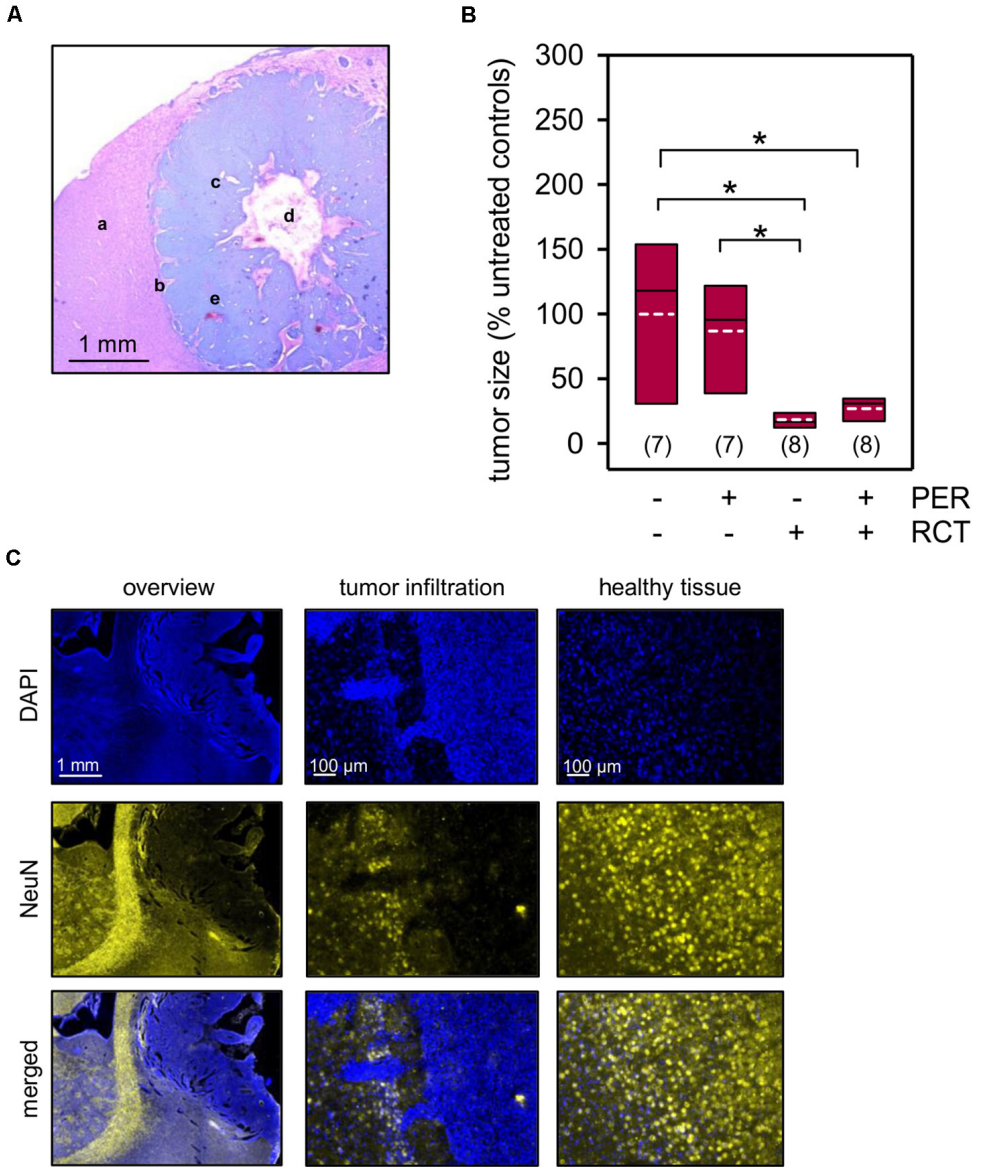


**FIGURE 3 |** Tumor size quantification. Animals with orthotopically-growing F98 glioma were sacrificed after 2 weeks and brains were prepared for further histological and morphological analysis. **(A)** Hematoxylin and eosin staining confirms the presence of F98 glioma. The sample picture shows healthy brain tissue (a), tumor infiltration (b), glioma cells (c), central necrosis (d), and microvascular hyperplasia (e). **(B,C)** The tumor volume was quantified in a three-dimensionally manner by NeuN expression (yellow) of the healthy brain tissue, whereas F98 glioma presented only little or no expression of the marker protein. Nuclei were counterstained with DAPI (blue); white dotted line represents arithmetic mean, black solid line represents median; *n* = number of animals per group; $^*p < 0.05$ [Kruskal–Wallis test followed by *post hoc* analysis (Dunn's test)].

attenuated to 7.7 ± 1.1 events per minute by adding 30 µM PER to the bath ($p < 0.05$, one-way ANOVA on ranks followed by Dunn's test), as expected from previous studies with rat and human glioma cell lines (Lange et al., 2019; Mayer et al., 2019). By further adding the NMDA receptor antagonist D-AP5 to the bath, the slices became electrically silent indicating that the network activity under our conditions was entirely glutamate-dependent. Similar results were obtained from the contralateral side suggesting that tumor infiltration in the corpus callosum or even parts of the contralateral hemisphere has occurred and affected glutamatergic network activity. Our results nicely demonstrate that PER, systemically applied prior to the brain preparation, had successfully been washed out before starting the *ex vivo* electrophysiological experiments.

Since we aimed to study neuroprotective effects of PER add-on to standard RCT in glioma *in vivo*, we provided the animals with subcutaneously localized osmotic pumps for continuous systemic PER treatment starting in parallel to the standard RCT. PER administration was continued until brain preparation on day 14–15 following tumor implantation, but was washed out before

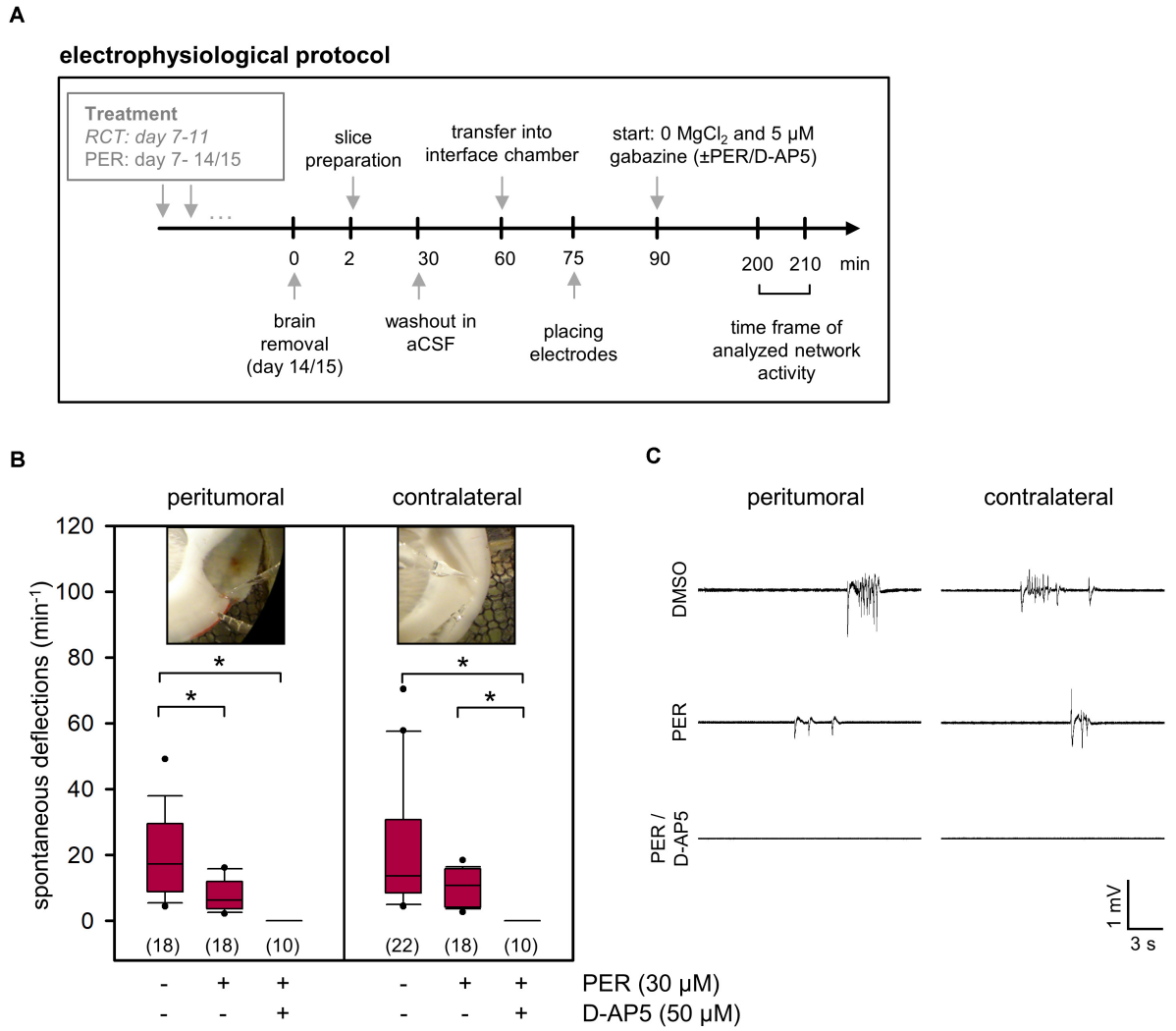


**FIGURE 4 |** *Ex vivo* effects of PER on network activity in acute F98 glioma slices. **(A)** Illustration of the electrophysiological protocol for investigation of PER action on network activity. **(B)** $1 \times 10^5$ F98 glioma cells were orthotopically injected into the neocortex of Fischer 344 rats. After 2 weeks the animals were sacrificed and brain slices were prepared for *ex vivo* application of vehicle control (DMSO), PER, NMDA receptor antagonist D-AP5, and combination thereof. Slices were challenged with aCSF with 0 µM $Mg^{2+}$ and 5 µM gabazine. Data are represented in box plots, $n$ = 10–22 measurements (slices were from a total of 16 rats with F98 glioma), *$p < 0.05$ (Kruskal–Wallis test with *post hoc* Dunn's test). Photographs indicate peritumoral or contralateral position of microelectrodes. **(C)** Sample traces of field potential recording illustrate deflections that were used for quantification.

electrophysiological experiments (for protocol see **Figure 4A**). Next, we quantified the physiological synaptic network activity in five different cohorts: sham-operated animals, untreated glioma-bearing animals as well as animals with glioma and tumor therapy (in three arms: PER, RCT, and RCT/PER). Two weeks after tumor implantation, the physiological glutamatergic network activity in the peritumoral tissue was significantly reduced compared to sham-operated rats (16.9 ± 1.5 deflections/min versus 33.9 ± 2.8 deflections/min, $p < 0.05$, one-way ANOVA on ranks followed by Dunn's test; **Figure 5A**) indicating severe disruption of synaptic connectivity. When glioma-bearing animals were treated with PER alone (13.1 ± 2.0 deflections/min, $p < 0.05$ versus sham, one-way ANOVA on ranks followed by Dunn's test) or RCT alone (17.4 ± 3.9 deflections/min, $p < 0.05$ versus sham, one-way ANOVA on ranks followed by Dunn's test), the induced physiological network activity was indistinguishable from that obtained in untreated F98 animals. This effect was specific for the peritumoral tissue, since on the contralateral side no differences were observed. However, the combination of the anticonvulsant PER together with RCT rescued the physiological network activity to 30.3 ± 4.7 deflections/min ($p < 0.05$ versus PER and $p < 0.05$ versus RCT, one-way ANOVA on ranks followed by Dunn's test; **Figure 5A**). These values were almost identical to those observed in sham-operated animals, hence indeed suggesting neuroprotective effects by combined *in vivo* PER and RCT treatment in the F98 glioma model. This finding is corroborated by the comparison of ipsilateral versus contralateral network activity for each experimental group. Of all groups,

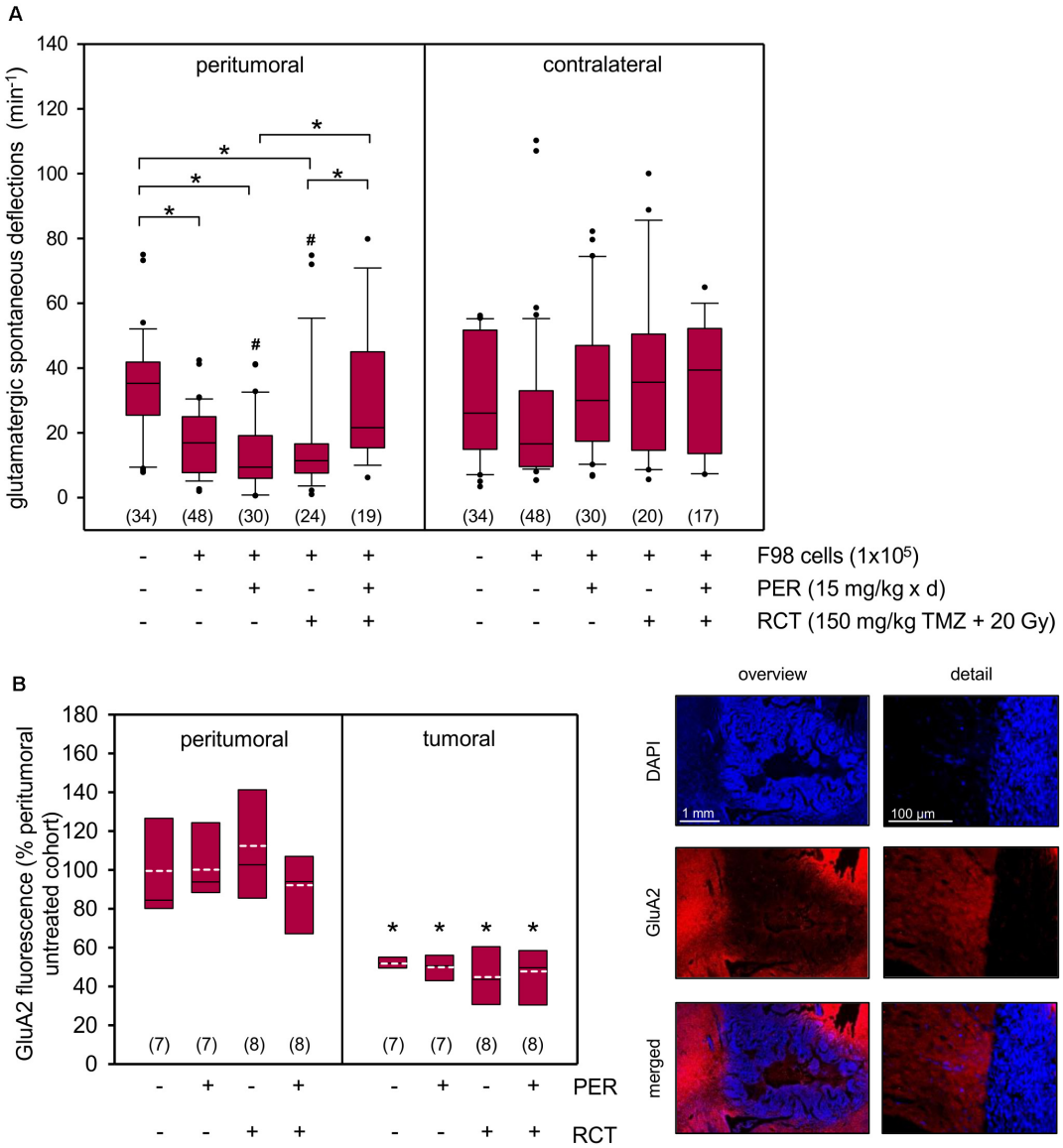


**FIGURE 5 |** Synergistic impact of PER and RCT on glutamatergic network activity and GluA2 expression in acute F98 glioma slices. **(A)** One week after stereotactic injection of 1 × $10^5$ F98 cells, RCT with fractionated irradiation (5 × 4 Gy), concurrent administration of temozolomide (5 × 30 mg/kg bw) and an anticonvulsive treatment with PER (15 mg/kg bw/day) was started. After a total of 14–15 days animals were sacrificed and brain slices were prepared for electrophysiological analysis. The slices were exposed to aCSF 0 mM $Mg^{2+}$ and 5 µM gabazine and field potential recordings were performed in surrounding tissue; $n$ = 17–48 number of measurements (brain slices were from a total of 35 rats); *$p < 0.05$ (Kruskal–Wallis test followed by *post hoc* analysis (Dunn's test); # < 0.05 versus contralateral equivalent ($U$ test). **(B)** AMPA receptor subunit GluA2 expression was determined in the tumor area and peritumoral tissue of the ipsilateral hemisphere. Glu2A (red) immunofluorescence was normalized to peritumoral immunofluorescence intensity of the vehicle controls without PER or RCT (see section "Materials and Methods"). Nuclei were counterstained with DAPI (blue); white dotted line represents arithmetic mean, black solid line represents median. Significant difference between peritumoral and glioma fluorescence for each group was determined ($n$ = 7–8 animals per group; *$p < 0.05$; $U$ test).

only monotherapy with PER or RCT (for both groups: $p < 0.05$ ipsilateral versus contralateral, $U$ test) presented lower values in ipsilateral recordings than contralateral, whereas a combination of RCT with adjuvant PER increased ipsilateral to contralateral network activity (**Figure 5A**). Interestingly, no differences were found in the cohort of untreated gliomas ($p = 0.097$, $U$ test).

The question was therefore, whether the low effect of PER on tumor growth was associated with the expression of AMPA receptors, which we know to promote disease progression. GluA2 was selected as surrogate marker for AMPA receptors, as expression of this subunit was found to be associated with excitotoxicity and tumor invasion (Ishiuchi et al., 2007; Wright and Vissel, 2012). Our data indicate that F98 glioma express GluA2 roughly 50% (47–55%) of peritumoral tissue and treatment with PER, RCT or combination thereof did not affect expression levels of the AMPA receptor subunit (**Figure 5B**).

Contralateral expression of GluA2 did not differ from ipsilateral peritumoral brain tissue (data not shown).

## DISCUSSION

In high grade glioma and glioblastoma, the preservation of health-related QOL is an important therapeutic endpoint. Neurocognitive impairments and especially seizures are common and an anticonvulsant treatment is often indicated. Our major finding was that adding the AMPA receptor antagonist PER to standard RCT preserved network activity significantly more efficiently than standard RCT or PER alone in the peritumoral tissue of F98 glioma-bearing rats. Preserving network activity implies intact neuronal structures and thus may be regarded as neuroprotection in a sense of preserved neural function. This could be of great interest since most patients are offered a therapy approach including a RT regime (Weller et al., 2017, 2019). Therefore, we hypothesize that a tumor volume reduction by RCT alone is not sufficient to significantly protect the surrounding neurons. It also needs a second mechanism such as attenuation of AMPA receptor-mediated neuronal excitation via PER to mediate neuroprotection. Samari et al. (2013) demonstrated that inhibition of $Ca^{2+}$ influx via NMDA receptors of irradiated neurons protects the cells from apoptosis. Furthermore, blocking of NMDA receptors prevents irradiation-induced abnormal glutamate signaling and synaptic remodeling (Duman et al., 2018). In addition, low doses of irradiation of glioma surrounding healthy brain tissue may lead to an enhanced migration and infiltration of glioma (Wank et al., 2018), which is presumed for F98 cells (Desmarais et al., 2016). PER may antagonize this key feature of glioma by blocking AMPA receptor-mediated migration (Ishiuchi et al., 2007; Piao et al., 2009).

The results of this study are in line with previous reports showing that F98 glioma progression can be decelerated by irradiation and concurrent administration of temozolomide (Wicks et al., 2015; De Meulenaere et al., 2019). The novel question in the focus of this study was whether PER can further improve the beneficial outcome. *In vitro* studies of glioblastoma and neuroblastoma cells indicated growth inhibition by the anticonvulsant (Lange et al., 2019; Nozawa et al., 2019) and a pilot study employing 12 patients suffering from glioblastoma correlated survival with PER plasma level (Izumoto et al., 2018). Here, no additional effect of PER on tumor size and survival was determined. Remarkably, in immunodeficient mice oral application of a high dose of PER reduced tumor cell density (Venkataramani et al., 2019). In that paper, slow-growing human glioma cells were used consistent with our previous study showing enhanced responsitivity to PER in human low-passage glioma cells (Lange et al., 2019). RCT reduced tumor size more than fivefold in comparison to untreated controls and animals had a 50% prolonged survival. One may speculate that potential effects of PER were disguised by the high impact of RCT. This negative finding is consistent with previous reports showing that AMPA receptor antagonists may fail to prolong survival in combination with RCT (Grossman et al., 2010; Iwamoto et al., 2010). Likewise, F98 cells could also be resistant to AMPA receptor inhibition. Glioblastoma often feature AMPA tetramers of GluA1 and GluA4 subunits (Corsi et al., 2019). It has been documented that F98 express all AMPA receptor subunits but GluA1 (Savaskan et al., 2011); a subunit that is associated with cell migration and adhesion in glioblastoma. In AMPA tetramers containing GluA2, $Ca^{2+}$ permeability depends on this subunit. Therefore, the GluA2 subunit was chosen as surrogate marker for AMPA receptors. This subunit is subjected to a glutamine/arginine (Q/R) site RNA editing. AMPA receptors with edited GluA2 subunit are $Ca^{2+}$ impermeable which also applies for F98 cells (Savaskan et al., 2011). In contrast, underedited GluA2 subunits allow the influx of $Ca^{2+}$ that eventually may promote an augmented excitotoxicity of tumor surrounding neurons (Ishiuchi et al., 2007). Our immunohistological analyses revealed that F98 gliomas express the AMPA receptor subunit GluA2 and this expression is unaffected by RCT or PER, respectively.

Furthermore, we asked if an adjuvant therapy of PER affects the glioma-associated epileptiform phenotype. Remarkably, a combination of RT and temozolomide alone attenuated interictal spike load to initial baseline levels. These data are in line with observations made in patients with low-grade glioma in which RCT may contribute to a better seizure control (Koekkoek et al., 2015). For the first time, we showed that PER acts in an anticonvulsive manner in a glioma-associated rodent model of epilepsy. That was somewhat expected, as in several pilot studies with small sample sizes of glioma patients with drug-resistant epilepsy, high response rates to PER with improved seizure control or even seizure-free conditions were achieved (Vecht et al., 2017; Dunn-Pirio et al., 2018; Izumoto et al., 2018; Maschio et al., 2018; Chonan et al., 2020). Given the strong impact of RCT on the epileptiform phenotype, it is not surprising that no additional inhibitory effects by adjuvant PER were observed in our experiments.

To conduct the *in vivo* experiments with respect to orthotopic glioma progression and onset of tumor-related seizures, a robust animal model was needed. F98 glioma in Fischer rats is a well-established glioma model (Belloli et al., 2013; Schültke et al., 2018; Wang et al., 2018), but to the best of our knowledge, no data about its tumor-associated epileptiform phenotype have been published so far (Kirschstein and Köhling, 2016). The results of our studies suggest that F98 glioma led to interictal epileptiform events (e.g., spikes and spike-waves), indicating a high susceptibility to develop seizures, which was also demonstrated by video-EEG analysis. In all tested rats, the untreated animals suffered from seizures, but the occurrence of seizures is distributed heterogeneously between the animals. We find interictal events to be a robust surrogate marker to indicate the severity of the epileptiform phenotype. Interictal events are known to arise during glioma progression and are a subclinical marker of epilepsy. A recently published study suggests that an increasing frequency of interictal events may be associated with progressive neurologic impairment in glioma (Montgomery et al., 2020). The *ex vivo* presence of an glioma-associated epileptic phenotype is consistent with previous reports on resected human tissue

(Köhling et al., 2006) and animal glioma models based on human genetic alterations (Hatcher et al., 2020). Together, our data demonstrate that the F98/Fischer 344 rat model is a suitable tool to investigate glioma-associated epilepsy in preclinical studies.

In conclusion, orthotopic implantation of F98 cells into the neocortex of Fischer 344 rats is a robust model of glioma progression. We have shown for the first time that this glioma model exhibits also an epileptiform phenotype. Furthermore, our data support the important role of glutamate and AMPA receptors in the context of glioma and its microenvironment. The administration of PER adjuvant to standard RCT led to neuroprotection in healthy glioma-surrounding brain tissue. This is important given the fact that radiotherapy is a crucial component in the treatment algorithms of glioma patients. However, PER failed to attenuate tumor growth or promote animal survival when administered adjuvant to RCT, but abolished the epileptiform phenotype of the rats.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Landesam für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Thierfelderstraße 18, 18059 Rostock.

## AUTHOR CONTRIBUTIONS

TK, FL, and ES designed the study. JH, TR, TS, JH, GR, KP, FL, and TK performed the experiments. ES and SK performed the irradiation. CL, JB, and VN analyzed the video-EEG recordings. GH provided materials to perform the experiments. FL wrote the manuscript. TK, ES, and RK contributed to parts of the manuscript and critically reviewed the final version of the manuscript. All authors analyzed and reviewed data.

## FUNDING

This research was funded by the Damp Stiftung, grant number 2016-20 to TK and FL and a grant from the University Medicine Rostock FORUN Program to FL (Project No.: 889010).

## ACKNOWLEDGMENTS

The authors wish to thank the team of the Oscar-Langendorff-Institute of Physiology and the medical physicists of the Department of Radiooncology at the Rostock University Medical Center.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.598266/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

MINI REVIEW
published: 30 November 2020
doi: 10.3389/fnins.2020.595664

# Deeper and Deeper on the Role of BK and Kir4.1 Channels in Glioblastoma Invasiveness: A Novel Summative Mechanism?

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OPEN ACCESS

**Edited by:**
*Yukihiro Ohno, Osaka University of Pharmaceutical Sciences, Japan*

**Reviewed by:**
*Michele Mazzanti, University of Milan, Italy*
*Luigi Catacuzzeno, University of Perugia, Italy*

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**Specialty section:**
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

**Received:** *17 August 2020*
**Accepted:** *09 November 2020*
**Published:** *30 November 2020*

**Citation:**
*Brandalise F, Ratto D, Leone R, Olivero F, Roda E, Locatelli CA, Grazia Bottone M and Rossi P (2020) Deeper and Deeper on the Role of BK and Kir4.1 Channels in Glioblastoma Invasiveness: A Novel Summative Mechanism? Front. Neurosci. 14:595664. doi: 10.3389/fnins.2020.595664*

In the last decades, increasing evidence has revealed that a large number of channel protein and ion pumps exhibit impaired expression in cancers. This dysregulation is responsible for high proliferative rates as well as migration and invasiveness, reflected in the recently coined term oncochannelopathies. In glioblastoma (GBM), the most invasive and aggressive primary brain tumor, GBM cells modify their ionic equilibrium in order to change their volume as a necessary step prior to migration. This mechanism involves increased expression of BK channels and downregulation of the normally widespread Kir4.1 channels, as noted in GBM biopsies from patients. Despite a large body of work implicating BK channels in migration in response to an artificial intracellular calcium rise, little is known about how this channel acts in GBM cells at resting membrane potential (RMP), as compared to other channels that are constitutively open, such as Kir4.1. In this review we propose that a residual fraction of functionally active Kir4.1 channels mediates a small, but continuous, efflux of potassium at the more depolarized RMP of GBM cells. In addition, coinciding with transient membrane deformation and the intracellular rise in calcium concentration, brief activity of BK channels can induce massive and rapid cytosolic water loss that reduces cell volume (cell shrinkage), a necessary step for migration within the brain parenchyma.

**Keywords: glioblastoma, Kir4.1, BK channel, cancer, channelopathy**

## INTRODUCTION

Glioblastoma (GBM, WHO grade IV astrocytoma) is the most common and malignant brain tumor (Brat et al., 2007; Furnari et al., 2007; Barbieri et al., 2018). In comparison to the majority of solid tumors, it is characterized by strong invasive and pro-angiogenic behavior associated with a poor prognosis with a median survival rate of about 15 months (Stupp et al., 2009). Because of the diffuse and aggressive invasiveness of GBM, it is generally not possible to achieve complete surgical resection, resulting in rapid relapse (Holland, 2001; Maher et al., 2001). Furthermore,

GBMs contain a small subpopulation of so-called cancer stem cells (CSCs), which are extremely resistant to radio and chemotherapy (Vescovi et al., 2006; Lathia et al., 2011). Death evasion minimizes the effect of all therapeutic strategies currently available, but enhanced invasiveness is the major feature that prevents successful treatment (Louis et al., 2007).

## GLIOBLASTOMA AS A CHANNELOPATHY: THE ROLE OF CHLORIDE AND POTASSIUM IONIC EQUILIBRIUM IN GBM CELL INVASIVENESS

Glioblastoma cells migrate through narrow spaces in the brain parenchyma (De Vleeschouwer and Bergers, 2017) that are usually smaller than the soma of the cell (around 8 μm in diameter, Liu et al., 2018). To make that possible, GBM cells undergo a reduction in their volume of about 30% facilitating migration and invasion (Demuth and Berens, 2004; Armento et al., 2017). Such shrinkage is achieved by modifying the osmotic equilibrium in the cell allowing a net release of cytoplasmic water. Increasing evidence suggests that specific ion channels and transporters are involved in modulating the cell volume. The two main ionic gradients reported to be altered in glioma cells are for chloride ($Cl^-$) and potassium ($K^+$) ions (Turner et al., 2014).

In contrast to other neurons in the brain, glioma cells have a higher cytosolic $Cl^-$ concentration (Habela et al., 2009). The chloride gradient is maintained and modulated by persistent activity of the $Na^+/K^+/Cl^-$ cotransporter 1 (NKCC1), hence its expression has been linked with GBM invasiveness and severity grade (Garzon-Muvdi et al., 2012). The overexpression of the NKCC1 cotransporter leads to abnormal accumulation of $Cl^-$ in the glioma cell cytosol (Haas and Sontheimer, 2010; Ben-Ari, 2017) so that upon opening of $Cl^-$ channels (Barbieri et al., 2018), the altered electrochemical gradient results in an outward flow of this ion with concomitant osmotic loss of water from the intracellular milieu (through the aquaporin channels). The net result is a loss of cellular volume (Luo et al., 2020).

Along with $Cl^-$ gradient, the altered $K^+$ flux, is also essential for invasion. In fact, due to the cytosolic calcium fluctuations during GBM cells migration, members of the family of $Ca^{2+}$-activated $K^+$ channels such as KCa3.1 (intermediate conductance $K^+$ channel) and the BK channel (large conductance $K^+$ channel), are overexpressed in 32% of glioma patients, and there is a linear correlation between the expression of these channels and the progression of the pathology. Due to their calcium sensitivity, it has been shown that such channels respond positively to bradykinin activation that increases intracellular $Ca^{2+}$, with a resulting efflux of $K^+$ and water (Reetz and Reiser, 1996; Catacuzzeno and Franciolini, 2018). As a consequence, the glioma cells reduce their total volume, which enables them to migrate through narrow spaces within the brain.

While the importance of the intermediate conductance $K^+$ channel in GBM progression has been recently summarized in several works (Catacuzzeno et al., 2012; D'Alessandro et al., 2018; Liu et al., 2019), the aim of this review is to underline the contributions of BK channels and Kir4.1 channels on GBM invasiveness, focusing on their biophysical properties and their osmo-electric effect at the RMP in GBM cells.

## THE BK CHANNEL: FROM STRUCTURE TO PHYSIOLOGY IN GLIOBLASTOMA CHANNELOPATHY

The BK channel is a tetrameric, large conductance $K^+$ channel, widely expressed in both neurons and glia across development and adulthood (for an extensive review see Lee and Cui, 2010). The BK channel is characterized by an outwardly rectifying current that shows both voltage and calcium-concentration sensitivity (Nardi and Olesen, 2008; Cui et al., 2009).

Overexpression of this channel has been reported in biopsies of glioblastoma patients (Liu et al., 2002; Catacuzzeno et al., 2015) and intriguingly the channel structure also seems to be altered, since Ransom et al. (2002) reported that GBM cells expressed a splicing variant of the channel on the *hSlo* (the gene linked to the encoding part of the alpha subunit) with a consequent increase in the sensitivity to intracellular calcium concentration.

Intracellular calcium dynamics are involved in the regulation of a wide number of processes in the brain that span from synaptic plasticity (Brandalise and Gerber, 2014; Brandalise et al., 2016a; Keck et al., 2017) to remodeling of cytoskeleton (Lebart and Benyamin, 2006; Correll et al., 2008). In GBM it has been demonstrated that calcium fluctuations from the intracellular stores (reticulum) along with different states of the RMP (depolarized versus hyperpolarized) are linked to GBM cell migration as well as to the proliferative state of the cell (Rondé et al., 2000; Ishiuchi et al., 2002; Catacuzzeno et al., 2011). Cyclic variation of both voltage and calcium concentration in GBM cells has led to the hypothesis that BK channels, in light of their overexpression, can be one of the key targets in triggering glioblastoma migration and the invasion process (Catacuzzeno et al., 2015).

## TRYING TO EXPLAIN THE BK ROLE AT THE GBM RMP

The implication of BK channels in GBM cell migration and invasion has been reported by various groups (Klumpp et al., 2018; Rosa et al., 2018). Blockade of BK channels with iberiotoxin (IbTx) or tetraethylammonium (TEA) in two- dimensional migration assays inhibits GBM cell motility (Soroceanu et al., 1999; Basrai et al., 2002; Weaver et al., 2006). Furthermore, the increase of intracellular calcium induced by extracellular menthol application significantly increases BK current and the migration of GBM cells and this effect was reversed by BK channel blockers (Wondergem and Bartley, 2009; Ratto et al., 2019).

However, despite the known functional upregulation of BK channels in GBM, their dependence on membrane potential deserves further examination. At resting cytoplasmic free calcium concentration (10–100 nM), BK channels open only at membrane

potentials above +10 mV (Lee and Cui, 2010), significantly more depolarized than the RMP of around −40 mV measured in GBM cells (Catacuzzeno et al., 2015). In other words, BK channels at RPM are in the closed state. Additionally, in the two-dimensional migration assay, there is a general consensus that blockade of BK channels does not significantly reduce migration of GBM cells. Nevertheless, the reduction of GBM cell invasion due to BK pharmacological blockade is effective only when the intracellular calcium concentration is raised (for example, by menthol or acetylcholine bath application) (Bordey et al., 2000; Kraft et al., 2003; Wondergem and Bartley, 2009). Therefore, although activating BK channels undoubtedly boosts GBM cell migration due to $K^+$ efflux, the blockade of the same conductance under resting conditions does not prevent GBM invasiveness.

## Kir4.1 IN GLOBLASTOMA: PRIMUM MOVENS OF RMP DEPOLARIZATION IN GBM CELLS?

Kir4.1, one of the inwardly rectifying potassium channels (coded by the KCNJ10 gene) is largely expressed in the glia cells of the brain (Nichols and Lopatin, 1997). In mature astrocytes, the high potassium permeability is mediated to a large extent by the Kir4.1 channel with two functional consequences: first, the negative RMP that is closer to the potassium equilibrium potential, and, second, the buffering of extracellular potassium after neuronal activity (Butt and Kalsi, 2006; Chever et al., 2010). Because a fraction of the channels is constitutively open, it has been proven that Kir4.1 plays a role in the homeostatic regulation of the RMP : for example, this is a crucial mechanism in cerebellar granule cell development during migration (Rossi et al., 1998; Brandalise et al., 2016b) as well as for glia maturation that settles the RMP at around −80 mV (Olsen and Sontheimer, 2008). Their peculiar current/voltage relation (**Figure 1A**) is due to weak, voltage-dependent rectification and to voltage-dependent pore block, from the internal side of the channel, by magnesium and other organic cations such as polyamine (Ruppersberg, 2000). At potentials more positive to the $K^+$ equilibrium, this block limits the amount of $K^+$ that flows through the channel. Kir4.1 is also involved in the regulation of other pathways in astrocytes such as the BDNF expression (Ohno et al., 2018).

In GBM, a downregulation of Kir4.1 during the early stage of the tumor progression has been reported (Olsen and Sontheimer, 2004; Ratto et al., 2019), which is correlated to the dramatic shift in RMP of GBM cells to more depolarized values around −40/−30 mV (Olsen and Sontheimer, 2004). Due to their internalization during GBM progression, very little attention has been paid to the residual activity of the Kir4.1 channels at RMP. However, recent investigations have provided evidence that justifies a reconsideration of the impact of this channel on the later progression of GBM:

- Despite the internalization and consequent functional downregulation of Kir4.1, there is still a significant fraction of this channel expressed in the membrane which, at the RMP of GBM cells, mediates a constitutive outward $K^+$ current (**Figure 1B**) that might play a role in the redistribution of cell volume and the consequent change in cell morphology.
- The fact that the channels are internalized but not degraded suggests that they still have a functional role as a readily available pool that is potentially re-inserted into the membrane in a relatively short time. Indeed, despite the fact that the mechanism is not yet understood, in Ratto et al., 2019, a calcium-dependent upregulation of Kir4.1-mediated current has been described within 9 min of menthol bath-application.
- The simultaneous block of Kir4.1 along with the BK channel seems to be an effective strategy for blocking GBM cell invasiveness in two-dimensional migration assays without additional perturbation (raising) of the cytosolic calcium concentration (Ratto et al., 2019). This suggests that the two classes of potassium channels are mutually involved in the shrinkage of the cell via regulation of the $K^+$ gradient.

## Kir4.1 AND BK CHANNELS: A SUMMATIVE LINK?

In the previous sections, we have summarized the literature on the role of BK and Kir4.1 channels in GBM invasiveness. We have linked the physiology of these two channels relating to the microenvironment of GBM cells (with particular interest in the $K^+$ electrical and osmotic gradient) raising some discrepancies between the channel biophysical properties and the electrochemical equilibrium range of GBM cells.

Now we tentatively propose how these two channels might work in concert during GBM cell invasion:

(1) Glia cells under physiological conditions: glia cells express a fairly high level of Kir4.1 that is known to be involved in ionic homeostasis by buffering ambient $K^+$ during neuronal activity. Moreover, glia cells have a strongly hyperpolarized RMP maintained mainly by Kir4.1 (the reversing potential of Kir4.1 in normal conditions is around −75/−80 mV). BK channels, on the other hand, are also expressed but are not activate at in the RMP (**Figure 1A**).

(2) Morphological changes in GBM cells: the severe alterations in $Cl^-$ and $K^+$ equilibrium in the cells prior to migration leads to a more depolarized RMP (−40 to −30 mV). Consequently, the reversal potential of Kir4.1 is no longer aligned with the RMP, and this sets the channel for a constitutively net outflow of $K^+$ ions (see **Figure 1B**). It is worth noting that in basal conditions, GBM cells seem to change their shape due to the efflux of water, but the total surface area of the cell is actually not reduced, probably due to the formation of lamellipodia. In this phase, BK channels are overexpressed in their spliced isoforms, which increases their sensitivity to the calcium concentration. Therefore, despite the fact that the RMP is still below the BK threshold, transient cytosolic

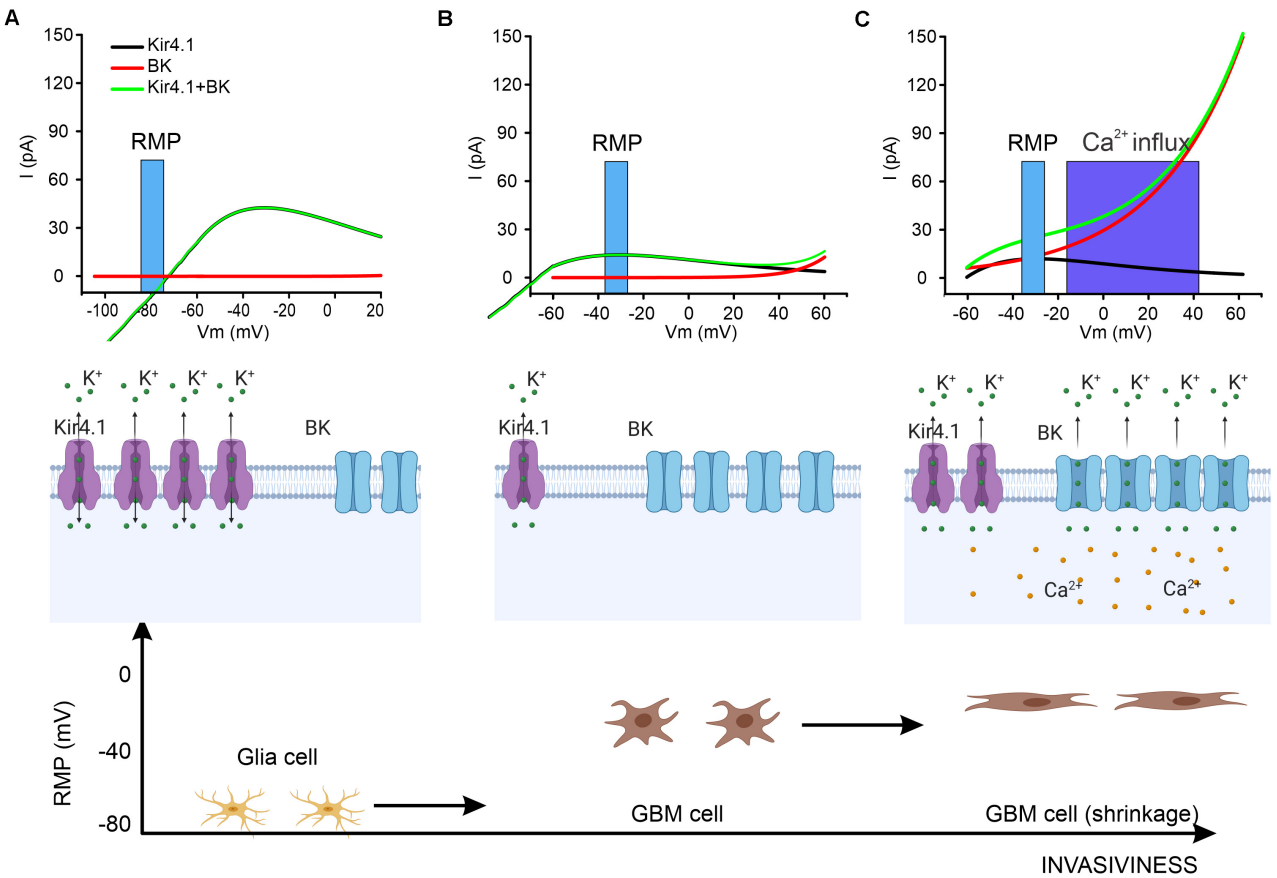


**FIGURE 1 |** A proposed action mechanism for BK and Kir4.1 channels. **(A)** In glial cells the RMP is set at hyperpolarized potentials so that Kir4.1 exerts a homeostatic equilibrium whereas BK channels are in the closed state. The **top panel** shows the kinetics of BK and Kir4.1 channel in the range of the RMP (blue bar). **Middle panel:** schematic of the expression of the two channels and the direction of the ionic potassium flow ($K^+$). **Bottom panel:** representation of glia cells in relationship with their RMP. **(B)** When glia transforms into GBM cells, the RMP shifts to a more depolarized potential **(top and bottom)** due to a downregulation of Kir4.1 channels while BK channels are overexpressed **(middle)**. **(C)** The increasing pressure on the GBM cells membrane during migration in the brain parenchyma exerts a double effect by directly activating BK channels and by raising the concentration of cytosolic calcium. This results in the opening of BK channels **(top and middle)** with consequent cell shrinkage **(bottom)** allowing the migration of the GBM cell.

calcium rise (Catacuzzeno et al., 2011; Li et al., 2020) could depolarize the cell and shift the activation curve to a more hyperpolarized potential allowing a transient opening of BK channels (**Figure 1C**). Under these conditions, BK channels can contribute efficiently and summate with Kir4.1 channels in controlling total efflux of potassium and consequent shrinkage. This scenario agrees with recent experimental evidence whereby blocking Kir4.1 and BK channels reduces GBM cell migration.

(3) Glioblastoma invasiveness in the brain parenchyma: When a GBM cell invades the extracellular matrix in the brain, the process requires a reduction of cytosolic volume. The increase in pressure that the extracellular matrix exerts on the GBM cell induces mechanical stress on the membrane, which has been suggested to activate BK channels (Zhao et al., 2010; Wawrzkiewicz-Jałowiecka et al., 2018). Moreover, this mechanical constraint might lead to a possible biochemical cascade with a consequent increase of intracellular calcium concentration that shifts the voltage activation curve of the channel to a more hyperpolarized potential (Charles et al., 1991). Overall, increased pressure on the GBM cell membrane results in greater functional BK channel activation that significantly increases the efflux of cytosolic water with consequent additional reduction in volume and capability to migrate efficiently (**Figure 1C**). Following the mechanical compression, the relief of pressure reduces the fraction of active BK channels and the balance of activation between the two channels returns to the state described in point 2 (**Figure 1B**).

## CONCLUSION

This review has not only the purpose of summarizing the most recent evidence on the role of BK and Kir4.1 channels in GBM cell-migration, but underlines the need for examining the roles of these channels in the context of the different functional states of GBM cells. The emerging perspective that considers GBM

as a channelopathy is a promising field (Litan and Langhans, 2015; Prevarskaya et al., 2018). However, many questions must be yet addressed for a more comprehensive understanding. The intracellular calcium concentration and its spontaneous oscillations seem to be the main actor behind the scenes as it modulates a large fraction of the ion channels implicated in GBM invasion. Furthermore, recent work has proposed that BK channels are modulated by mechanical stress on the membrane indicating that the dynamics of GBM cell membrane changes must be investigated. Finally, $K^+$ equilibrium is a result of the interactions between a large variety of channels and pumps; in this review we focused on BK and Kir4.1 on account of their high conductance and their roles at RMP, respectively. However, for deeper insight into GBM cell invasiveness, future investigations should take into account other channels as well, such as intermediate conductance calcium-activated potassium channels and leak channels. In conclusion, a large number of potassium channels play a key role in GBM progression. However, despite that potassium channels offer a surface-accessible therapeutical target, the fact that they are largely expressed in other cell types such as neurons, glia and cardiomyocytes imposes a strong monitoring of the acceptable toxicity threshold induced by potentials specific blockers as a side effect.

## AUTHOR CONTRIBUTIONS

FB and PR: study concepts and design. FB, PR, ER, and DR: manuscript preparation. FO and CL: manuscript editing. RL and MG: manuscript review. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the Italian Ministry of Education, University and Research (MIUR): Dipartimenti di Eccellenza Program (2018–2022) and the Department of Biology and Biotechnology "L. Spallanzani," University of Pavia, Fondo di Ricerca Giovani (FRG, University of Pavia), and Crowdfunding funds.

## ACKNOWLEDGMENTS

We thank experts from BioMed Proofreading® LLC for English editing and Prof. Urs Gerber for final revision of the manuscript. **Figure 1** is created with "BioRender.com."

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 17 December 2020
doi: 10.3389/fnins.2020.578316

# Drug Repositioning Screen on a New Primary Cell Line Identifies Potent Therapeutics for Glioblastoma

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Glioblastoma is a malignant brain cancer with limited treatment options and high mortality rate. While established glioblastoma cell line models provide valuable information, they ultimately lose most primary characteristics of tumors under long-term serum culture conditions. Therefore, established cell lines do not necessarily recapitulate genetic and morphological characteristics of real tumors. In this study, in line with the growing interest in using primary cell line models derived from patient tissue, we generated a primary glioblastoma cell line, KUGBM8 and characterized its genetic alterations, long term growth ability, tumor formation capacity and its response to Temozolomide, the front-line chemotherapy utilized clinically. In addition, we performed a drug repurposing screen on the KUGBM8 cell line to identify FDA-approved agents that can be incorporated into glioblastoma treatment regimen and identified Topotecan as a lead drug among 1,200 drugs. We showed Topotecan can induce cell death in KUGBM8 and other primary cell lines and cooperate with Temozolomide in low dosage combinations. Together, our study provides a new primary cell line model that can be suitable for both *in vitro* and *in vivo* studies and suggests that Topotecan can offer promise as a therapeutic approach for glioblastoma.

**Keywords: glioblastoma, primary cell line, drug repurposing, Topotecan, Temozolomide**

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***Edited by:***
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***Specialty section:***
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

***Received:*** *30 June 2020*
***Accepted:*** *18 November 2020*
***Published:*** *17 December 2020*

***Citation:***
*Senbabaoglu F, Aksu AC, Cingoz A, Seker-Polat F, Borklu-Yucel E, Solaroglu İ and Bagci-Onder T (2020) Drug Repositioning Screen on a New Primary Cell Line Identifies Potent Therapeutics for Glioblastoma. Front. Neurosci. 14:578316. doi: 10.3389/fnins.2020.578316*

## INTRODUCTION

Glioblastoma (grade IV astrocytoma) is the most common of all malignant brain and CNS tumors (Ohgaki and Kleihues, 2005). Relative survival estimates for glioblastoma patients are quite low; 5.1% of patients survive 5 years post diagnosis. The hallmarks of glioblastoma can be defined as uncontrolled cell proliferation, diffuse infiltration, tendency for necrosis, angiogenesis, resistance to apoptosis, and genomic instability. The aggressive nature and heterogeneity of glioblastoma make treatment very difficult: Extensive tumor infiltration into the surrounding healthy brain tissue, tumor mass formed by distinct cell types and resistance to available therapy options are major challenges for successful treatment (Cancer et al., 2008; Ostrom et al., 2015; Mansouri et al., 2017).

Current therapies include surgery, radiation therapy, and administration of Temozolomide (TMZ). TMZ is usually administered at the beginning of treatment along with radiotherapy for 6 weeks, followed by six cycles of TMZ alone for 5 days every 28 days. With this standard

regimen, the median survival reaches 14.6 months compared to 12 months observed for patients treated with radiotherapy alone (Stupp et al., 2009). This aggressive trimodal regimen is still faint against glioblastoma, recurrence occurs within months in 90% of patients (Stummer et al., 2005; Stupp et al., 2009). Upon recurrence, many patients undergo further surgical resection if possible; and therapy options include re-challenging with alkylating agents such as TMZ, platinum-based drugs, or VEGF inhibitors (Pavon et al., 2014; Messaoudi et al., 2015). Yet, none of these options can currently cure glioblastoma.

For years, glioblastoma cell lines such as U87MG, U251, and T98G, have been studied extensively, providing valuable information about this cancer type. Cell line-based models are reproducible with reliable growth rates for unlimited number of divisions (Huszthy et al., 2012). However, cell line-based models also have shortcomings: First, the serum-containing medium modifies both their genomes and transcriptomes. Prolonged cell culture changes the genetic, epigenetic, and morphological characteristics of cancers. For example, most glioblastoma lines lack epidermal growth factor receptor (EGFR) amplification, while 40% of the primary tumor specimens contain this mutation. In addition, prolonged cell culture leads to progressive hypermethylation of the MGMT promoter, approximately 80% of glioblastoma cell lines are MGMT hypermethylated, as compared to a 40% frequency of promoter hypermethylation in the initial clinical diagnosis (Carlson et al., 2011). Second, upon orthotopic implantation to the brain, most cell lines fail to develop the defining morphological features of glioblastoma, such as diffuse infiltration into surrounding healthy tissue and microvascular proliferation (Lee et al., 2006; Xie et al., 2015). Third, including serum in the cell culture media reduces tumor-propagating and stem cell-like feature of tumor cells due to cellular differentiation (Singh et al., 2003; Seidel et al., 2014). Thus, serum-based 2D monolayer cultures are not the best option to investigate therapeutic effects of a new finding in glioblastoma.

Glioblastoma cell culture techniques have been improved in the last years by culturing glioblastoma cells in serum-free medium that was originally established for neural stem cells (NSCs) (Singh et al., 2003; Galli et al., 2004). Neurobasal media, a DMEM derivative with reduced osmolarity and lower glutamine concentrations, are supplemented with biotin, insulin, transferrin, FGF and EGF (Brewer et al., 1993; Ledur et al., 2017). In this defined medium, NSCs and glioblastoma cancer stem cell like cells maintain their self-renewal ability and display no differentiation (Singh et al., 2003; Quiñones-Hinojosa et al., 2007). Additionally, in this serum-free NSC media, glioblastoma cells grow as spheres, resembling neurospheres, and they can keep a semi-3D environment in which cells deposit ECM to create their own unique microenvironment (10). Orthotopic transplantation of spheroid glioblastoma cells generates secondary tumors, which retain the features of the primary tumors that the cells are originally derived from. Moreover, direct orthotopic transplantation of patient derived glioblastoma cells show that these xenograft models represent the histopathology, genetic and phenotypic properties of the corresponding patient's primary tumor (Xie et al., 2015). Thus, while investigating glioblastoma, it is essential to work with primary cell cultures and/or xenografts if possible.

Drug repurposing or repositioning is the application of known drugs to other uses, which eliminates years required to develop a new drug and provides a marked economic benefit (Cha et al., 2018). In addition, a repurposed drug already comes with extensive preclinical and clinical knowledge, which can reduce cytotoxicity issues for the newly desired application. Drug repurposing has provided many potential candidates for various diseases in the past. For example, a screen in 1994 revealed Gefitinib (AstraZeneca) and Erlotinib (Roche) as specific inhibitors of the EGFR, which were approved by Food and Drug Administration (FDA) in 2003, and are currently used in the treatment of many cancers (Fry et al., 1994).

In this study, we first generated a new primary glioblastoma cell line, called KUGBM8, and characterized its *in vitro* and *in vivo* growth properties as well as its response to TMZ. Providing a new cell line model, we conducted a high throughput screen with a 1,200 FDA-approved drug library as part of a drug repurposing approach. We identified Topotecan, a Topoisomerase inhibitor, as the lead agent that inhibited the growth of KUGBM8 cells, suggesting its clinical potential for glioblastoma therapy.

# MATERIALS AND METHODS

## Patient Information

A patient within the range of 40–45 year-old was presented with a 2 months history of headache, right hemiparesis and impairment of vision. Magnetic resonance imaging (**Figure 1A**) studies revealed a hyperintense lobulated tumor in the left parieto-occipital lobe. The patient underwent a left parieto-occipital craniotomy. The tumor was removed gross totally. Histopathology of the tumor confirmed the diagnosis of glioblastoma. The postoperative course was uneventful, and patient was discharged from the hospital on the fifth postoperative day.

## Establishing Primary Glioblastoma Cell Line KUGBM8

Protocol of primary cell line formation was adapted from Xie et al. (2015) and conducted according to the guidelines and approval of the Koç University Institutional Review Board (2014.079.IRB2.022). Surgically removed tumor specimens were placed in DMEM containing 2.5% Fetal Bovine Serum (FBS) and 1% Pen-strep prior to transfer on ice. Fresh tumor samples were washed with 1X PBS containing 1% Pen-strep several times and minced with a scalpel.

The dissected tissue was incubated in a mixture of 1:1 Accutase (Stem Cell Technologies) and Trypsin (Gibco) for 10 min at 37°C for chemical dissociation. The dissociated cells were washed twice with DMEM/F12, followed by centrifugation at 600 rpm for 8 min. Tumor sample was then divided into two parts, one part was transferred into wells of a 6-well plate (depending on the size of the tumor specimen) and supplemented with DMEM containing 10% FBS and 1% Pen-strep. The

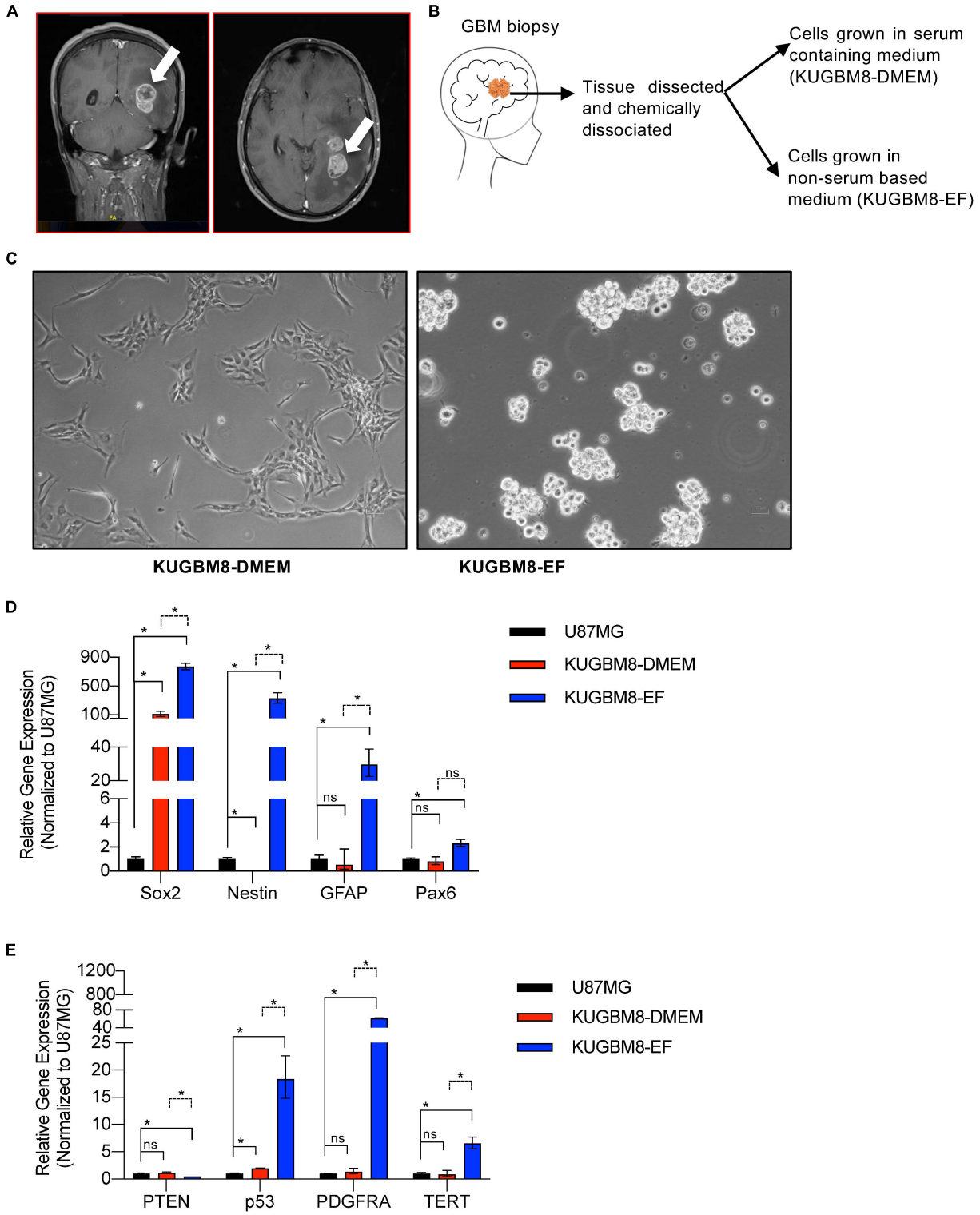


**FIGURE 1 |** Establishment of primary KUGBM8 cell lines. **(A)** MRI brain gadolinium-enhanced T1 weighted image (coronal and sagittal view, respectively) showing hyperintense lesion (arrows) in the left parieto-occipital lobe. **(B)** Schematics of cell line establishment from the tumor. **(C)** Representative photos of KUGBM8-DMEM and KUGBM8-EF cell lines taken by 10X light microscopy. **(D)** Gene expression levels of neural stem cell marker genes detected by qRT-PCR. Values are normalized to the housekeeping gene GAPDH. **(E)** Gene expression levels of cancer related genes detected by qRT-PCR. Values are normalized to the housekeeping gene GAPDH. * denotes $p < 0.05$, ns denotes $p > 0.05$, *t*-test.

attachment of the cells from the dissected tissue was observed and medium was changed 2–3 times a week. These cells were named KUGBM8-DMEM. Second part of the dissociated tissue was plated to 25 $cm^3$ flasks with EF medium [Neurobasal medium (Gibco, Cat. No. 21103-049), L-Glutamine (Gibco), B27 and N2 supplements (Gibco), Pen-Strep (0.5%, Gibco), Heparin (Stem Cell Technologies), FGF (20 ng/ml, Gibco, PHG0266), and EGF (20 ng/ml, Peprotech, AF-100-15)]. After sphere formation (5–7 days), spheres in suspension were centrifuged and divided into single cells with accutase. Resuspended cells were placed in a new flask containing fresh media. These cells were named KUGBM8-EF. Cells were kept in 37°C humidified incubator with 5% $CO_2$. Cells were routinely checked for mycoplasma contamination (Lonza Mycoplasma Detection Kit).

## Cell Line Oncopanel Testing Capture

Patient derived cell lines KUGBM8-DMEM was KUGBM8-EF was studied by Oncopanel testing capture (Illumina's TruSight Cancer Kit, Illumina), by screening 94 genes and 284 SNPs as described before (Börklü-Yücel et al., 2020). Briefly, sequencing was performed using MiSeq sequencer (Illumina) to produce 2 × 150 bp reads. Raw reads were mapped against human reference genome hg19 using BWA-MEM algorithm, de-duplicated using Picard and variant calling was executed using GATK best practices pipeline. Quality filtered variants with a minimum of 20X coverage and no more than 10% Mapping Quality Zero (MAPQ0), were annotated using ANNOVAR with avSNP release of 142, 1,000 genomes release of 2014 along with NIH-NHLBI 6500 exome database version 2. MAF filter ($< 0.01$) was set based on ExAC, NIH 6500, gnomAD, and 1,000 genomes data. Pathogenicity evaluation was performed based on the inheritance mode, database entries (HGMD, ClinVar, CentoMD), *in silico* prediction tools (SIFT, Polyphen2, MutationTaster) and ACMG recommendations. All intronic variants located outside the boundaries of 10 bp from the exons and synonymous exonic ones were filtered out. Putative splicing variants were analyzed using Human Splicing Finder (HSF). Sequencing data is deposited to SRA database (PRJNA644513).

## Cell Culture

Human glioblastoma cells U87MG was supplied and authenticated by American Tissue Type Culture Collection (ATCC) and cultured in DMEM (Gibco) with 10% Fetal Bovine Serum (Gibco) and 1% Pen-Strep (Gibco). GBM4 and GBM8 patient derived glioblastoma cells were kind gifts from Dr. Hiroaki Wakimoto (Massachusetts General Hospital, Boston, MA) and cultured in Neurobasal medium (Gibco, Cat. No. 21103-049), L-Glutamine (Gibco), B27 and N2 supplements (Gibco), Pen-Strep (0.5%, Gibco), Heparin (Stem Cell Technologies), FGF (20 ng/ml, Gibco, PHG0266), and EGF (20 ng/ml, Peprotech, AF-100-15). KUGBM8-EF and KUGBM8-DMEM cells were established and cultured as described above.

## Quantitative Real Time PCR (qRT-PCR)

U87MG, KUGBM8-DMEM, and KUGBM8-EF cells were collected, and total RNA was isolated using Macherey-Nagel RNA Nucleospin isolation kit (MN GbmH & Co.) according to manufacturer's protocol. 1 μg of RNA was used to establish cDNA with M-MLV Reverse Transcriptase (Invitrogen). Quantitative real time PCR was performed with PikoReal 96 Real Time PCR system (Thermo Fisher Scientific) by using LightCycler 480 SYBR Green I Master (Roche). The primers used in these experiments are listed in **Supplementary Table 1**. Relative gene expression levels were detected by normalization to GAPDH expression.

## Testing Tumor Forming Capacities and Temozolomide Response *in vivo*

SCID mice cared in appropriate conditions of Koç University Animal Facility were used and the institution boards of Koç University (HADYEK#2014-22) approved all protocols. Firefly Luciferase (Fluc) and mCherry expressing stable KUGBM8-DMEM and KUGBM8-EF cells were generated by viral transduction as described (Bagci-Onder et al., 2012). Initially, mCherry expression was examined under fluorescence microscopy and their Fluc activity was validated by utilizing *in vitro* luminescence assay. Increasing number of Fluc-mCherry expressing cells (0–50.000/well) were seeded on 96-well black-bottom plates and luciferase activity was detected by adding 1,000 μg/ml D-Luciferin. Bioluminescence measurements were taken every week.

For tumor implantation, 4 mice for each condition were used. A total of 120,000 cells per mouse were injected in 7 μl PBS stereotactically into the brain (From bregma, AP: −2 mm, ML: 1.5 mm and from dura V: 2 mm). After injection, tumor growth was visualized using IVIS Lumina III Bioluminescence Imager. Accordingly, Fluc activity of tumors was measured by injecting mice with 150 μg/g body weight of D-Luciferin intraperitoneally. After repeated measurements of tumor growth for 75 days, mice were sacrificed, and brains were recovered with perfusion. Quantification of tumor progression was performed with GraphPad PRISM software. For histological analyses, 10 micron thick cryo-sections from tumors were stained with hematoxylin and eosin and imaged with Leica M205 FA Stereo microscope (Leica Microsystems).

For Temozolomide *in vivo* treatment, after tumor formation as gaged by Fluc imaging, mice were treated with 2 mg/kg DMSO or Temozolomide for 5 consecutive days. Growth was assessed by Fluc activity as described above. Statistical measurements were performed using ANOVA.

## Drug Screening and Cell Viability Measurement

The drug library composed of 1,200 FDA (Food and Drug Administration of United States) approved drugs was purchased from Prestwick Chemical (France) as described (Senbabaoglu et al., 2016). Individual drugs, namely Astemizole, Camptothecine (S, +) Quinacrine dihydrochloride dihydrate, Mitoxantrone dihydrochloride, Doxorubicin, and Topotecan were supplied from Prestwick Chemicals.

For the high throughput drug screen, 384-well plates (Corning) were employed. Accordingly, KUGBM8-EF cells were automatically seeded to 384-well plates as 1,500 cells/40 μl/well via Multidrop$^{TM}$ Combi Reagent Dispenser (Thermo Fisher Scientific). Next day, 50 μM drug plates were freshly prepared

**TABLE 1 |** Pathology results of the surgically excised tumor tissue.

| Marker | Result |
|---|---|
| p16 | Positive (score 4) |
| EGFR | Positive (score 3) |
| Ki-67 | Positive (%52) |
| IDH1 | Negative |
| PTEN | Negative (score 32) |
| MGMT | Positive (methylated %80–100) |
| OLIG2 | Negative |
| GFAP | Positive |
| NeuN | Negative |
| p53 | Negative |

within EF medium and added to 384 well plates with an automated pipettor (Matrix$^{TM}$ Multichannel Electronic Pipettes, Thermo Fisher Scientific) for a final concentration of 5 μM. Each treatment was performed in quadruplicates. Cell viability was determined 3 days later. Changes in cell viability were measured by normalization to untreated controls. Statistical measurements were performed using *t*-test and $IC_{50}$ of Temozolomide was calculated by Graphpad Prism with non-linear regression analysis.

For the chosen hits, cell viability was further investigated on black 96-well plates (Corning) with different dosages. Cell viability was detected after 3 days after treatment by CTG.

## Western Blot Experiments

Western blots were performed as described previously (Senbabaoglu et al., 2016). Cells were treated with Topotecan for 24 h to check PARP (Cell Signaling/9,542) cleavage. α-Tubulin (Abcam/ab15246) was used as a loading control.

# RESULTS

## Establishment of Patient-Derived Primary Glioblastoma Cell Lines From Fresh Tumor Tissues

KUGBM8-DMEM and KUGBM8-EF cell lines were derived from a glioblastoma patient. MRI brain gadolinium-enhanced T1-weighted image (coronal and sagittal view, respectively) showed hyperintense lesion (arrows) in the left parieto-occipital lobe (**Figure 1A**). The excised tumor tissue was freshly dissociated and divided into two parts to establish two different cell lines, one that grows under serum-supplied culture conditions (KUGBM8-DMEM) and one growing in an anchorage-independent manner in medium devoid of serum and enriched with neurosphere supplements (KUGBM8-EF) (**Figure 1B**). The tumor tissue pathological analysis revealed that MGMT promoter was hypermethylated, EGFR was amplified, and PTEN was deleted in the original tumor (**Table 1**). After cell lines were established, KUGBM8-DMEM cells exhibited typical characteristics of two-dimensional cancer cell line cultures, where cells flattened in a monolayer on the bottom of the culture vessel. On the contrary, KUGBM8-EF cells displayed growth as three-dimensional spheroids (**Figure 1C**).

To examine the genetic alterations in these established lines, we performed onco-profiling of KUGBM8-DMEM and KUGBM8-EF cells in early passages, and genes were filtered by minor allele frequency (MAF). *MUTYH, PTEN, RB1, TP53,* and *TSC1* (**Table 2**) genes were found to be commonly mutated in these two cell lines, supporting the proposed role of *TP53*, *PTEN,* and *RB1* in the pathogenesis of human glioblastomas (Chow et al., 2011). Defects in *TSC1*, hamartin, has been proven to induce mTORC1 activity, which in turn triggers glioma development when combined with other oncogenic signals (Yamada et al., 2014), such as variations in the known tumor suppressor genes *PTEN, TP53,* and *RB1*. Biallellic germline mutations of *MUTYH* have primarily been related to colorectal adenomas, but a recent report states that monoallelic germline *MUTYH* mutations may induce an increase in the risk of malignant brain tumors (Kline et al., 2016).

It is widely known that cell culture conditions can alter the genetics and metabolism of cancer cells (Ackermann and Tardito, 2019). To minimize such alterations and resemble the features of patient tumors as much as possible, our newly generated primary cell lines were only used up to passage 10 during the experiments. We also tested how these cells compare to the widely used established cell lines, such as U87MG, for their expression of several genes from the NSC lineage. NSC markers *SOX2*, *NESTIN*, PAX6 (Lathia et al., 2015; Zhang and Jiao, 2015) as well as the well-established astrocyte marker *GFAP* were expressed significantly higher in KUGBM8-EF cells than in KUGBM8-DMEM and U87MG cells (**Figure 1D**). Next, we checked the gene expression of some cancer related genes (*PTEN, P53, PDGFRA,* and *TERT*) (**Figure 1E**) and observed significant fold change differences among KUGBM8-DMEM and KUGBM8-EF. For example, high expression of *PDGFR* and *TERT* as well as low expression of *PTEN* was evident in KUGBM8-EF cells compared to KUGBM8-DMEM or U87MG cells.

**TABLE 2 |** Genetic alterations observed in KUGBM8-EF cells.

| Gene | Transcript number | Nucleotide change | Aminoacid change | Zygosity | MAF | Predicted effect | References |
|---|---|---|---|---|---|---|---|
| MUTYH | NM_001128425 | c.C799T | p.Q267X | Het | 0.0002 | Pathogenic | PubMed: 17703316 |
| PTEN | NM_000314 | c.1026+1G>A | ? | Het | – | Pathogenic | PubMed: 15372512 |
| RB1 | NM_000321 | c.C1654T | p.R552X | Het | – | Pathogenic | PubMed: 7704558 |
| TP53 | NM_000546 | c.G404T | p.C135F | Het | – | Likely pathogenic | – |
| TSC1 | NM_000368 | c.C1798T | p.Q600X | Het | – | Likely pathogenic | – |
| TSC1 | NM_000368 | c.G1219A | p.V407M | Het | 0.0002 | VUS | – |

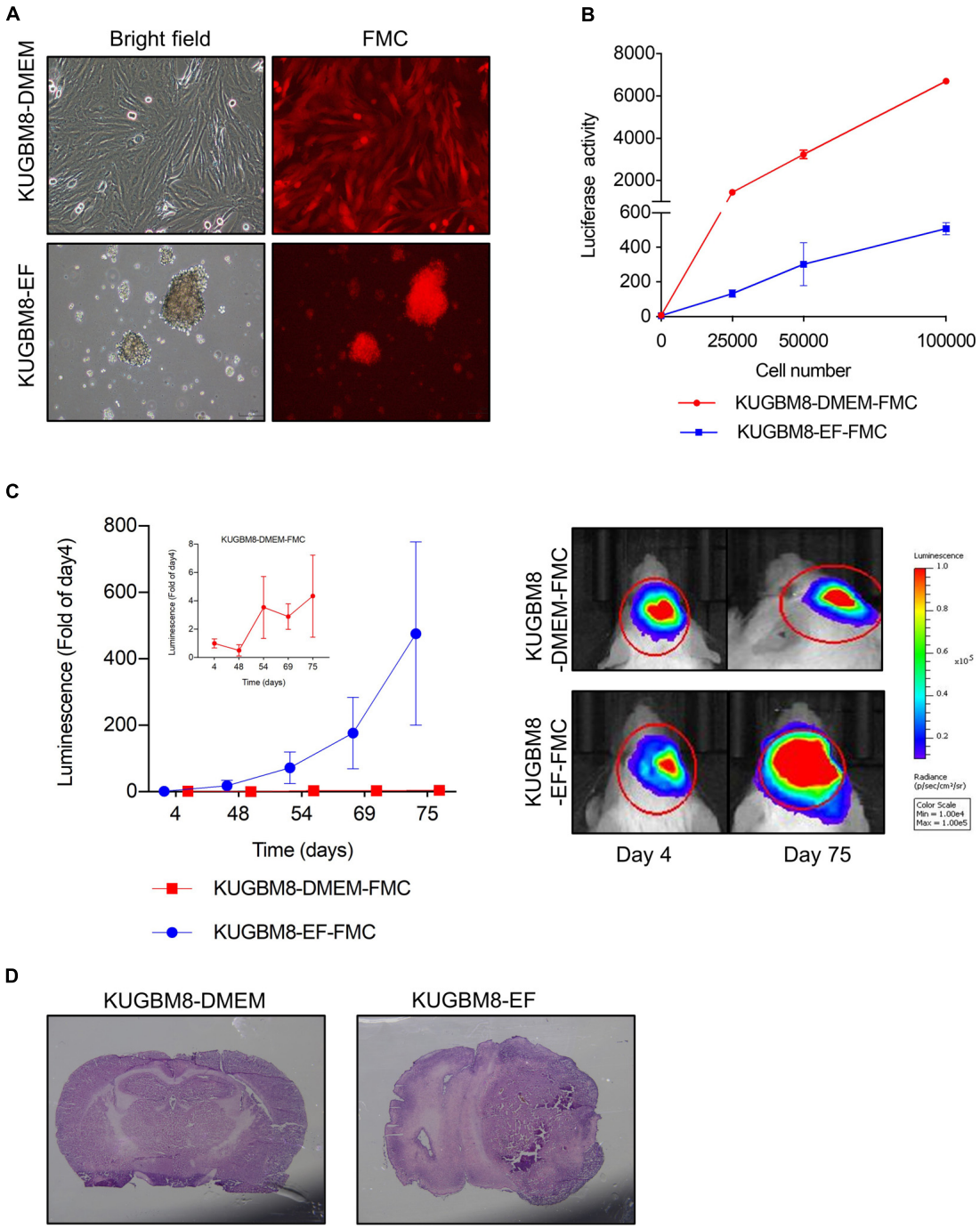


**FIGURE 2 |** Characterization of KUGBM8 cell lines *in vivo.* **(A)** Representative photos of fluc-mcherry transduced KUGBM8-DMEM and KUGBM8-EF cell lines. **(B)** Plot demonstrating *in vitro* bioluminescence of KUGBM8-DMEM-FMC and KUGBM8-EF-FMC cell lines. **(C)** *In vivo* tumor growth curve of established cell lines. KUGBM8-EF and KUGBM8-DMEM cells were intracranially implanted into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice and assessed for tumor growth for 75 days. Representative images of bilateral tumors of same mice from 4 to 75 days displaying normalized bioluminescent efficiencies acquired (blue to red indicates lower to higher radiance as photons/s/cm2/steradian). *N* = 4 per group. **(D)** Representative histological examination of tumors removed at the end of last imaging session, stained by H&E.

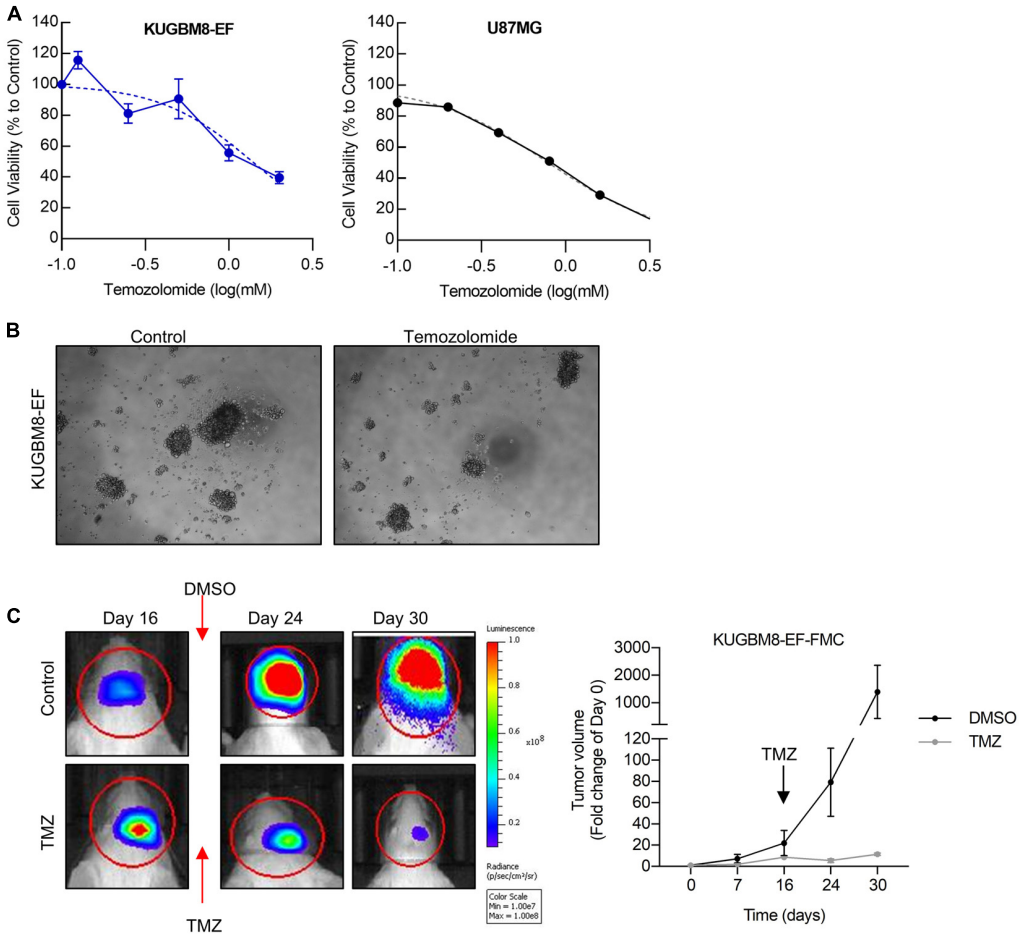


**FIGURE 3 |** Temozolomide response of KUGBM8 cell line. **(A)** Dose response curve of KUGBM8-EF and U87MG to Temozolomide treatment for 5 days. $IC_{50}$ was calculated by Graphpad Prism with non-linear regression analysis. **(B)** Representative photos of KUGBM8-EF after 4 days of treatment with 250 μM Temozolomide. **(C)** The efficacy of Temozolomide (TMZ) was tested *in vivo* in KUGBM8-EF implanted NOD/SCID mice. Cells were treated with pulsed TMZ (2 mg/kg) for 5 days and growth rates were detected by non-invasive bioluminescence imaging for 30 days. $N = 3$ mice per group. Statistical analysis between DMSO and TMZ treated mice were done by ANOVA, $p < 0.05$.

## Patient Derived Primary Glioblastoma Cell Lines Are Capable of Forming Tumors *in vivo*

One important feature of cancer cell lines for studying tumorigenesis is their ability to form tumors *in vivo*. To test the tumor forming capacity of KUGBM8 cells, we labeled each cell line with Firefly Luciferase (Fluc) and mCherry encoding vectors and generated stable KUGBM8-DMEM and KUGBM8-EF cells by viral transduction (termed KUGBM8-DMEM-FmC and KUGBM8-EF-FmC, respectively). Both cell lines preserved their unique morphologies and preserved their viability upon transduction. While their mCherry expression was comparable as gauged by fluorescence microscopy (**Figure 2A**), their Fluc activities were different as measured by *in vitro* luminescence assays. The KUGBM8-DMEM-FmC cells displayed more Fluc activity/cell compared to KUGBM8-EF-FmC cells (**Figure 2B**).

For tumor implantation, each cell line was stereotaxically implanted into the same sites in the brain and tumor growth was monitored with non-invasive bioluminescence imaging over 75 days. While KUGBM8-DMEM-FmC cells did not grow to form any tumors, KUGBM8-EF-FmC cells were highly tumorigenic (**Figure 2C**). Histological examination of brain sections validated the presence of tumors derived KUGBM8-EF-FmC cells (**Figure 2D**). Hence, given their tumorigenic properties, we continued our experiments with KUGBM8-EF as a clinically relevant model.

## Patient Derived Primary Glioblastoma Cell Lines Respond to Temozolomide *in vitro and in vivo*

As frontline therapeutic for glioblastoma is TMZ, we first examined whether our new primary cell line is affected by TMZ treatment. TMZ response varies greatly for cell lines in the literature depending on the cell viability assay used (Lee, 2016). Temozolomide *in vitro* (125–2,000 μM) for 5 days and cell viability was detected using an ATP based reagent. 50% cell

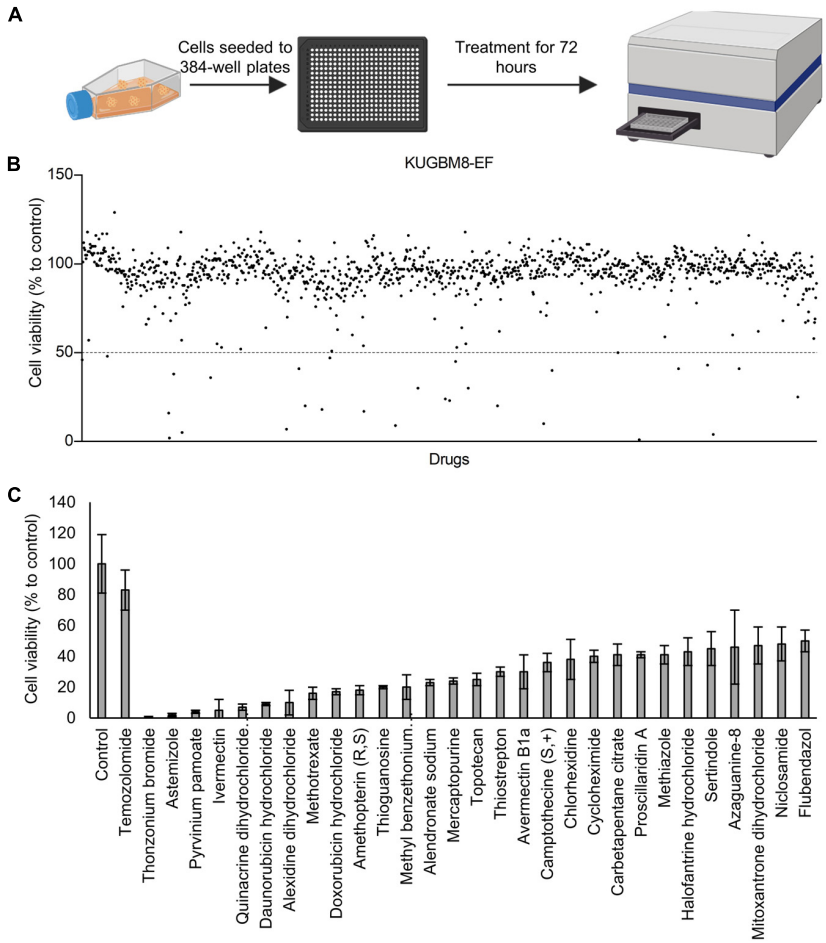


**FIGURE 4 |** Drug repurposing screen performed on KUGBM8-EF cells**. (A)** Schematics of the screen. **(B)** Cell viability results detected by Cell Titer Glo (CTG) after 72 h of treatment. Drug treated cells were normalized to untreated wells. $N = 4$ for each condition. **(C)** Hits determined by 50% cell viability threshold. Error bars represent Normalized Standard Deviation. All hits have a significant $p$-value ($p < 0.05$) compared to control group, $t$-test.

inhibition dosage ($IC_{50}$) was found as 1.38 mM (**Figures 3A,B**) for KUGBM8-EF cells. With similar conditions $IC_{50}$ for U87MG was found to be 787 μM (**Figure 3A**).

Next, we sought to study the dynamics of TMZ response of KUGBM8-EF *in vivo*. After establishing tumors *in vivo*, we treated mice with 2 mg/kg of TMZ for 5 days following the treatment regimen previously performed for U87MG model (Qi et al., 2013; Woo et al., 2014). In line with our *in vitro* results, we observed significant attenuation of tumor growth in TMZ-treated mice compared to DMSO-treated control group (**Figure 3C**). Taken together, these findings suggest that we generated a new patient derived primary glioblastoma model that is suited for studying therapy response *in vitro* and *in vivo*.

## Screen Among 1,200 FDA-Approved Drugs Reveals Topotecan as a Lead Drug for Primary Glioblastoma Cell Line

To find new drugs that can be incorporated into glioblastoma treatment, we utilized our newly generated KUGBM8-EF cell line and a chemical library of 1,200 FDA and EMEA approved drugs. The library is composed of drugs from 15 different therapeutic classes, namely, endocrinology, cardiovascular, immunology, diagnostic, metabolism, allergology, dermatology, gastroenterology, hematology, ophthalmology, neuromuscular, infectiology, respiratory, central nervous system, and oncology.

Previous work, including ours, used this FDA-approved drug library in a variety of cancer screens in concentrations of 2–10 μM (Yip et al., 2006; Finlay et al., 2010; Senbabaoglu et al., 2016); accordingly, we chose 5 μM as optimal for this screen. KUGBM8-EF cells were treated with the library and viability was detected at 3 days (**Figure 4A**). Percent viability was determined by normalization to untreated controls, and a cut-off value of 50% was applied to select for the "hit" drugs (**Figure 4B**). Temozolomide at 250 μM dosage was included in the screen as a treatment control. 29 out of 1,200 drugs reduced KUGBM8-EF cell viability below 50% when normalized to control groups. These drugs were Thonzonium bromide (1 ± 0%), Astemizole (2 ± 1%), Pyrinium pamoate (4 ± 1%), Ivermectin (5 ± 7%), Quinacrine dihydrochloride

**TABLE 3 |** Table of hits and their therapeutic classes and effects.

| Chemical name | %Cell viability ± %SD | Therapeutic class | Therapeutic effect |
|---|---|---|---|
| Alexidine dihydrochloride | 10 ± 8 | Infectiology | Antibacterial |
| Methyl benzethonium chloride | 20 ± 8 | Infectiology | Antibacterial |
| Thiostrepton | 30 ± 3 | Infectiology | Antibacterial |
| Cycloheximide | 40 ± 4 | Infectiology | Antibacterial |
| Daunorubicin hydrochloride | 9 ± 1 | Infectiology, oncology | Antibacterial, antineoplastic |
| Doxorubicin hydrochloride | 17 ± 2 | Infectiology, oncology | Antibacterial, antineoplastic |
| Chlorhexidine | 38 ± 13 | Infectiology | Antibacterial, antiseptic |
| Ivermectin | 5 ± 7 | Infectiology | Antihelmintic |
| Avermectin B1 | 30 ± 11 | Infectiology | Antihelmintic |
| Methiazole | 41 ± 6 | Infectiology | Antihelmintic |
| Niclosamide | 48 ± 11 | Infectiology | Antihelmintic |
| Quinacrine dihydrochloride | 7 ± 2 | Infectiology, metabolism | Antihelmintic, Antiparasitic |
| Astemizole | 2 ± 1 | Allergology | Antihistaminic |
| Halofantrine hydrochloride | 43 ± 9 | Metabolism | Antimalarial |
| Amethopterin (R,S) | 18 ± 3 | Immunology, oncology | Antineoplastic |
| Methotrexate | 16 ± 4 | Oncology | Antineoplastic |
| Thioguanosine | 20 ± 1 | Metabolism | Antineoplastic |
| Topotecan | 25 ± 2 | Oncology | Antineoplastic |
| Camptothecine (S,+) | 36 ± 6 | Oncology | Antineoplastic |
| Azaguanine-8 | 46 ± 24 | Oncology | Antineoplastic |
| Mitoxantrone dihydrochloride | 47 ± 12 | Oncology | Antineoplastic |
| Alendronate sodium | 23 ± 2 | Metabolism | Antiosteoporetic |
| Sertindole | 45 ± 11 | Central Nervous System | Antipsychotic |
| Thonzonium bromide | 1 ± 0 | Dermatology | Antiseptic |
| Carbetapentane citrate | 41 ± 7 | Central nervous system | Antispastic, local anesthetic |
| Mercaptopurine | 24 ± 2 | Immunology, oncology | Immunosuppressant |
| Pyrvinium pamoate | 4 ± 1 | Metabolism | |
| Proscillaridin A | 41 ± 2 | Cardiovascular | |
| Flubendazol | 50 ± 7 | Metabolism | |

dihydrate (7 ± 2%), Daunorubicin hydrochloride (9 ± 1%), Alexidine dihydrochloride (10 ± 8%), Methotrexate (16 ± 4%), Doxorubicin hydrochloride (17 ± 2%), Amethopterin (18 ± 3%), Thioguanosine (20 ± 1%), Methyl benzethonium chloride (20 ± 8%), Alendronate sodium (23 ± 2%), Mercaptopurine (24 ± 2%), Topotecan (25 ± 4%), Thiostrepton (30 ± 3%), Avermectin (30 ± 11%), Camptothecine (S, +) (36 ± 6%), Chlorhexidine (38 ± 13%), Cycloheximide (40 ± 4%), Carbetapentane citrate (41 ± 7%), Proscillaridin A (41 ± 2%), Methiazole (41 ± 6%), Halofantrine hydrochloride (43 ± 9%), Sertindole (45 ± 11%), Azaguanine-8 (46 ± 24%), Mitoxantrone dihydrochloride (47 ± 12%), Niclosamide (48 ± 11%), and Flubendazol (50 ± 7%) (**Figure 4C** and **Table 3**). When we categorized the hits into groups for their therapeutic effects, we observed that the major hit drugs belonged to infectiology and oncology (**Table 3**).

Among the 26 hits, we chose Astemizole, Quinacrine, Doxorubicin, Mitoxantrone, and Topotecan for further studies. KUGBM8-EF cells responded to these drugs in a dose-dependent manner (**Figure 5A**). We then chose to continue with Topotecan, a topoisomerase 1 inhibitor, which has been shown as a radiation sensitizer in glioma cells (Pinel et al., 2006; Lesimple et al., 2009; Kortüm et al., 2016) and has blood brain barrier permeability (Baker et al., 1995; Stewart et al., 2004). Next, to investigate the mode of cell death in KUGBM8-EF cells, we checked cleaved PARP protein expression as an indicator of apoptosis. Accordingly, when KUGBM8-EF cells were treated with low doses of Topotecan (0.3 μM), cleaved PARP levels were low, however, with high dose Topotecan treatment (5 μM), cleaved PARP was markedly observed (**Figure 5B**). These results suggested that Topotecan induces apoptosis in KUGBM8-EF cells.

To investigate whether Topotecan can induce cell death in other primary Glioblastoma cell lines to similar degrees, we treated two different primary cell lines, GBM4 and GBM8, alongside KUGBM8-EF. We observed cell death and dissociation of spheres starting from day 3 of treatment in all cell lines (**Figure 5C**). When we measured cell viability after 5 days upon Topotecan treatment, we observed that cell viability was decreased below 25% for all three cell lines (**Figure 5D**). These results indicate that, our new cell line model, KUGBM8-EF, acts similarly to other known primary cells (Wakimoto et al., 2009).

Lastly, we investigated whether Topotecan can cooperate with TMZ and confers any additive effects on these primary cell lines. When we combined TMZ (250 μM for KUGBM8-EF, 65 μM for GBM4, and 10 μM for GBM8) with low

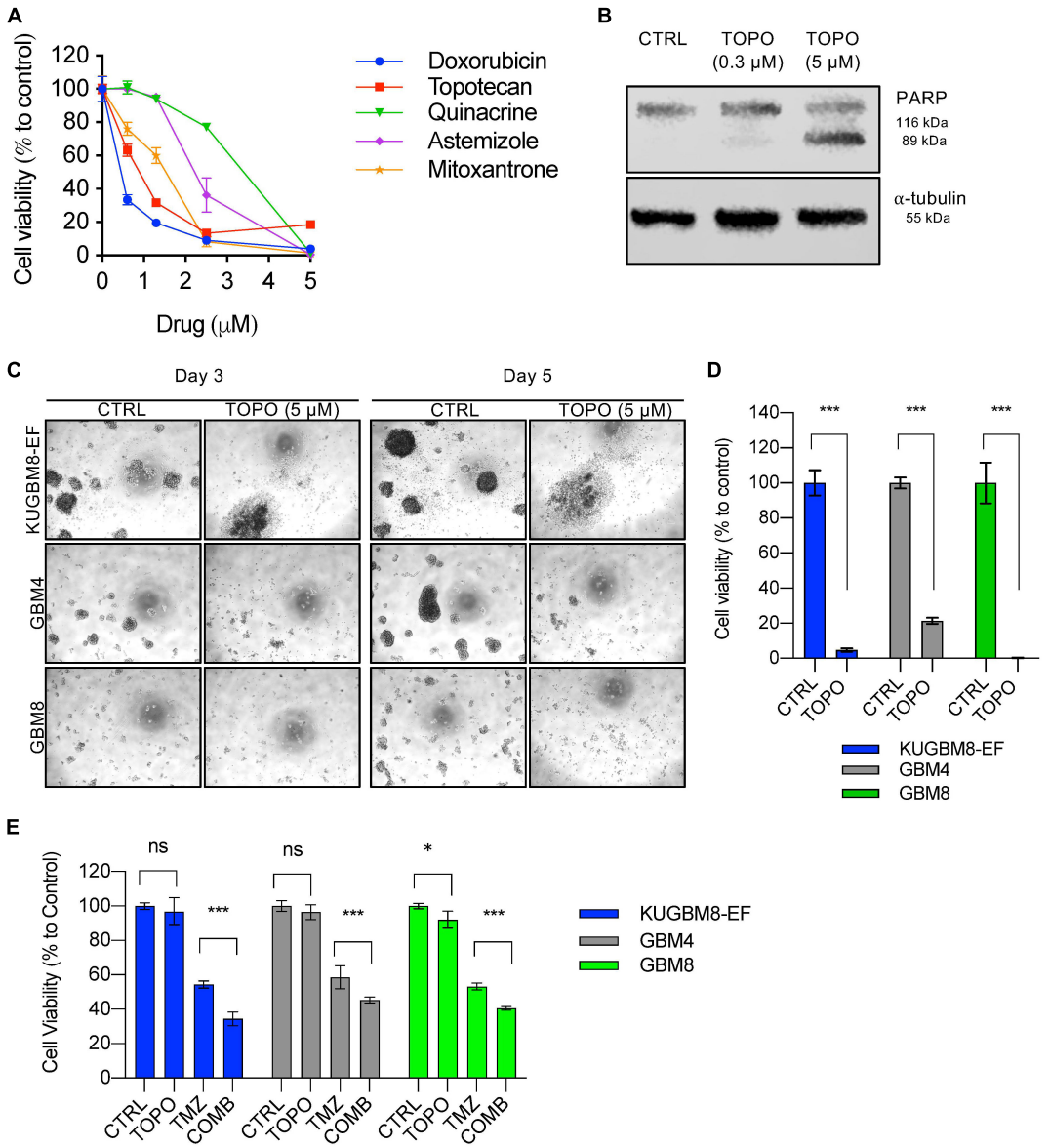


**FIGURE 5 |** Hit validation revealed Topotecan as a potential apoptosis inducer in GBM **(A)** Dose response curves of selected hits treated for 3 days in 96-well format. **(B)** Detection of PARP cleavage after TOPO (0.3 or 5 μM) treatment for 24 h. α-tubulin was used as a loading control. **(C)** Representative photos of Topotecan (5 μM) response of 3 different primary GBM cell lines (KUGBM8-EF, GBM4, and GBM8) after 3 or 5 days of treatment. **(D)** Cell viability graphs of Topotecan (5 μM) treatment for 5 days on KUGBM8-EF, GBM4, and GBM8 cells. **(E)** The effect of low dose Topotecan (0.3 μM) combination with TMZ (250 μM for KUGBM8-EF, 65 μM for GBM4, and 10 μM for GBM8) on different primary GBM cell lines. Cell viability experiments were detected by CTG and viability was normalized to untreated controls. Error bars represent normalized standard deviation. n.s. stands for $p > 0.05$, * stands for $p < 0.05$, *** stands for $p < 0.0001$, *t*-test.

dose Topotecan (0.3 μM) we observed significant cooperation between TMZ and Topotecan in all cell lines (**Figure 5E**). Taken together, Topotecan can be a good candidate to incorporate in glioblastoma treatment in the future.

## DISCUSSION

For high throughput drug screening *in vitro*, feasible and cost-effective *in vitro* models are required. Established cell lines utilized for many years in cancer research have traditionally been cultured in the presence of serum. However, recent studies have shown that serum-based 2D cancer cell line models have different drug response patterns when compared to 3D cell line models (Horning et al., 2008; Hongisto et al., 2013). Especially for glioblastoma, serum based cell line culturing has been shown to cause depletion of cancer stem cell like cells (Singh et al., 2003; Seidel et al., 2014) and poor tumor growth abilities in mice (Lee et al., 2006; Xie et al., 2015). In this study, we established a patient-derived glioblastoma cell

line, KUGBM8-EF, which was grown as spheres in neurobasal medium with supplements. Further analysis has shown that stem cell markers such as *SOX2, NESTIN,* and *PAX6* were highly expressed in KUGBM8-EF cells. In addition, KUGBM8-EF had high capacity for tumor growth in mice brain. Thus, we presented that this non-serum-based 3D cultures of KUGBM8-EF cells can be a useful option for drug screening. Since a repurposed drug comes with systemic toxicity knowledge, cytotoxicity problems can be circumvented when a drug is used for a novel indication. Therefore, we aimed to investigate potential glioblastoma therapy drugs out of a 1,200 FDA-approved drug library utilized at 5 μM dosage for 3 days. We identified 26 drugs that can reduce cell viability below the 50% threshold. Some of them were previously suggested as therapeutic candidates for glioblastoma such as Astemizole (Lee et al., 2016; Sales et al., 2016), Pyrivinium pamoate (Hothi et al., 2012; Lamb et al., 2015; Venugopal et al., 2015), Ivermectin (Liu et al., 2016), Doxorubicin hydrochloride (Jiang et al., 2014), Campthothecine (Lee et al., 2013), Niclosamide (Wieland et al., 2013), Proscillaridin A (Denicolaï et al., 2014), and Topotecan (Patel et al., 2013; Bernstock et al., 2017). Unfortunately, TMZ was not able to decrease cell viability less than 50%, even with the 250 μM dosage added manually to the screen, attesting to the important need for identifying novel therapy options. Topotecan is a topoisomerase I inhibitor derived from Campthothecin, and is approved by FDA for cancer treatments such as cervical, ovarian and small cell lung cancer (Pommier, 2006; Paton et al., 2010). Topotecan is also a blood brain barrier permeable agent (Baker et al., 1995; Stewart et al., 2004), which is a major concern for treatment options for brain cancers. Indeed, there is considerable clinical interest in using Topotecan as it is being tested in phase II clinical trials in combination to TMZ and/or other agents in recurrent gliomas (NCT00002814, 2011; NCT00541138, 2011; NCT00610571, 2012; NCT01931098, 2020). In accordance with its well-established effects in the literature, we demonstrated that Topotecan can induce cell death in various primary glioblastoma cell lines including our new one, KUGBM8-EF. In addition, Topotecan can be combined with TMZ in low dosages to reduce cell viability *in vitro*.

Together, our study adds a new primary glioblastoma cell line model to the growing list of primary lines. Our identification of Topotecan as a lead drug in this model suggests the utility of KUGBM8-EF cells as tool to discover novel glioblastoma therapeutics in the future.

## CONCLUSION

In this study we established new primary glioblastoma cell lines, KUGBM8-DMEM and KUGBM8-EF, utilizing different medium conditions. Here, we showed that culturing freshly derived tumor tissue in serum-free neurogenic medium provides a better alternative to culturing these cells with serum. We also showed that KUGBM8-EF cells can readily grow *in vivo* and respond to frontline TMZ chemotherapy, suggesting its future applications to study the biology of glioblastoma. Performing a high throughput screen with non-serum based KUGBM8-EF with clinically approved drugs, we identified 26 lead drugs that can significantly reduce cell viability Among these, Topotecan, a topoisomerase inhibitor with blood brain barrier permeability, revealed the most efficacy as a single agent or in combination with TMZ.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Koç University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Koç University Institutional Review Board.

## AUTHOR CONTRIBUTIONS

FS and TB-O conceived and planned the experiments, and took lead in writing the manuscript. FS, ACA, AC, FS-P, EB-Y, and İS carried out the experiments and data interpretation. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

## FUNDING

The authors gratefully acknowledge use of the services and facilities of the Koç University Research Center for Translational Medicine (KUTTAM), funded by the Presidency of Turkey, Presidency of Strategy and Budget. The content was solely the responsibility of the authors and does not necessarily represent the official views of the Presidency of Strategy and Budget.

## ACKNOWLEDGMENTS

We thank Zeynep Kahya Yesil for technical assistance. We also thank Dr. Hiroaki Wakimoto (Massachusetts General Hospital, Harvard University) for providing GBM4 and GBM8 primary glioblastoma cell lines.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.578316/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 22 March 2021
doi: 10.3389/fnins.2021.589906

# A New Platinum-Based Prodrug Candidate for Chemotherapy and Its Synergistic Effect With Hadrontherapy: Novel Strategy to Treat Glioblastoma

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**Specialty section:**
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

**Received:** 31 July 2020
**Accepted:** 08 February 2021
**Published:** 22 March 2021

**Citation:**
Ferrari B, Roda E, Priori EC, De Luca F, Facoetti A, Ravera M, Brandalise F, Locatelli CA, Rossi P and Bottone MG (2021) A New Platinum-Based Prodrug Candidate for Chemotherapy and Its Synergistic Effect With Hadrontherapy: Novel Strategy to Treat Glioblastoma. Front. Neurosci. 15:589906. doi: 10.3389/fnins.2021.589906

Glioblastoma (GBM) is the most common tumor of the central nervous system. Current therapies, often associated with severe side effects, are inefficacious to contrast the GBM relapsing forms. In trying to overcome these drawbacks, (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV), also called Pt(IV)Ac-POA, has been recently synthesized. This new prodrug bearing as axial ligand (2-propynyl)octanoic acid (POA), a histone deacetylase inhibitor, has a higher activity due to (i) its high cellular accumulation by virtue of its high lipophilicity and (ii) the inhibition of histone deacetylase, which leads to the increased exposure of nuclear DNA, permitting higher platination and promoting cancer cell death. In the present study, we investigated the effects induced by Pt(IV)Ac-POA and its potential antitumor activity in human U251 glioblastoma cell line using a battery of complementary techniques, i.e., flow cytometry, immunocytochemistry, TEM, and Western blotting analyses. In addition, the synergistic effect of Pt(IV)Ac-POA associated with the innovative oncological hadrontherapy with carbon ions was investigated, with the aim to identify the most efficient anticancer treatment combination. Our *in vitro* data demonstrated that Pt(IV)Ac-POA is able to induce cell death, through different pathways, at concentrations lower than those tested for other platinum analogs. In particular, an enduring Pt(IV)Ac-POA antitumor effect, persisting in long-term treatment, was demonstrated. Interestingly, this effect was further amplified by the combined exposure to carbon ion radiation. In conclusion, Pt(IV)Ac-POA represents a promising prodrug to be incorporated into the treatment regimen for GBM. Moreover, the synergistic efficacy of the combined protocol using chemotherapeutic Pt(IV)Ac-POA followed by carbon ion radiation may represent a promising approach, which may overcome some typical limitations of conventional therapeutic protocols for GBM treatment.

**Keywords: platinum(IV) chemotherapeutics, hadrontherapy, carbon ions irradiation effects, tumor resistance, glioblastoma (GBM), glioblastoma, *in vitro***

## INTRODUCTION

Glioma is a broad category of glial brain and spinal cord tumors which originate in the glial cells that surround and support neurons in the brain, including astrocytes, oligodendrocytes, and ependymal cells. About 33% of all brain tumors are gliomas, accounting for about 80% of the total malignant central nervous system (CNS) tumors in adults (Hanif et al., 2017).

Among these, glioblastoma (GBM) is one of the most common and aggressive primary brain tumor (Davis, 2016), characterized by diffuse infiltration of the adjacent brain parenchyma and development of drug resistance to standard treatment (Chen et al., 2018). So far, GBM remains associated with an extremely aggressive clinical course, and only 0.05–4.7% of patients survive 5 years from diagnosis (Ostrom et al., 2018).

Cellular pleomorphism with nuclear atypia, high mitotic activity, and microvascular proliferation distinguish GBM from other lower-grade gliomas (Hambardzumyan and Bergers, 2015). In addition, the inter- and intra-patient tumor heterogeneity causes several obstacles, limiting the improvement of an early diagnosis and treatment protocols. The tumor microenvironment (TME) plays a crucial role in mediating tumor progression and invasiveness, contributing to tumor aggression and poor prognosis (Yekula et al., 2020). Recent studies have shown that differentiated tumor cells may have the ability to dedifferentiate acquiring a stem-like phenotype in response to microenvironment stresses such as hypoxia. Acidic extracellular pH and nitric oxide were also shown to be involved in stemness preservation (Dahan et al., 2014).

Currently, the standard of care consists of surgical resection followed by radiotherapy (RT) and concomitant and adjuvant chemotherapy. Despite this aggressive treatment regimen, the median survival is only around 15 months, and the 2-year survival rate is only 26.5% (von Neubeck et al., 2015; Chen et al., 2018). Indeed, due to the location of GBM origin and its infiltrative growth (Urbańska et al., 2014), complete surgical resection of the tumor is often not possible other than with a high risk of neurological damages for the patient (Goldbrunner et al., 2018). The main chemotherapeutic agent employed for GBM treatment is temozolomide (TMZ) (Strobel et al., 2019); however, intrinsic or acquired resistance to TMZ often defines the poor efficacy of this drug. Notably, the expression of $O^6$-methylguanine-DNA methyl-transferase (MGMT) unmethylated promoter in some patients is one of the principal mechanisms responsible for this chemoresistance, reducing mean patients' survival (Chen et al., 2018).

Cisplatin (*cis*-dichlorodiammineplatinum, CDDP) is another alkylating agent used as an anticancer chemotherapy drug employed to treat various types of malignancies, including GBM (Dasari and Tchounwou, 2014; Pérez et al., 2019). Despite initial benefits, CDDP is often associated with severe systemic toxicity, which occurs especially after long-term treatment (Karasawa and Steyger, 2015; Chovanec et al., 2017). Among these adverse side effects, neurotoxicity assumed increasing clinical importance as it is dose-cumulative and becomes limiting in long-lasting therapies, thus decreasing CDDP's clinical use in GBM treatment (Staff et al., 2019).

In the attempt to circumvent all the above reported drawbacks, a large number of "non-classical" platinum complexes have been prepared and tested for anticancer activity. In this view, the synthesis of new platinum(IV)-based prodrugs, Pt(IV) may pose great advantages allowing the addition of one or two adjuvant/synergistic agents to the parent cytotoxic Pt(II) square-plane scaffold in axial position. The axial ligands can be used to improve the physical and chemical properties of the complex, designing multifunctional prodrugs, often called "combo" (Gabano et al., 2014; Kenny et al., 2017; Sabbatini et al., 2019). In this way, they can improve the lipophilicity and, consequently, influence passive diffusion, promoting a synergistic intracellular accumulation (Ravera et al., 2015; Raveendran et al., 2016).

In addition, these Pt(IV) derivatives, acting as prodrugs, are rather inert to ligand substitution or hydrolysis, minimizing the off-target interactions and the side effects on healthy cells typical of the more reactive Pt(II) progenitors. Pt(IV) prodrugs can then be reduced in the hypoxic intracellular milieu of tumor cells to the resultant cytotoxic Pt(II) metabolites with the simultaneous loss of the adjuvant or synergistic agents from the axial positions (Najjar et al., 2017; Sabbatini et al., 2019).

In this perspective, the innovative complex (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato) platinum(IV), named Pt(IV)Ac-POA, has been recently synthesized starting from the CDDP which is oxidized with hydrogen peroxide to obtain an intermediate octahedral compound, which is then esterified with the addition of two long-chain carboxylic acids. Pt(IV)Ac-POA contains a different medium-chain fatty acid–histone deacetylase inhibitor (MCFA-HDACi), namely 2-(2-propynyl)octanoate (POA), along with an inert acetate (Ac) as axial ligands (Gabano et al., 2017). This prodrug, bearing as axial ligand POA, has a higher activity because of the high cellular accumulation due to its high lipophilicity and to the inhibition of histone deacetylase that leads to the increased exposure of nuclear DNA, thereby permitting higher platination levels at DNA and promoting cancer cell death (Gabano et al., 2017; Novohradsky et al., 2017). Indeed, the HDACi activity of free POA has been established as an inducer of a strong histone H3 acetylation (at lysine 9 level), presumably acting at the HDAC8 level, contributing to increasing the overall anti-proliferative activity (Oehme et al., 2009; Gabano et al., 2017; Sabbatini et al., 2019). Some experimental studies demonstrated that the new prodrug Pt(IV)Ac-POA exhibited promising antitumor activity both *in vitro*, on different human tumor cell lines, as well as *in vivo*, already at concentrations lower than those standardly used for CDDP, also supporting a reduction of undesirable cytotoxicity on healthy tissues (Gabano et al., 2017; Rangone et al., 2018; Ferrari et al., 2020).

Combined with conventional and innovative chemotherapeutic protocols, radiotherapy has always played an important role in the treatment of deep-seated tumors (Liauw et al., 2013; Gupta et al., 2020). However, it has non-eliminable weak points that derive from the physical nature of the photons, being unable to be completely focused on tumor mass, also damaging healthy tissues adjacent to the target, and so increasing

the risk of detrimental damage. In the past decades, endless progress of hadrontherapy (HT) occurred, bringing technical innovations both in clinical and scientific research (Rossi, 2015; Marvaso et al., 2017). Hadrontherapy displayed less invasiveness than conventional radiotherapy, being also more effective compared to X-ray radiotherapy (Combs et al., 2013). While megavoltage photons used in conventional radiotherapy deposit energy uniformly through the tissue, with the exception of a buildup region in superficial tissue, where a relative dose-sparing occurs (Tinganelli and Durante, 2020), protons and carbon ions, releasing energy at the inverse of their velocity, present a pronounced peak, called the Bragg peak, at a deeper point (Ebner and Kamada, 2016). Indeed, carbon ion beams allow an improved dose distribution, leading to the concentration of enough dose within a target volume while minimizing the dose in the adjacent healthy tissues (Kamada et al., 2015). Furthermore, in contrast to X-rays, protons and ions are heavy particles, so they can penetrate the tissue without deviating much from the initial direction, and with their electric charge, they tear electrons from the tissue molecules, depositing most of their energy in the last centimeters of the path, providing a higher action (Kamada et al., 2015) and, in the case of carbon ions, achieving a greater relative biological effectiveness (RBE). Carbon ions are also less dependent on the oxygen enhancement ratio (OER), which in radiobiology refers to the increased effect of ionizing radiation due to the presence of oxygen. This would allow carbon ion radiotherapy (CIRT) to eradicate hypoxic glioblastoma cells, for example following an anti-angiogenic therapy. The induction of apoptosis, autophagy, and cellular senescence is a set of mechanisms underlying the killing of glioblastoma cells mediated by the irradiation of carbon ions (Jinno-Oue et al., 2010; Tomiyama et al., 2010). Furthermore, it has been demonstrated that such radiations would be able to inhibit the migratory capacity of glioma cells through a reduction in the expression of integrins (Rieken et al., 2012). It has also recently been hypothesized that carbon ion radiation can overcome the intrinsic radioresistance of cancer stem cells (Pignalosa and Durante, 2012); besides, it may be a promising therapy for pediatric brain tumors, decreasing side effects related to CNS sensibility (Laprie et al., 2015; Combs, 2018).

Based on all these previously reported knowledge, the present work aimed at characterizing *in vitro* the action of the recently synthesized platinum-based compound Pt(IV)Ac-POA using human U251 cell lineage as one of the GBM-specific models, typically characterized by drug resistance properties (Naidu et al., 2010; Liu et al., 2015; Lin et al., 2018). Specifically, a first experimental phase was conducted to identify the Pt(IV)Ac-POA efficient cytotoxic concentration and the involved cell death pathways after both short- and long-term exposure, also assessing the potential clonogenicity impairment. Then, the second step of the study was devoted to addressing the potential synergistic action of Pt(IV)Ac-POA treatment followed by carbon ion radiation, with the final goal of assessing the efficacy and feasibility of a therapeutic protocol combining chemotherapy and carbon ion radiation therapy in acute and long-term GBM treatment.

# MATERIALS AND METHODS

## Cell Culture

Human U251 MG cell line (Sigma-Aldrich, Milan, Italy) was cultured in Eagle's minimal essential medium (EMEM) supplemented with 2 mM l-glutamine, 1% non-essential amino acid (NEAA), 1% sodium pyruvate, 10% fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cell culture was maintained at 37°C in a humidified atmosphere (95% air/5% $CO_2$). All cell culture reagents were purchased from Celbio S.p.a. and Euroclone S.p.a. (Pero, Milan, Italy).

## Experimental Design

### Pt(IV)Ac-POA Dose Selection: Cell Viability and Proliferation Evaluated by MTS Assay

In order to select the proper Pt(IV)Ac-POA dose to be used in all the following analyses, as a first experimental step, a range of Pt(IV)Ac-POA concentrations was evaluated through the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay.

Briefly, the cell viability test was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) kit. A volume of 200 μl of cells was suspended at a density of 5,000 cells/well, transferred to a 96-well plate (0.2 ml per well), and incubated at 37°C for 24 h in a humidified atmosphere containing 5% of $CO_2$. Subsequently, the culture medium was replaced with a fresh medium to then carry out the requested treatment. As a control, the cells were incubated with the culture medium alone. For Pt(IV)Ac-POA exposure, a range of concentrations, ranging from 1 to 40 μM, was prepared by dissolving the prodrug in the specific culture medium. The dose range was chosen based on previous works in which the effects of the prodrug Pt(IV)Ac-POA were assessed on tumor cell lines of the nervous system, i.e., B50 neuroblastoma and C6 glioma rat cells, respectively (Rangone et al., 2018; Ferrari et al., 2020). Forty-eight hours after exposure, the culture medium was replaced with fresh medium, the MTS solution (20 μl/well) was added to each well in the darkness, and the plates were then incubated for about 3 h at 37°C. At the end of the incubation time, the quantification was performed by measuring the samples' absorbance at 490 nm with the ELx808TM Absorbance Microplate Reader (Bio-Tek Instruments, Inc.). Data were expressed as a percentage of control.

Percentage cell viability was calculated using the following formula:

$$\textit{Cell viability}\ (\%) = (\textit{Abs}490\ \textit{treated cells}/\textit{Abs}490\ \textit{control cells}) \times 100$$

### Pharmacological Treatment and Exposure Conditions

Forty-eight hours before the experiments, the cells were seeded on glass coverslips (20,000 cells) for fluorescence microscopy evaluations or grown in 75-$cm^2$ plastic flasks for flow cytometry, Western blotting, and transmission electron microscope (TEM) ultrastructural analysis. Cell exposure to chemotherapeutic drugs was performed at 37°C.

To compare the efficacy of the prodrug Pt(IV)Ac-POA to that observed after the conventional CDDP treatment (Teva Pharma, Milan, Italy), a 40 μM CDDP concentration was selected based on previous *in vitro* investigations (Bottone et al., 2008; Grimaldi et al., 2019) as well as *in vivo* experimental studies (Bottone et al., 2008; Cerri et al., 2011), employing a single subcutaneous injection (5 μg/g, b.w.) in 10-day-old rats, corresponding to the therapeutic dose already employed in clinical practice (Bodenner et al., 1986; Dietrich et al., 2006).

Cell lines were exposed to the diverse platinum compounds according to the following protocols:

(i) Standard acute test: 48-h continuous treatment (CT) to Pt(IV)Ac-POA or CDDP.

(ii) Standard acute test (48-h CT) to Pt(IV)Ac-POA or CDDP, followed by a 7-day recovery phase in drug-free normal EMEM, namely "recovered" (REC) condition.

### Carbon Ion Radiotherapy

Before the experiments, U251 cells were seeded on culture flasks or flasks sterile on slide 18 × 50 mm (200,000 cells) (Thermo Scientific$^{TM}$ Nunc$^{TM}$ Lab-Tek$^{TM}$) for fluorescence microscopy (**Figure 1Ab**). Then, the cells were treated with platinum compounds for 48-h CT. At the end of this drug exposure, U251 cells were irradiated with the clinical carbon ion beam at the CNAO Foundation in Pavia. In detail, the flasks were positioned in a water phantom placed at a depth of 15 cm, corresponding to the central position of a 6-cm-wide (from 12 to 18 cm depth in water) spread-out Bragg peak (SOBP) (**Figures 1Ac,d**). The cells were vertically irradiated with a horizontal beam according to the protocol envisaged for clinical use of carbon ion therapy at CNAO (Facoetti et al., 2015; Mirandola et al., 2015). The SOBP, homogeneous in terms of the dose absorbed in water, was obtained with a modulation of the pencil beam using 31 different energies in the range from 246 to 312 MeV/u. The linear energy transfer (LET) at a depth of 15 cm is equivalent to about 46 keV/u. For this study, the cellular samples were irradiated at room temperature with 2 or 4 Gy physical dose. The irradiation times varied between 15 and 30 min to reach a dose of 2 or 4 Gy, respectively. These radiation doses were selected based on previous literature data considering treatments carried out *in vitro* and related to the fractionated focal irradiation in daily fractions of 2 Gy given 5 days per week for 6 weeks, for a total of 60 Gy used in GBM patients and other tumors (Stupp et al., 2005; Dhermain, 2014; Roach et al., 2018; Ma et al., 2019).

At the end of the experiment, for the analysis of the "standard acute test" condition, pellets for protein extraction were immediately obtained from flasks, while the cells of the flasks sterile on slide were fixed with 4% formalin for 20 min and post-fixed with 70% ethanol at −20°C for at least 24 h for immunocytochemical procedures. In parallel, to evaluate the "REC" condition, at the end of the irradiation, the medium was discarded and replaced with a drug-free fresh medium, followed by a 7-day recovery phase, and then the samples were collected for protein extraction and immunohistochemical procedures as described above.

### Clonogenic Cell Survival Assay

Clonogenic assay was performed as previously reported (Shen et al., 2015; Ballestreri et al., 2018; Riaz et al., 2019). Briefly, U251 cells were treated as indicated [40 μM CDDP or 1–40 μM Pt(IV)Ac-POA] for 48-h CT. Next, they were removed using trypsin–EDTA 0.10%, seeded in six-well plates, and incubated for 10 days undisturbed, allowing them to form colonies in drug-free medium. To investigate the effects of 10 μM Pt(IV)Ac-POA plus carbon ion radiation, the cells were irradiated with 1, 2, or 4 Gy physical dose after exposure to Pt(IV)Ac-POA (10 μM). The cells were then plated in six-well plates at low densities and colonies were counted after 10 days.

After this 10-day time window, the colonies were gently washed with phosphate-buffered saline (PBS), fixed, and stained with crystal violet solution (0.5% in $H_2O$/methanol, 1:1) (Sigma Chemical Co., St. Louis, MO, United States). Stained colonies, defined as groups of ≥ 50 cells, were scored using an Olympus CKX41 inverted microscope combined with an Olympus MagniFire digital camera. Plating efficiency (PE) was calculated as the number of colonies counted divided by the number of cells seeded, considering the colonies formed by control cells as 100%. The average of these values was reported as "surviving fraction."

## Flow Cytometry

After 48-h CT to the diverse chemotherapeutics, the cells were detached by mild trypsinization (0.25% in PBS, with 0.05% EDTA) to obtain single-cell suspensions to be processed for flow cytometry with a Partec PAS-III flow cytometer (Münster, Germany), equipped with argon laser excitation (power, 200 mW) at 488 nm. Data were analyzed with the built-in software (Flowmax, Partec).

### Cell Cycle Analysis

The cells were washed in PBS, permeabilized in 70% ethanol for 10 min, treated with RNase A 100 U/ml, and then stained for 10 min at room temperature (RT) with propidium iodide (PI) 50 μg/ml (Sigma-Aldrich, Milan, Italy) 1 h before flow cytometry analysis. PI red fluorescence was detected with a 610-nm long-pass emission filter. At least 20,000 cells per sample were measured to obtain the distribution among the different phases of the cell cycle and the percentage of apoptotic cells.

### Annexin V Assay: Apoptosis Identification

Single-cell suspensions, obtained as described above, were incubated with annexin V–fluorescein isothiocyanate (FITC) (Annexin V-FITC Apoptosis Detection Kit, Abcam, Italy) for 10 min in the dark at RT. PI was used as a counterstain to discriminate necrotic/dead cells from apoptotic ones. Fluorescence was revealed by means of flow cytometry at 488 nm excitation and with 530/30 (FITC) and 585/42 nm (PI) band-pass emission filters.

## TEM Ultrastructural Investigation

Control and treated cells were harvested by mild trypsinization (0.25% trypsin in PBS containing 0.05% EDTA) and collected by centrifugation at 800 rpm for 5 min in fresh tubes. The samples

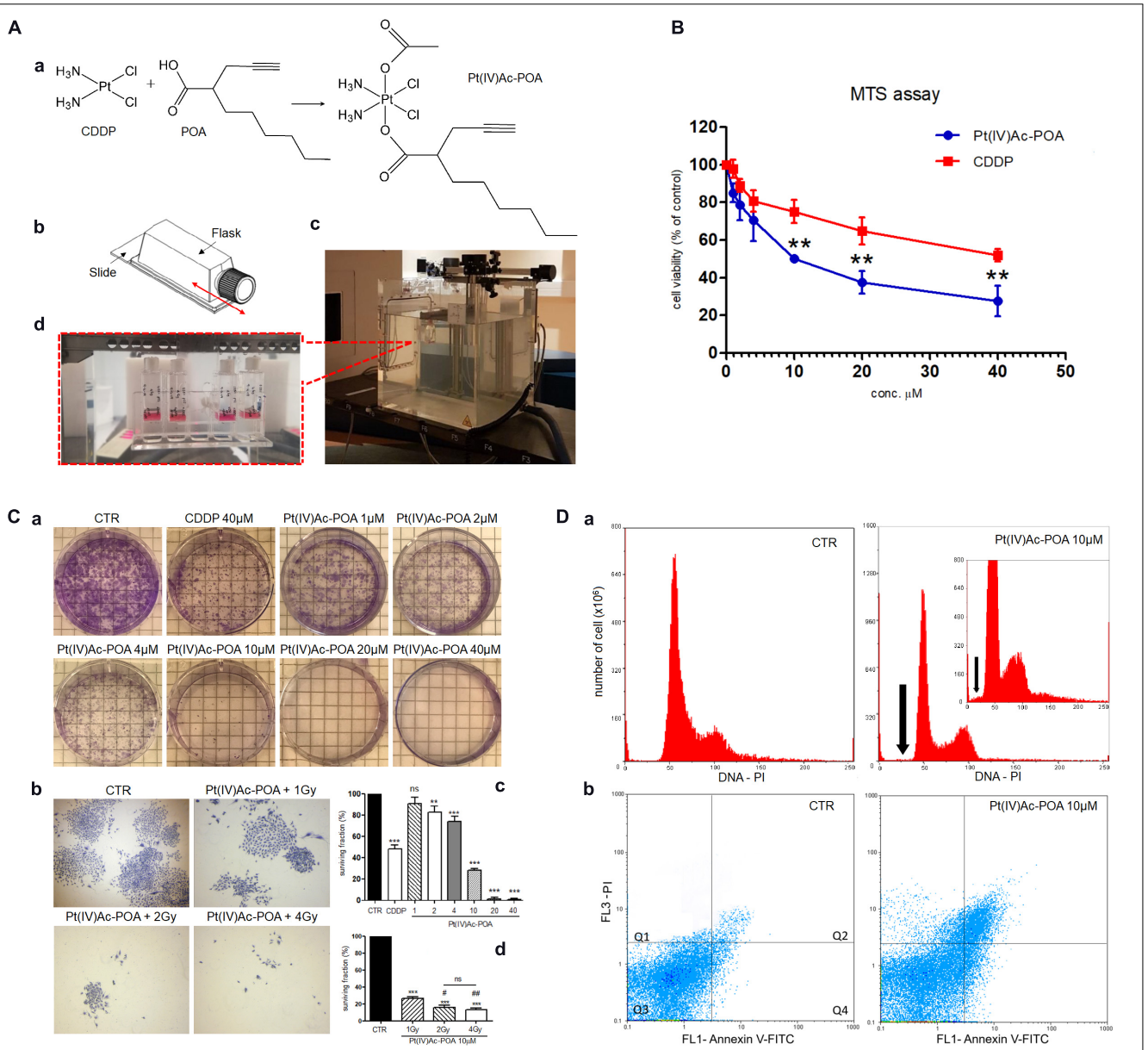


**FIGURE 1 | (A)** Schematic representation of (*OC*-6-44)-acetatodiamminedichlorido (2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA] synthesis process and carbon ion irradiation. **(a)** Cisplatin (*cis*-dichlorodiammineplatinum, CDDP), 2-(2-propynyl)octanoic acid (POA), and the related Pt(IV) mixed derivative (Pt(IV)Ac-POA). **(b)** Flask sterile on slide used for U251 cell culture irradiation. **(c)** Equipment setup in the irradiation room facility at the CNAO Foundation. **(d)** Flasks positioned in a water phantom placed at a depth of 15 cm, corresponding to the central position of a 6-cm-wide spread-out Bragg peak (SOBP), even before irradiation with a horizontal beam. **(B)** Effects of Pt(IV)Ac-POA and CDDP on cell viability and proliferation of human U251 MB cell lineage. Cell viability/proliferation obtained using MTS assay after standard acute exposure, i.e., 48-h continuous treatment (CT), to increasing concentrations (1–40 μM) of either Pt(IV)Ac-POA or CDDP. The relative cell viability is expressed as a percentage relative to the untreated control cells. Data represent the mean ± SD. Statistical analysis by Student's *t*-test: different from CDDP (**$p < 0.01$). **(C)** Clonogenic cell survival assay showing the dose–response effect after 48-h CT to increasing concentrations of Pt(IV)Ac-POA alone **(a,c)** and combined with increasing carbon ion radiation doses (1–4 Gy) **(b,d)**. **(c,d)** Histograms showing the U251 surviving fraction (in percent). Statistical analysis by one-way ANOVA followed by Dunnett's test (histograms in **(c)** or *post hoc* Bonferroni's test (histograms in **d**). Statistical significance calculated as follows: *control *vs*. each experimental condition; # Pt(IV)Ac-POA + 1 Gy *vs*. Pt(IV)Ac-POA CDDP + 2 or 4 Gy; *ns*, not significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; #$p < 0.05$; ##$p < 0.01$; ns: not significant. **(D)** Flow cytometry data after annexin V and propidium iodide (PI) staining. **(a)** Cytograms showing the DNA content after PI staining in U251 MB cell lineage: controls *vs*. treated cells [10 μM Pt(IV)Ac-POA 48-h CT]. *Insert* shows the cytogram of the 10 μM Pt(IV)Ac-POA-treated sample compared to a control sample with an identical number of analyzed cells ($y = 800 \times 10^6$). The *black arrow* indicates the sub-$G_1$ peak corresponding to dead cells. **(b)** Dual-parameter cytograms of FITC-labeled annexin V (FL1) *vs*. PI staining (FL3) in controls (CTR) and cells exposed to 10 μM Pt(IV)Ac-POA 48-h CT. Quadrants Q1, Q2, Q3, and Q4 show necrotic, late apoptotic, viable, and early apoptotic cells, respectively.

were immediately fixed with 2.5% glutaraldehyde (Polysciences, Inc., Warrington, PA, United States) in culture medium (2 h at room temperature), centrifuged at 2,000 rpm for 10 min, and washed in PBS. Later, the samples were post-fixed in 1% $OsO_4$ (Sigma Chemical Co., St. Louis, MO, United States) for 2 h at room temperature and washed in water. The cell pellets were pre-embedded in 2% agar, and dehydrated with increasing concentrations of acetone (30, 50, 70, 90, and 100%, respectively). Finally, the pellets were embedded in Epon resin and polymerized at 60°C for 48 h. Ultrathin sections were obtained with ultramicrotome Rechter, then located on nickel grids, and stained with uranyl acetate and lead citrate. Sections were observed under a Zeiss EM 900 TEM (Carl Zeiss S.p.A., Milan, Italy) operating at 80 kV. The plates, after being developed, have been computerized through an Epson Perfection 4990 photo scanner at a resolution of 800 dpi and then processed using the Epson Scan software.

## Western Blotting

Control and treated cells were washed twice with PBS and lysed in RIPA (radioimmunoprecipitation assay) buffer (1 M Tris-HCl, pH 7.6, 0.5 M EDTA, pH 8, 5 M NaCl, 100% NP40 Nonidet), with the addition of protease and phosphatase inhibitors at 4°C for 30 min. Proteins were quantified using the Bradford reagent (Sigma-Aldrich, Milan, Italy). Samples were electrophoresed in a 15% SDS-PAGE minigel and transferred onto a nitrocellulose membrane (BioRad, Hercules, CA, United States) by semidry blotting for 1.30 h under a constant current of 60 mA. The membranes were saturated for 30 min with PBS containing 0.2% Tween–20 and 5% skim milk and incubated overnight with the antibody reported in **Table 1**. After several washes with PBS-Tween, the membranes were incubated for 30 min with the proper secondary antibody conjugated with horseradish peroxidase. Immunoreactive bands were detected with the reagent Luminata$^{TM}$ Crescendo (Merk Millipore, Billerica, MA, United States), according to the appropriate instructions, and revealed on Amersham HyperfilmTM ECL (GE Healthcare, Little Chalfont, United Kingdom) slabs. ImageJ software was used to obtain the density bar chart of the protein bands which are normalized to the respective actin and the loading control. At least three independent experiments were carried out.

## Immunofluorescence Reactions

Control and treated cells were grown on coverslips, fixed with 4% formalin (20 min), and post-fixed with 70% ethanol at −20°C for at least 24 h. The samples were rehydrated for 10 min in PBS and then immunolabeled with primary antibodies diluted in PBS for 1 h at RT in a dark moist chamber. The cells were then washed three times with PBS and incubated for 45 min with the proper secondary antibody diluted in PBS. The cells were therefore counterstained for DNA with 0.1 μg/ml of Hoechst 33258 (Sigma-Aldrich, Milan, Italy) for 6 min, washed with PBS, and finally mounted in a drop of Mowiol (Calbiochem-Inalco, Italy) for fluorescence microscopy. For each experimental condition, three independent experiments were carried out.

An Olympus BX51 microscope equipped with a 100-W mercury lamp was used under the following conditions: 330–385 nm excitation filter (excf), 400 nm dichroic mirror (dm), and 420 nm barrier filter (bf) for Hoechst 33258; 450–480 nm excf, 500 nm dm, and 515 nm bf for the fluorescence of Alexa 488; 540 nm excf, 580 nm dm, and 620 nm bf for Alexa 594. Images were recorded with an Olympus MagniFire camera system and processed with the Olympus Cell F software. The primary and secondary antibodies used for immunofluorescence reactions are summarized in **Table 2**.

### Immunocytochemical Evaluations

For the immunofluorescence quantifications, three independent experiments were performed for each experimental condition. After reactions, image acquisition was performed by Cell F software and the analysis was achieved using ImageJ software. For each condition, 11 quadrants were evaluated for a random analysis. The channels of each fluorescence have been split to obtain the single images in a grayscale, where the minimum value is 0 (black) and the maximum value is 255 (white). Then, the mean values were normalized to the control and expressed as a percentage.

## Statistical Analysis

In the present study, data are presented as the mean ± SEM or mean ± SD over the mean experimental values of each of three independent experiments. The statistical analyses were carried out using Student's *t*-test or one-way ANOVA, followed by either *post hoc* Bonferroni's test or Dunnett's test, performed using GraphPad Prism Inc. 5.03 (GraphPad Software Inc., La Jolla, CA, United States) and R software. A $\rho$ value $< 0.05$ was considered statistically significant.

# RESULTS

The Pt(IV)Ac-POA concentration range presently tested on U251 cells was chosen based on previous *in vitro* data obtained with

**TABLE 1 |** Antibodies employed for Western blotting analyses.

| Antigen | Primary antibody | Dilution in PBS | Secondary antibody | Dilution in PBS |
|---|---|---|---|---|
| *PARP-1* | Rabbit monoclonal anti-PARP-1 (Cell Signaling Technology, Danvers, United States) | 1:1000 | Goat anti-rabbit horseradish peroxidase (Dako, Milan, Italy) | 1:2000 |
| *p62/SQSTM1* | Mouse monoclonal anti- p62/SQSTM1 (Abcam, Cambridge, United States) | 1:1000 | Goat anti-mouse horseradish peroxidase (Dako, Milan, Italy) | 1:2000 |
| *Actin* | Mouse monoclonal anti-actin (Developmental Studies Hybridoma Bank, Iowa City, United States) | 3:500 | Goat anti-mouse horseradish peroxidase (Dako, Milan, Italy) | 1:2000 |

**TABLE 2 |** Primary/secondary antibodies and respective dilution used for immunofluorescence experimental procedures.

| Antigen | Primary antibody | Dilution in PBS | Secondary antibody | Dilution in PBS |
|---|---|---|---|---|
| *Caspase-3* | Rabbit monoclonal anti-caspase-3 (Cell Signaling Technology, Danvers, United States) | 1:200 | Alexa 594-conjugated anti-rabbit antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *Caspase-8* | Rabbit monoclonal anti-caspase-8 (Cell Signaling Technology, Danvers, United States) | 1:100 | Alexa 594-conjugated anti-rabbit antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *PARP-1* | Rabbit monoclonal anti-PARP-1 (Cell Signaling Technology, Danvers, United States) | 1:200 | Alexa 594-conjugated anti-rabbit antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *RIP1* | Rabbit polyclonal anti-RIP1 (Santa Cruz Biotechnology) | 1:200 | Alexa 594-conjugated anti-rabbit antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *MLKL* | Mouse monoclonal Anti-MLKL Antibody, clone 3H1 (Sigma-Aldrich) | 1:200 | Alexa 594-conjugated anti-mouse antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *LC3B* | Rabbit polyclonal anti-LC3B (Cell Signaling Technology, Danvers, United States) | 1:400 | Alexa 594-conjugated anti-rabbit antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *p62/SQSTM1* | Mouse monoclonal anti- p62/SQSTM1 (Abcam, Cambridge, United States) | 1:100 | Alexa 488-conjugated anti-mouse antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *AIF* | Rabbit polyclonal anti-AIF (Cell Signaling Technology, Danvers, United States) | 1:200 | Alexa 594-conjugated anti-rabbit antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *Golgi* | Human autoimmune serum recognizing proteins of Golgi Apparatus[a] | 1:200 | Alexa 594-conjugated anti-human antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *Mitochondria* | Human autoimmune serum recognizing the 70 kDa E2 subunit of the pyruvate dehydrogenase complex[b] | 1:200 | Alexa 594-conjugated anti-human antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *α-tubulin* | Mouse monoclonal anti-α-tubulin (Cell Signaling Technology, Danvers, United States) | 1:1000 | Alexa 488-conjugated anti-mouse antibody (Alexa Fluor, Molecular Probes, Invitrogen) | 1:200 |
| *Actin* | Alexa 488-Phalloidin /Alexa 594-Phalloidin (Molecular Probes, Invitrogen) | 1:500 | — | — |

[a]*Santin et al., 2011*; [b]*Bottone et al., 2008*.

conventional CDDP (40 μM) and new platinum(II) compounds such as [Pt(*O*,*O*′-acac)(γ-acac)(DMS)]. The MTS assay data revealed that both CDDP and Pt(IV)Ac-POA (**Figures 1A,B**) caused a dose-dependent cytotoxicity, with the prodrug showing a significant more marked effect already at 10 μM, which became more pronounced at the higher doses, i.e., 20 and 40 μM. In particular, the Pt(IV)Ac-POA concentration of 10 μM was found to be a half maximal inhibitory concentration, able to induce cell death, causing a significant decrease (about 50%) in the number of living proliferating cells (**Figure 1B**). In addition, the clonogenic assay results revealed that (i) 40 μM CDDP was able to cause a 50% colony formation reduction, while (ii) exposure to increasing Pt(IV)Ac-POA concentrations induced a dose-dependent colony formation inhibition, with a surviving fraction of 30% after the 10 μM dose and a further clonogenic impairment (>90%) at the higher tested doses (20 and 40 μM) (**Figures 1Ca,c**).

Based on these results, the concentration of 10 μM Pt(IV)Ac-POA was selected, under the standard acute test condition (48-h CT), and then employed for all the following analyses.

## Cell Cycle Distribution and Cell Death

Concerning the cytofluorimetric analysis (**Figure 1D**), the graphics in **Figure 1Da** illustrate the DNA distribution in U251 control and 48-h CT cells exposed to 10 μM Pt(IV)Ac-POA. In the control condition, the cells were normally distributed among the different cell phases ($G_1$, S, and $G_2$). Differently, after 10 μM Pt(IV)Ac-POA 48-h CT, a slight decrease (14.73 ± 0.51%) of the S phase was observed. In appraising the cytograms, the presence of a sub-$G_1$ peak was evidenced in the treated sample only, indicating high mortality of the cell population. Accordingly, the annexin V/PI staining confirmed that, compared to the control condition, after 10 μM Pt(IV)Ac-POA 48-h CT, an increase in the number of apoptotic cells was identified (**Figure 1Db**). In particular, the biparametric cytograms showed that the control (CTR) cell population was predominantly viable (97.51 ± 0.11%), with only 0.47 ± 0.09% and 1.22 ± 0.03% of early and late apoptotic cells, respectively. In contrast, in the treated sample, a strong reduction of living cells (50.13 ± 0.08%), paralleled by an augment of early (21.52 ± 0.12%) and late (25.74 ± 0.43%) apoptosis, was observed. A slight increase of necrotic cells (2.61 ± 0.04%) was also detected in the treated sample only.

## Ultrastructural Features by TEM

Based on our previous *in vitro* findings (Gabano et al., 2017; Rangone et al., 2018; Ferrari et al., 2020) demonstrating the possible activation of different mechanisms after exposure to Pt(IV)Ac-POA, morphological changes were analyzed by electron microscopy. In the control condition, cells were characterized by the presence of the nucleus showing a decondensed chromatin, a well-organized endoplasmic reticulum (ER), located in the perinuclear area, and medium-sized mitochondria (**Figure 2A**), together with the presence of cytoplasmic protrusions around the cell body. Differently, after 10 μM Pt(IV)Ac-POA 48-h CT, different ultrastructural alterations were detected. Specifically, typical

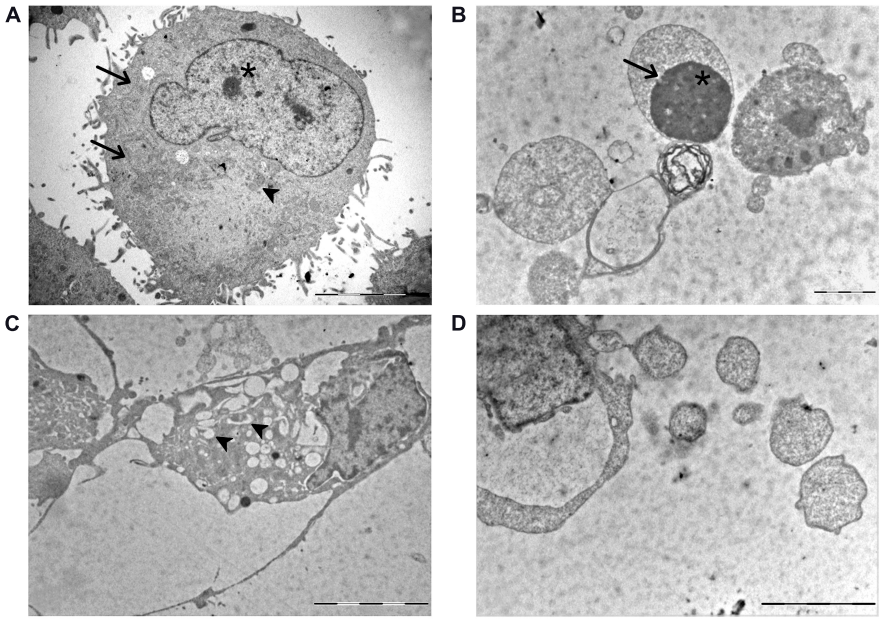


**FIGURE 2 |** TEM ultrastructural analysis. **(A)** U251 control cells: *arrowhead* indicates the mitochondria with a physiological feature; *asterisk* identifies a well-visible nucleolus with a decondensed chromatin. A well-organized endoplasmic reticulum (ER) is also visible in the perinuclear zone (*thin arrows*). **(B–D)** U251 cells after 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA] 48-h continuous treatment (CT). **(B)** Chromatin condensation (*asterisk*) and absence of the nuclear envelope (*thin arrows*) in apoptotic cells. **(C)** Several vacuoles engulfed with cell debris (*arrowheads*) in autophagic cells. **(D)** Typical necroptotic features accompanied by a partially preserved cytoplasmic membrane. *Scale bar*, 5 μm.

features representative of different cell death pathways were identified: (i) chromatin condensation and disappearance of the nuclear envelope, as a distinctive apoptosis hallmarks (**Figure 2B**); (ii) increased number of vacuoles containing cell debris, as a crucial element of autophagy (**Figure 2C**); and (iii) progressive chromatin condensation paralleled by a partially preserved cytoplasmic membrane, typically ascribable to necroptosis (**Figure 2D**).

## Apoptotic Cell Death Pathway Evaluation

Based on the distinctive features detected by TEM, the expressions and possible changes of the different proteins mainly involved in the diverse cell death pathways were assessed by immunofluorescence. Together with the standard acute test, the "REC" condition, previously mentioned in *Materials and Methods* (see section "Pharmacological Treatment and Exposure Conditions"), was included in these evaluations in order to reveal potential long-term defense strategies played by tumor cells several days after 10 μM Pt(IV)Ac-POA or 40 μM CDDP 48-h CT, during which the cells were maintained in drug-free normal EMEM. CDDP exposure was performed as a reference parameter, being the conventional treatment, already investigated in several studies (Grimaldi et al., 2016, 2019; Peng et al., 2018; Islam and Abdelilah Aboussekhra, 2019; Kuo et al., 2019).

The activation of the intrinsic and extrinsic apoptotic pathways was investigated focusing on cleaved caspase-3 and caspase-8, respectively (**Figure 3**). An increased immunopositivity was observed for both evaluated proteins, after both 10 μM Pt(IV)Ac-POA and 40 μM CDDP 48-h CT, compared to the control cells characterized by a lack of labeling. Notably, a progressive increase of immunofluorescence was detected in the REC samples when compared both to the controls as well as to their respective 48-h CT. Indeed, the greatest apoptogenic effect was observed in the REC samples, with the highest outcomes in those exposed to 10 μM Pt(IV)Ac-POA. The actin cytoskeleton immunolabeling revealed a well-defined structural organization in the control cells. In contrast, after both 10 μM Pt(IV)Ac-POA and 40 μM CDDP 48-h CT, some typical cellular degeneration features were detected, characterized by the cytoskeleton collapsing around the highly degraded nuclei. These alterations were also detectable in the Pt(IV)Ac-POA REC sample, while a partial restoration of normal cell morphology was observed in CDDP REC cells. The consequent quantitative measurements of caspase-3 and caspase-8 immunofluorescence confirmed an increased immunopositivity in cells after 10 μM Pt(IV)Ac-POA compared to both 40 μM CDDP 48-h CT and control conditions (**Figure 3**). A strong increase in caspase-3 and caspase-8 immunopositivity was also observed both in Pt(IV)Ac-POA REC samples as well as in CDDP REC cells, with the former showing the greatest increase in immunopositive cell density. The quantification values are summarized in **Table 3** with their respective significance.

Afterward, the activation of the cleaved caspase-3 substrate, i.e., poly[ADP-ribose] polymerase 1 (PARP-1), was investigated (**Figure 4**). In U251 control cells, PARP-1 was localized at the nuclear level, and the well-organized tubulin cytoskeleton ensured a normal cell morphology. After both 10 μM Pt(IV)Ac-POA and 40 μM CDDP 48-h CT, the cells underwent

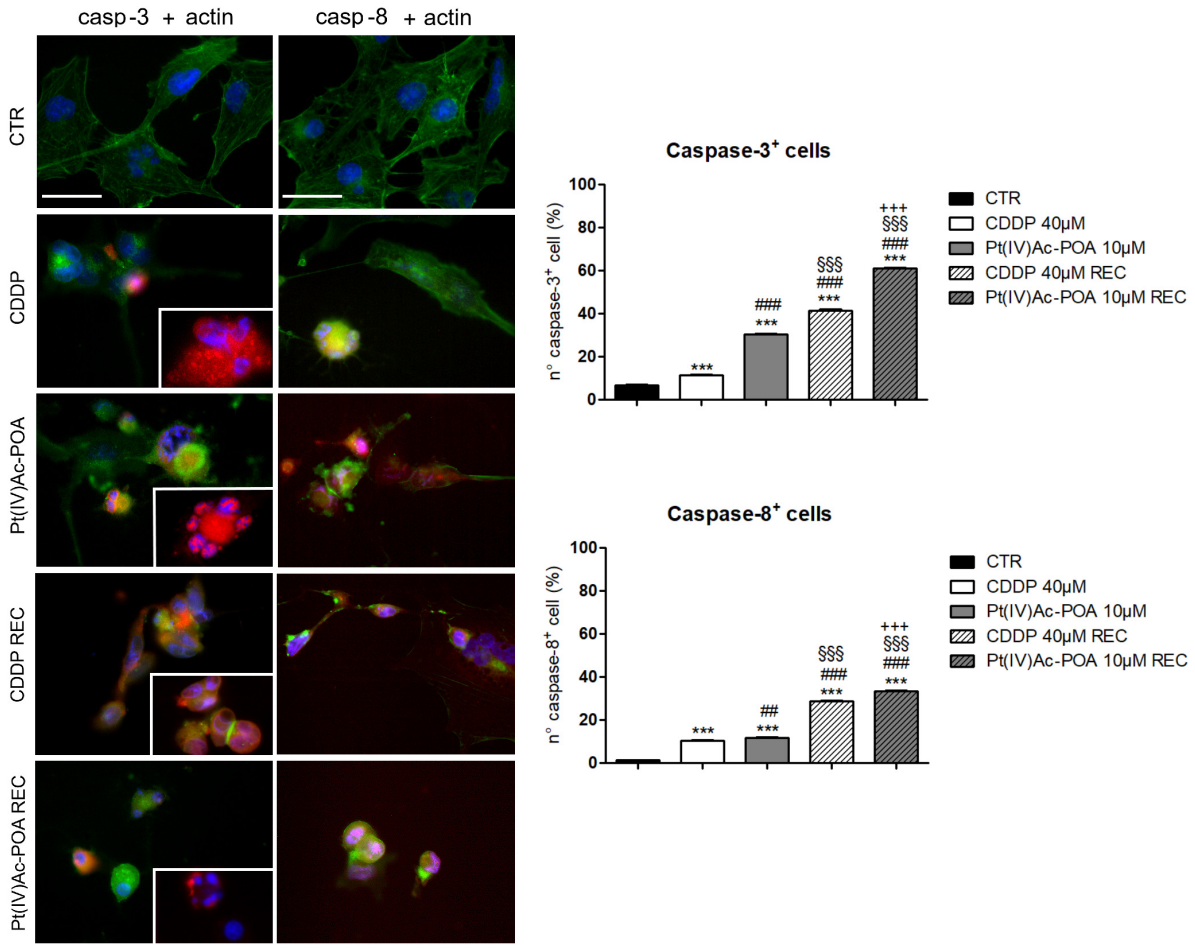


**FIGURE 3 | Left panel:** Apoptotic pathway investigated by fluorescence microscopy in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. Double immunolabeling for active caspase-3 and caspase-8 (*red fluorescence*) and actin (*green fluorescence*). Nuclear counterstaining with Hoechst 33258 (*blue fluorescence*). *Inserts*: high-magnification micrographs showing caspase-immunopositive nuclei and collapsed cytoskeleton. *Scale bar*, 40 μm. **Right panel:** Histograms showing the percentage value of caspase-3- and caspase-8-immunopositive cells, respectively. Statistical significance calculated as follows: *control *vs*. each experimental condition; $^{\#}$CDDP *vs*. other treatments; $^{\S}$Pt(IV)Ac-POA *vs*. REC conditions; $^{+}$CDDP REC conditions *vs*. Pt(IV)AC-POA REC. $^{***}p < 0.001$. $^{\#\#\#}p < 0.01$; $^{\#\#}p < 0.001$; $^{\S\S\S}p < 0.001$; $^{+++}p < 0.001$.

apoptosis, as indicated by the presence of several degraded nuclei. Interestingly, different cell death phases were observed: (i) late apoptosis, characterized by the lack of PARP-1 immunofluorescence in the nuclei, and (ii) early apoptosis, in which PARP-1, or rather the p89 fragment, moved from the nucleus to the cytoplasm. This latter phenomenon was especially evident after 10 μM Pt(IV)Ac-POA. On the other hand, after 40 μM CDDP 48-h CT, cells with a healthy phenotype still showed a nuclear PARP-1 signal. In the CDDP REC condition, the fluorescent "spot-like" PARP-1 signal was observed. In this condition, apoptosis was also observed, paralleled by some features resembling an ongoing phagocytosis process. In the Pt(IV)Ac-POA REC condition, apoptosis occurrence was observed in several cells, while some cells, presenting PARP-1 nuclear expression, displayed a strongly altered tubulin cytoskeleton, probably indicating possible cellular damage (**Figure 4A**). Lastly, Western blot analyses showed PARP-1 activation after both chemotherapeutic treatments, considering all exposure conditions, compared to the controls. It should be noted that in the Pt(IV)Ac-POA REC condition, an evident reduction in PARP-1 expression was detected, nonetheless associated with its activation. This data could suggest a strong, long-lasting Pt(IV)Ac-POA-induced effect in reducing PARP-1 expression, this protein being pivotally involved in DNA repair (**Figure 4B**).

Concerning caspase-8, a critical mediator of the extrinsic apoptotic pathway, the RIP1 (receptor-interacting protein kinase 1) protein expression and changes were investigated by immunofluorescence to confirm the activation of this apoptotic pathway and the possible preliminary activation of necroptosis (**Figure 5**). In control cells, RIP1 fluorescence signal was homogeneously detectable in the entire cytoplasm, but

**TABLE 3 |** Quantitative evaluation of cleaved caspases-3 and 8 immunopositivity.

| % | *CTR* | *CDDP* | *Pt(IV)Ac-POA* | *CDDP REC* | *Pt(IV)Ac-POA REC* |
|---|---|---|---|---|---|
| *Casp-3* | 6.66 ± 0.47 | 11.22 ± 0.27 | 30.48 ± 0.27 | 41.27 ± 0.57 | 61.02 ± 0.20 |
| *Casp-8* | 1.19 ± 0.09 | 10.34 ± 0.22 | 11.59 ± 0.27 | 28.57 ± 0.40 | 33.33 ± 0.34 |
| **Caspase-3** | | | | | |
| **Bonferroni's Multiple Comparison Test** | **ρ value** | | | | |
| CTR *vs.* 40 μM CDDP | *** | | | | |
| CTR *vs.* 10 μM Pt(IV)Ac-POA | *** | | | | |
| CTR *vs.* 40 μM CDDP *REC* | *** | | | | |
| CTR *vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA | *** | | | | |
| 40 μM CDDP *vs.* 40 μM CDDP *REC* | *** | | | | |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| 10 μM Pt(IV)Ac-POA *vs.* 40 μM CDDP *REC* | *** | | | | |
| 10 μM Pt(IV)Ac-POA *vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| 40 μM CDDP *REC vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| **Caspase-8** | | | | | |
| **Bonferroni's Multiple Comparison Test** | **ρ value** | | | | |
| CTR *vs.* 40 μM CDDP | *** | | | | |
| CTR *vs.* 10 μM Pt(IV)Ac-POA | *** | | | | |
| CTR *vs.* 40 μM CDDP *REC* | *** | | | | |
| CTR *vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA | * | | | | |
| 40 μM CDDP *vs.* 40 μM CDDP *REC* | *** | | | | |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| 10 μM Pt(IV)Ac-POA *vs.* 40 μM CDDP *REC* | *** | | | | |
| 10 μM Pt(IV)Ac-POA *vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |
| 40 μM CDDP *REC vs.* 10 μM Pt(IV)Ac-POA *REC* | *** | | | | |

*$\rho < 0.05$; **$\rho < 0.01$; ***$\rho < 0.001$.

being completely lacking at the nuclear level. After 40 μM CDDP 48-h CT, a rearrangement of the fluorescence signal was observed, mainly localized around the fragmented nuclei in the majority of cells; a RIP1 cytoplasmic expression was perceived only in some clusters of cells displaying characteristics similar to those observed in the control condition. Notably, after 10 μM Pt(IV)Ac-POA, a significant increase in RIP1 signal was identified around the degraded nuclei. Furthermore, the tubulin cytoskeleton appeared strongly damaged compared to both the control and CDDP conditions. In the REC condition, after both treatments, non-viable cells were observed, with a marked increase in RIP1 fluorescence signal, mostly concentrated around the visibly damaged nuclei. In particular, in the Pt(IV)Ac-POA REC condition, cells displayed evident pyknotic features.

Based on the knowledge that (i) in necrosome formation, RIP1, or rather RIP3, interacts with mixed lineage kinase domain-like (MLKL) that is essential to induce necroptosis and (ii) MLKL conformational change promotes its translocation to the plasma membrane, causing its permeabilization, but also to the nucleus in the early necroptosis stage (Weber et al., 2018), the possible MLKL translocation was presently investigated (**Figure 6**). In control cells, cytoplasmic labeling was detected, while after both acute drug treatments the MLKL fluorescence signal was rearranged in cells characterized by evident fragmented nuclei. After 40 μM CDDP 48-h CT, the MLKL signal was localized around the damaged nuclei. A similar effect was observed after 10 μM Pt(IV)Ac-POA 48-h CT; besides, in this condition, a strong alteration of the actin cytoskeleton was detectable. After 10 μM Pt(IV)Ac-POA 48-h CT and in the REC condition after either CDDP or Pt(IV)Ac-POA, a significant increase of MLKL expression was detected, particularly evident in cells showing overt altered features. The accumulation of MLKL fluorescence in the degraded cell nuclei of the Pt(IV)Ac-POA REC sample has to be noted.

Subsequently, the activation of the caspase-independent cell death pathway was investigated by immunofluorescence, focusing on the apoptosis-inducing factor (AIF) protein and the mitochondria (**Figure 7**). In control cells, AIF expression was observed at the mitochondria level, as sustained by the colocalization of the two fluorescence signals. Following 48-h CT with either 40 μM CDDP or 10 μM Pt(IV)Ac-POA, AIF progressively lost its physiological localization, moving into the cytoplasm and then into the nucleus.

After 40 μM CDDP 48-h CT or 10 μM Pt(IV)Ac-POA 48-h CT, the translocation of AIF to the nucleus was particularly

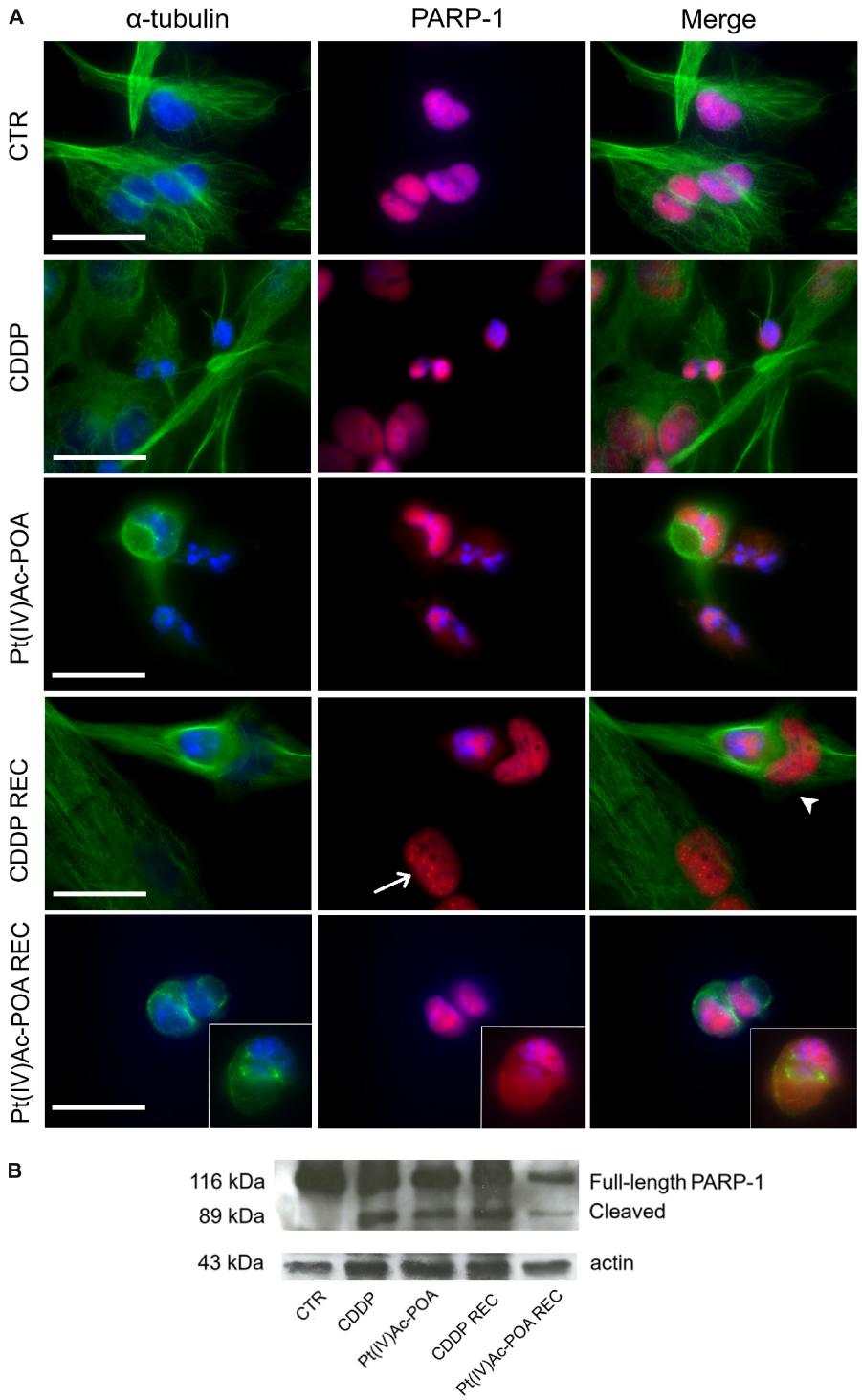


**FIGURE 4 |** Apoptotic pathway investigated using fluorescence microscopy. **(A)** Double immunofluorescence detection of poly[ADP-ribose] polymerase 1 (PARP-1, *red fluorescence*) and α-tubulin (*green fluorescence*) in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Inserts*: high-magnification micrographs showing PARP-1 translocation from the nucleus to the cytoplasm. *Thin arrow* indicates PARP-1 "spot-like" labeling, while *arrowhead* designates phagocytic cell. Cytoskeletal alterations are also noticeable. *Scale bar*, 20 μm. **(B)** Western blotting data showing full-length PARP-1 (116 kDa) and cleaved PARP-1 (89 kDa) bands, respectively, compared to the loading control and actin (43 kDa).

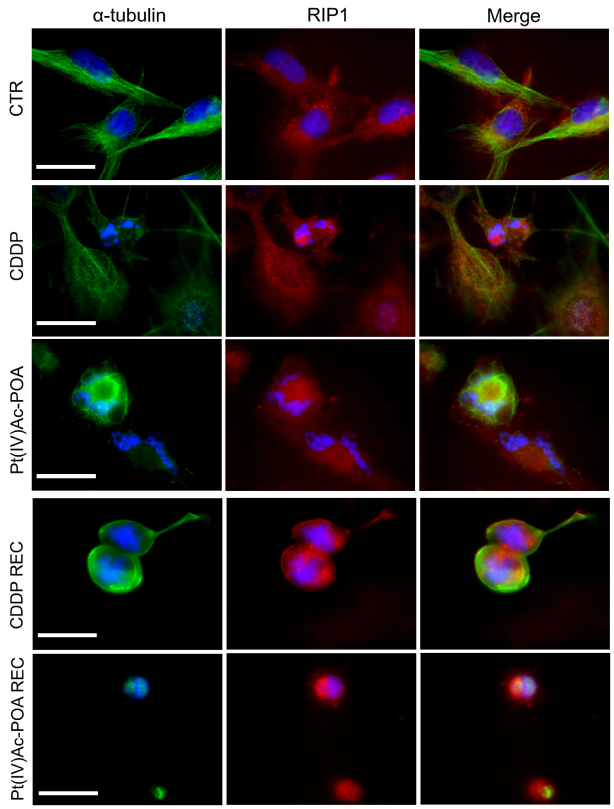


**FIGURE 5 |** Extrinsic apoptotic pathway evaluation by double immunocytochemical detection of receptor-interacting protein kinase 1 (RIP1, *red fluorescence*) and α-tubulin (*green fluorescence*) in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm.

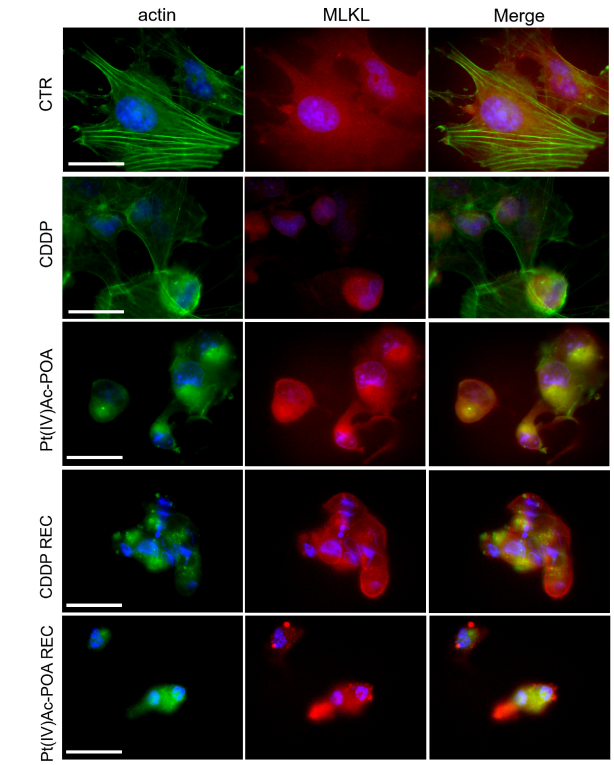


**FIGURE 6 |** Investigation of necroptotic pathway using fluorescence microscopy in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato) platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. Double immunolabeling detection of mixed lineage kinase domain-like (MLKL, *red fluorescence*) and actin (*green fluorescence*). DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm.

evident. This effect was particularly manifest in cells treated with Pt(IV)Ac-POA, in which the loss of colocalization of the two fluorescence signals was evident. Diversely, in the CDDP REC condition, the AIF translocation process was less discernible, suggesting a possible return to the normal mitochondrial localization, which could, therefore, be associated with a cellular recovery mechanism. In contrast, in the Pt(IV)Ac-POA REC sample, AIF remained in the perinuclear area without any sign of restoration of the physiological mitochondrial localization.

## Autophagic Pathway Activation

To deepen the above-reported results revealing the presence of typical autophagic features, p62/SQSTM1 and LC3B, being two specific markers of autophagy, were then evaluated. In control cells, the two fluorescence signals did not colocalize; in particular, p62/SQSTM1 was detected both in the cytoplasm and the nucleus, while LC3B was predominantly identified in the cytoplasm. Differently to the condition observed after 10 μM Pt(IV)Ac-POA 48-h CT, which was similar to that observed in the controls, after 40 μM CDDP 48-h CT, the colocalization of the two protein signals was detectable, suggesting an activation of the autophagic process. Notably, in the REC condition after CDDP only, the cells displayed a strongly modified morphology, showing an increased size and a significant enhancement of the basal expressions of both p62/SQSTM1 and LC3B, whose fluorescence signals did not colocalize (**Figure 8A**). Differently, the Pt(IV)Ac-POA REC samples exhibited a degraded morphology associated with the colocalization of the two fluorescent markers. Further, Western blotting analyses (**Table 4**) demonstrated that, despite a significant reduction in the expression levels of p62/SQSTM1 when comparing the Pt(IV)Ac-POA REC condition to 40 μM CDDP 48-h CT, any statistically significant difference was obtained comparing the control to all other treatments/exposure conditions (**Figure 8B**).

## Pt(IV)Ac-POA Effects on Cytoplasmic Organelles

Previous investigations demonstrated that platinum compounds may affect intracellular organelles. Experimental studies using different neuronal cell lineages highlighted a significant effect

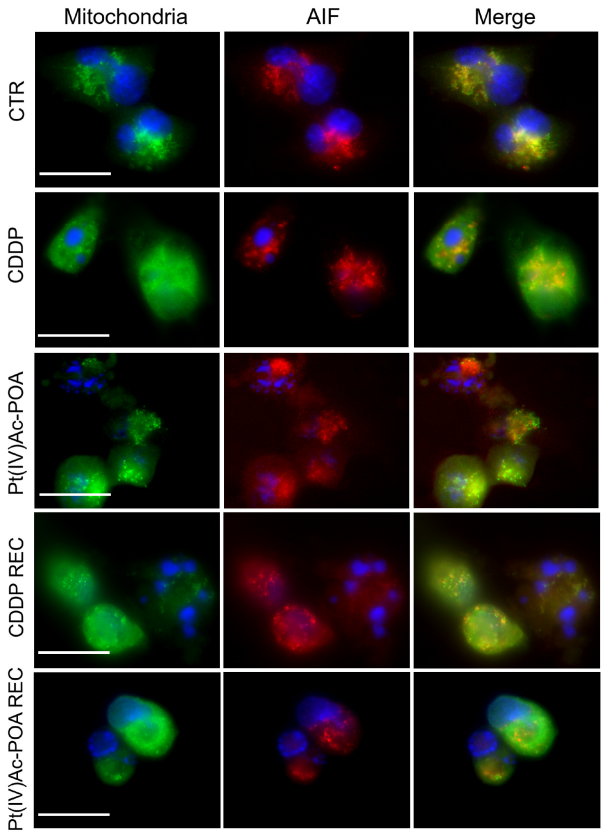


**FIGURE 7 |** Caspase-independent cell death pathway activation investigated by fluorescence microscopy. Double immunostaining for mitochondria (*green fluorescence*) and apoptosis-inducing factor (AIF, *red fluorescence*) in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm.

of cisplatin and platinum(II)-based compound on the Golgi apparatus and mitochondria (Santin et al., 2012, 2013; Grimaldi et al., 2016; Ferrari et al., 2020). Therefore, in the present study, we explored whether the above-mentioned cytoplasmic organelles may be a target of the novel Pt(IV)Ac-POA.

In control cells, Golgi apparatus immunofluorescence showed a normal appearance, with homogeneous distribution in the perinuclear zone and, partially, throughout the cell body. Additionally, the actin-positive cytoskeleton maintained the normal shape and internal organization.

In contrast, after 40 μM CDDP 48-h CT or 10 μM Pt(IV)Ac-POA 48-h CT, a degeneration of Golgi cistern arrangement and cytoskeletal structure was detected. In detail, a degradation of the Golgi apparatus occurred, resulting in the presence of diffuse and homogeneous immunofluorescence localized around the fragmented nuclei. This effect was significantly more marked in the Pt(IV)Ac-POA REC samples, where the cytoskeleton and Golgi apparatus were even no longer distinguishable. Differently, in the CDDP REC condition, despite the presence of some shrunken cells, restoration of physiological features was perceived, resembling the characteristics observed in the control condition. Specifically, a well-defined actin cytoskeletal immunofluorescence as well as the Golgi apparatus immunolabeling were clearly observable (**Figure 9**).

A similar trend was observed when evaluating the double immunofluorescence for the mitochondria and tubulin cytoskeleton (**Figure 10**). In the control conditions, the mitochondria were evenly distributed throughout the entire cell cytoplasm, arranged according to the microtubule distribution pattern of the well-organized cytoskeleton. After 10 μM Pt(IV)Ac-POA 48-h CT, a striking damaging effect was detected, with the mitochondria showing a small and rounded organization, being often clustered within dying cells. This effect retained a long-lasting duration, being even measurable in the respective REC condition. After 40 μM CDDP 48-h CT, several cells displayed characteristics similar to those observed in controls; nonetheless, a worsened situation was perceived in the respective REC condition. Notably, after all different exposure conditions, CDDP treatment was demonstrated to cause less intense effects than those measured after exposure to Pt(IV)Ac-POA.

## Irradiation With Carbon Ions

Initial experimental studies have then been conducted on U251 cells using carbon ion irradiation. The effects were assessed using different experimental conditions, with the goal to investigate the action of hadrontherapy alone and combined with the two chemotherapeutics under investigation, i.e., CDDP and Pt(IV)Ac-POA. The same drug concentrations previously employed for the above reported *in vitro* experiments were employed in order to compare the effects caused by the combined protocol (drug exposure + hadrontherapy) with those obtained after chemotherapeutic treatments alone. Specifically, clonogenic cell survival as well as apoptotic and autophagic markers, already determined after chemotherapeutic therapy alone, were assessed after 48-h CT to either CDDP or Pt(IV)Ac-POA followed by carbon ion irradiation at doses of 2 and 4 Gy. Preliminary data concerning the REC conditions were also obtained.

### Clonogenic Cell Survival Assay: Standard Acute Treatment With 10 μM Pt(IV)Ac-POA Plus Increasing Carbon Ion Irradiation Doses

Compared to the data obtained after exposure to the prodrug alone, the clonogenicity of U251 was further impaired when exposed to the combined treatment, i.e., 10 μM Pt(IV)Ac-POA + carbon ion irradiation at increasing doses (i.e. 1, 2, and 4 Gy). Specifically, a synergistic effect was measured when 10 μM Pt(IV)Ac-POA was combined with two of the tested radiation doses, i.e., 2 and 4 Gy, reducing to the colony formation of about 85%, with surviving fractions of 16 and 13% after 2 and 4 Gy, respectively (**Figures 1Cb,d**). Notably, these data demonstrated that the synergistic effect played by the combined treatment with 10 μM Pt(IV)Ac-POA + carbon ion radiation was similar using either 2 or 4 Gy. Diversely, the combined exposure to 10 μM Pt(IV)Ac-POA + 1 Gy induced a clonogenicity impairment similar to that observed after treatment with the prodrug alone

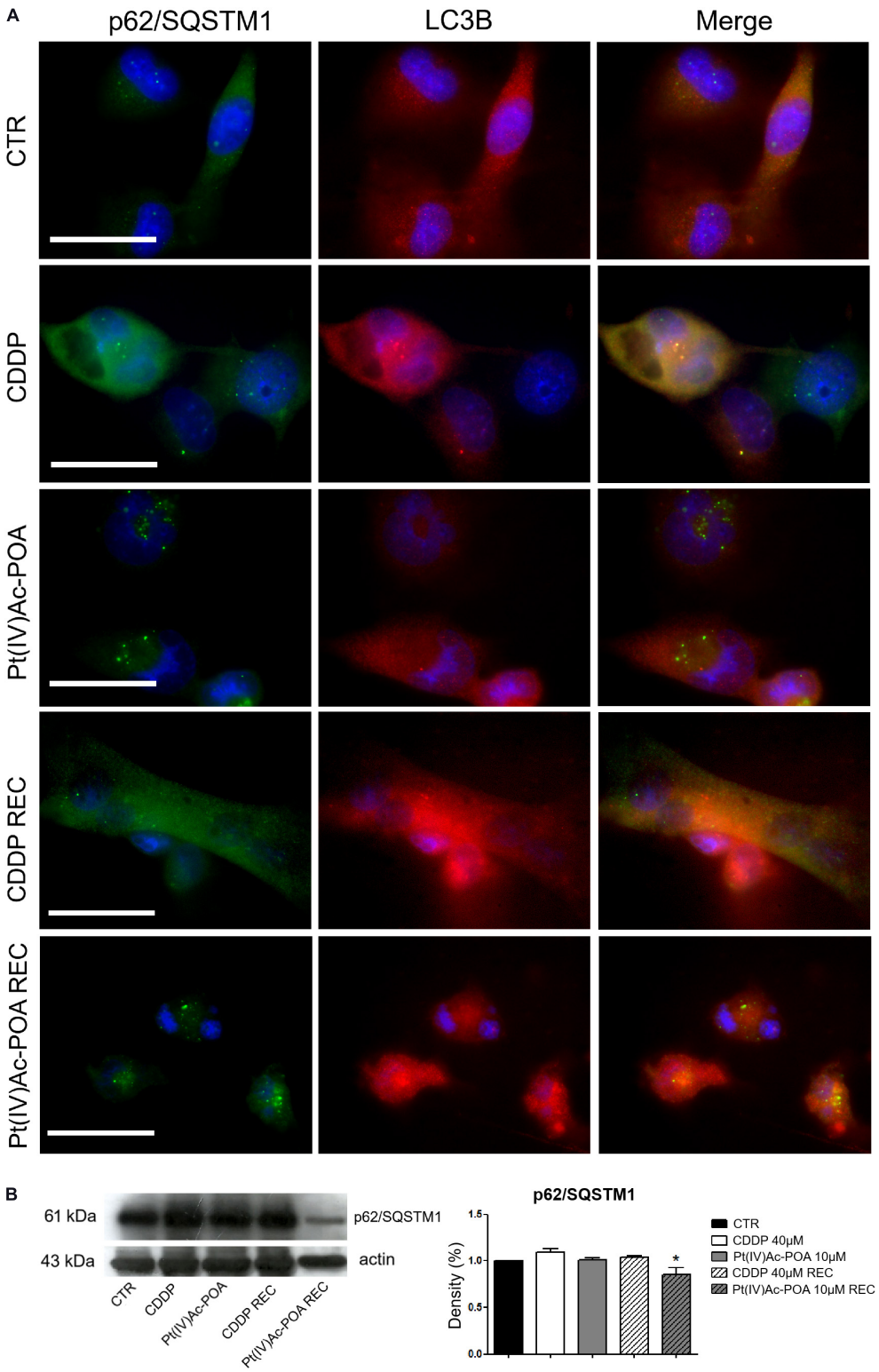


**FIGURE 8 |** Immunofluorescence study of autophagy activation. **(A)** Double immunolabeling for p62/SQSTM1 (*green fluorescence*) and LC3B (*red fluorescence*). DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm. **(B)** Western blotting data: p62/SQSTM1. Histograms representing density band quantification of p62/SQSTM1 in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. Statistical significance: *$\rho < 0.05$, 40 μM CDDP 48-h CT *vs*. the Pt(IV)AC-POA REC condition.

**TABLE 4 |** Protein (p62/SQSTM1) band density quantification after Western blotting experiments.

| % | CTR | CDDP | Pt(IV)Ac-POA | CDDP REC | Pt(IV)Ac-POA REC |
|---|---|---|---|---|---|
| ***p62/SQSTM1*** | 1.00 ± 0.00 | 1.09 ± 0.04 | 1.01 ± 0.02 | 1.04 ± 0.02 | 0.85 ± 0.08 |

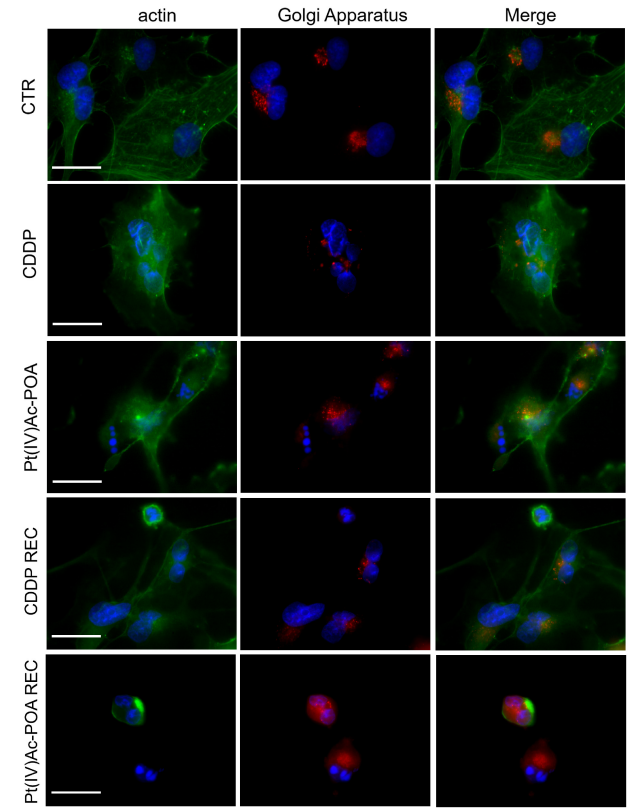


**FIGURE 9 |** Intracellular organelle investigation by immunofluorescence. Double immunocytochemical detection of the Golgi apparatus (*red fluorescence*) and actin (*green fluorescence*) in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bars*, 20 μm.

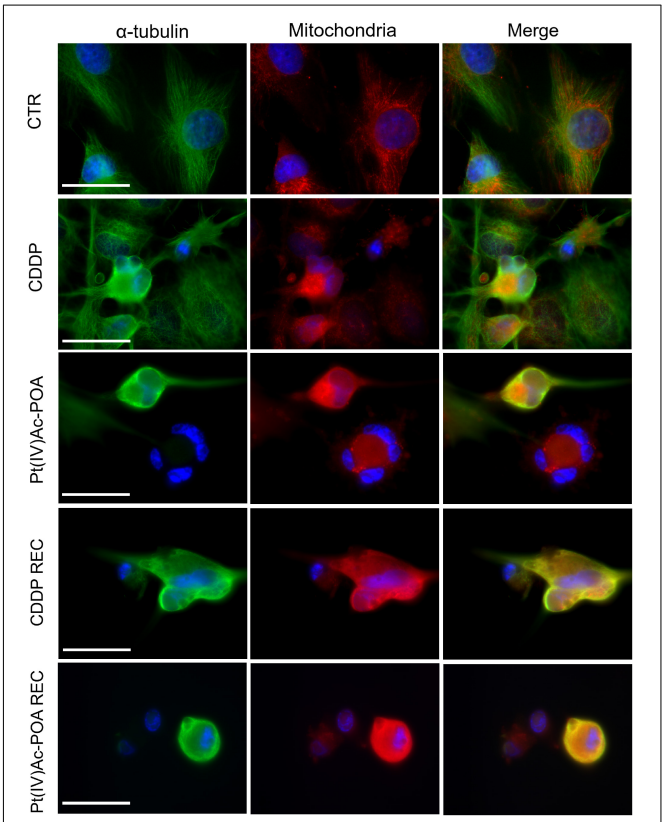


**FIGURE 10 |** Intracellular organelle investigation by immunofluorescence. Double immunolabeling for the mitochondria (*red fluorescence*) and α-tubulin (*green fluorescence*) in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl) octanoato)platinum(IV) [Pt(IV)Ac-POA], and in the recovered (REC) conditions. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bars*, 20 μm.

(26% *vs*. 30%) (**Figures 1Cb,d**). These findings supported that the clonogenicity of glioblastoma cells was impaired synergistically when Pt(IV)Ac-POA was combined with hadrontherapy.

## Acute Exposure Condition—Apoptotic Pathway Evaluation: Caspase-3 and PARP-1 Immunofluorescence Staining

As illustrated in **Figure 11**, caspase-3 immunofluorescence increased in the samples exposed to carbon ion radiation (10.71 ± 0.02% and 15.00 ± 0.31% for 2 and 4 Gy, respectively) compared to the controls (0.94 ± 0.15%). Compared to the former samples, immunopositivity slightly increased after 40 μM CDDP 48-h CT (15.71 ± 0.30%) or 10 μM Pt(IV)Ac-POA 48-h CT (18.12 ± 0.39%). Notably, the number of caspase-3-positive cells significantly increased after the combined treatment, i.e., chemotherapeutics exposure followed by carbon ion radiation. Specifically, the samples firstly treated with 40 μM CDDP or 10 μM Pt(IV)Ac-POA and then exposed to 4 Gy showed a strong increase of caspase-3 immunofluorescence (50.00 ± 0.99% and 90.24 ± 0.47%, respectively) compared to the cells exposed to drug treatments followed by the 2 Gy dose (20.83 ± 0.43% and 65.16 ± 0.94%, respectively). In detail, the greatest effect was measured in the samples exposed to Pt(IV)Ac-POA followed by carbon ion radiation at a dose of 4 Gy. **Table 5** summarizes the significant values of caspase-3-immunopositive cell quantification.

The results pertaining to PARP-1 expression are shown in **Figure 12**. In control cells, PARP-1 was localized at the nuclear

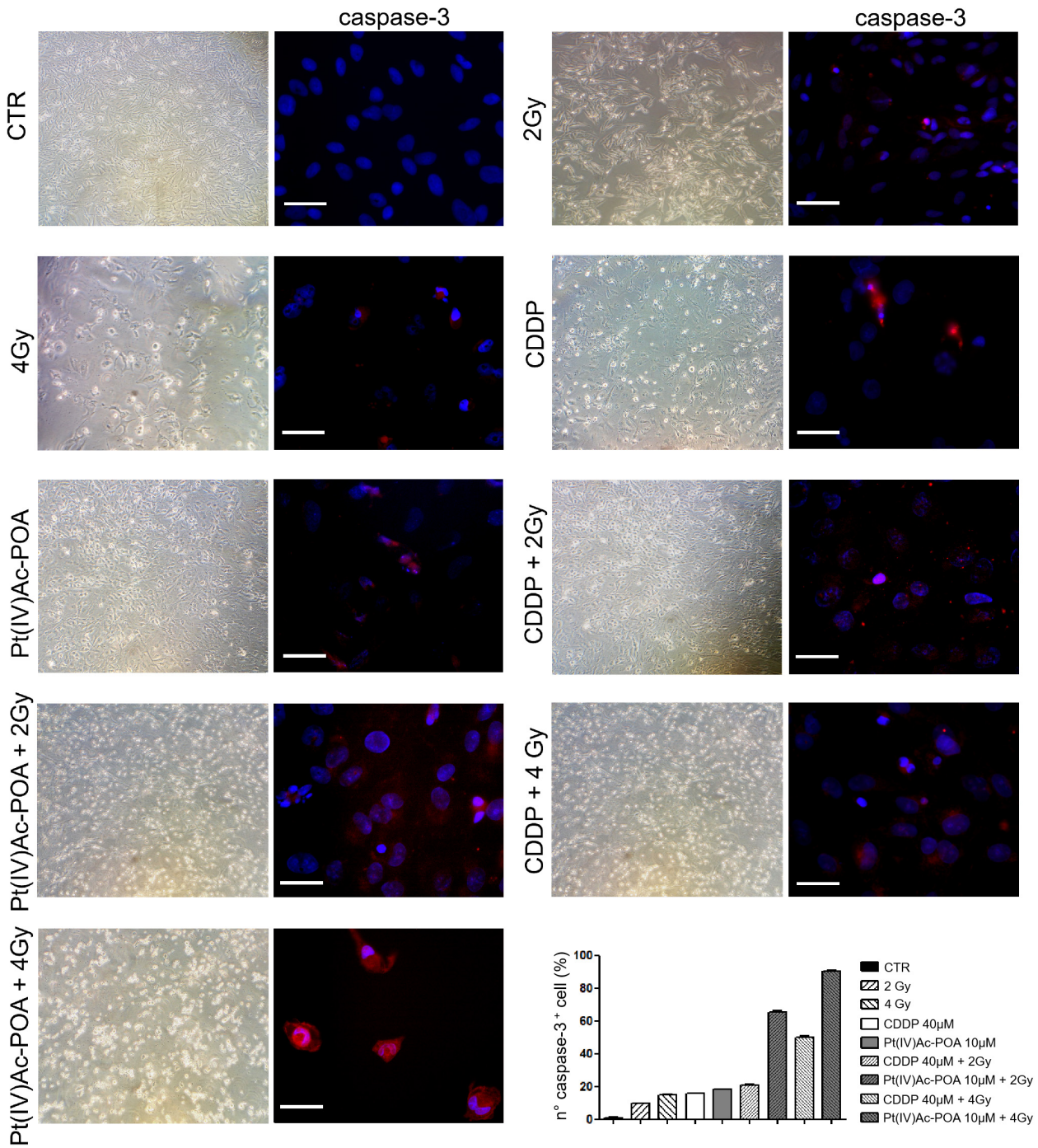


**FIGURE 11 |** Different experimental conditions: chemotherapeutics exposure alone and followed by carbon ion irradiation. The **left column** shows phase-contrast micrographs of U251 cells cultured in flask sterile on slide in different experimental conditions before being processed for immunofluorescence reactions. The **right column** displays caspase-3 immunolabeling (*red fluorescence*) in the control (CTR), differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA] alone or followed by carbon ion radiation at 2 or 4 Gy dose. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 40 μm. Histograms (**lower right part**) representing the percentage values of caspase-3-immunopositive cells.

level, and the well-organized tubulin cytoskeleton supported the maintenance of the normal cell morphology. A similar immunopositivity trend was detected in the samples exposed to carbon ion radiation alone (applying both 2 and 4 Gy doses), in which PARP-1 immunofluorescence was observed in the nuclei accompanied by the presence of an organized

**TABLE 5 |** Caspase-3 immunofluorescence: summary of statistical significances.

| Bonferroni's Multiple Comparison Test | ρ value |
|---|---|
| CTR *vs.* 2 Gy | *** |
| CTR *vs.* 4 Gy | *** |
| CTR *vs.* 40 μM CDDP | *** |
| CTR *vs.* 10 μM Pt(IV)Ac-POA | *** |
| CTR *vs.* 40 μM CDDP + 2 Gy | *** |
| CTR *vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| CTR *vs.* 40 μM CDDP + 4 Gy | *** |
| CTR *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 2 Gy *vs.* 4 Gy | *** |
| 2 Gy *vs.* 40 μM CDDP | *** |
| 2 Gy *vs.* 10 μM Pt(IV)Ac-POA | *** |
| 2 Gy *vs.* 40 μM CDDP + 2 Gy | *** |
| 2 Gy *vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| 2 Gy *vs.* 40 μM CDDP + 4 Gy | *** |
| 2 Gy *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 4 Gy *vs.* 40 μM CDDP | *ns* |
| 4 Gy *vs.* 10 μM Pt(IV)Ac-POA | ** |
| 4 Gy *vs.* 40 μM CDDP + 2 Gy | *** |
| 4 Gy *vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| 4 Gy *vs.* 40 μM CDDP + 4 Gy | *** |
| 4 Gy *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA | *ns* |
| 40 μM CDDP *vs.* 40 μM CDDP + 2 Gy | *** |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| 40 μM CDDP *vs.* 40 μM CDDP + 4 Gy | *** |
| 40 μM CDDP *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 10 μM Pt(IV)Ac-POA *vs.* 40 μM CDDP + 2 Gy | * |
| 10 μM Pt(IV)Ac-POA *vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| 10 μM Pt(IV)Ac-POA *vs.* 40 μM CDDP + 4Gy | *** |
| 10 μM Pt(IV)Ac-POA *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 40 μM CDDP + 2 Gy *vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| 40 μM CDDP + 2 Gy *vs.* 40 μM CDDP + 4 Gy | *** |
| 40 μM CDDP + 2 Gy *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 10 μM Pt(IV)Ac-POA + 2 Gy *vs.* 40 μM CDDP + 4 Gy | *** |
| 10 μM Pt(IV)Ac-POA + 2 Gy *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 40 μM CDDP + 4 Gy *vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |

*Different experimental conditions (CTR, control, 40 μM CDDP and 10 μM Pt(IV)Ac-POA 48 h-CT alone or combined with 2 or 4 Gy dose irradiation); ρ values: *ρ < 0.05; **ρ < 0.01; ***ρ < 0.001, ns, not significant.*

tubulin cytoskeleton. After 40 μM CDDP 48-h CT or 10 μM Pt(IV)Ac-POA 48-h CT, various cells displayed degraded nuclei and a loss of their usual elongated shape due to cytoskeletal alterations. Interestingly, the cells treated with Pt(IV)Ac-POA alone or combined with carbon ion exposure showed different cell death phases, i.e., early and late apoptosis. Differently, the cells exposed to CDDP alone conserved a nuclear PARP-1 immunofluorescence signal. The most striking morphological alterations were identified in the samples exposed to combined therapy, i.e., early exposure to the compounds followed by ion radiation. After 40 μM CDDP 48-h CT associated with 2 Gy radiation, any marked increase in the cells with degraded nuclei was measured; nevertheless, the cytoskeleton showed structural abnormalities. Notably, when the CDDP treatment was associated with 4 Gy radiation dose, the effect was amplified, distressing not only the cytoskeleton but also the nucleus. The greatest cytotoxic effect was, however, observed after 10 μM Pt(IV)Ac-POA 48-h CT followed by 2 or 4 Gy radiation exposure. Important morphological alterations were detected already at 2 Gy, and the effect lasted and increased even at the 4-Gy dose. Based on the significant data obtained after radiation exposure at 4 Gy, this dose was selected to conduct Western blot determinations (**Figure 13A**). Accordingly to the above reported data concerning the first experimental step, Western blotting analyses demonstrated, yet again, no significant difference in the PARP-1 expression levels (including the cleaved fragment p89) in the cells either exposed to CDDP or Pt(IV)Ac-POA after 48-h CT. Notably, U251 irradiated with the 4-Gy dose alone, as well as the samples exposed to 40 μM CDDP 48-h CT followed by 4 Gy carbon ion radiation, showed similar full-length PARP-1 expression patterns; differently, cleaved PARP-1 was detected in 4-Gy-irradiated cells only. In these latter conditions, the expression of the cleaved PARP-1 fragment was strongly decreased compared to the cells exposed either to 40 μM CDDP 48-h CT or to 10 μM Pt(IV)Ac-POA 48-h CT. In the cells exposed to 10 μM Pt(IV)Ac-POA followed by 4 Gy carbon ion radiation, a reduced expression of full-length PARP-1 was observed paralleled by the presence of cleaved PARP-1.

### Acute Exposure Condition—Autophagic Pathway Activation: p62/SQSTM1 Expression

Based on the significant data obtained after radiation exposure at 4 Gy, this dose was selected to conduct subsequent Western blotting analyses. The obtained data, shown in **Figure 13B**, demonstrated an increased, though not statistically significant, expression of p62/SQSTM1 protein in the CDDP-treated sample compared to the controls, suggesting a homeostatic imbalance in the activation of the autophagic pathway. On the contrary, when 40 μM CDDP 48-h CT was followed by 4 Gy radiation exposure, a significant decrease of the p62/SQSTM1 protein expression was measured, indicating the protein involvement in autophagy activation. In the sample exposed to 4 Gy radiation alone, the cells did not display any appreciable difference compared to the controls. After 10 μM Pt(IV)Ac-POA 48-h CT and after the combined exposure to 10 μM Pt(IV)Ac-POA 48-h CT + 4 Gy, the cells showed a p62/SQSMT1 level similar to that observed in the controls. **Table 6** summarizes the Western blotting quantitative data.

### REC Conditions: Apoptotic Pathway Evaluation: Caspase-3 and PARP-1 Assessment

Based on the above-described results, we focused the initial evaluation in the REC condition on the assessment of some specific markers representative of apoptosis. Concerning caspase-3 expression (**Figure 14**), the samples exposed to carbon ion radiation only displayed immunopositive cell frequencies of 2.72 ± 0.60% and 4.57 ± 0.32% after the 2 and 4 Gy doses, respectively, compared to the controls (0.43 ± 0.18%); notably, the presence of cells in active mitosis

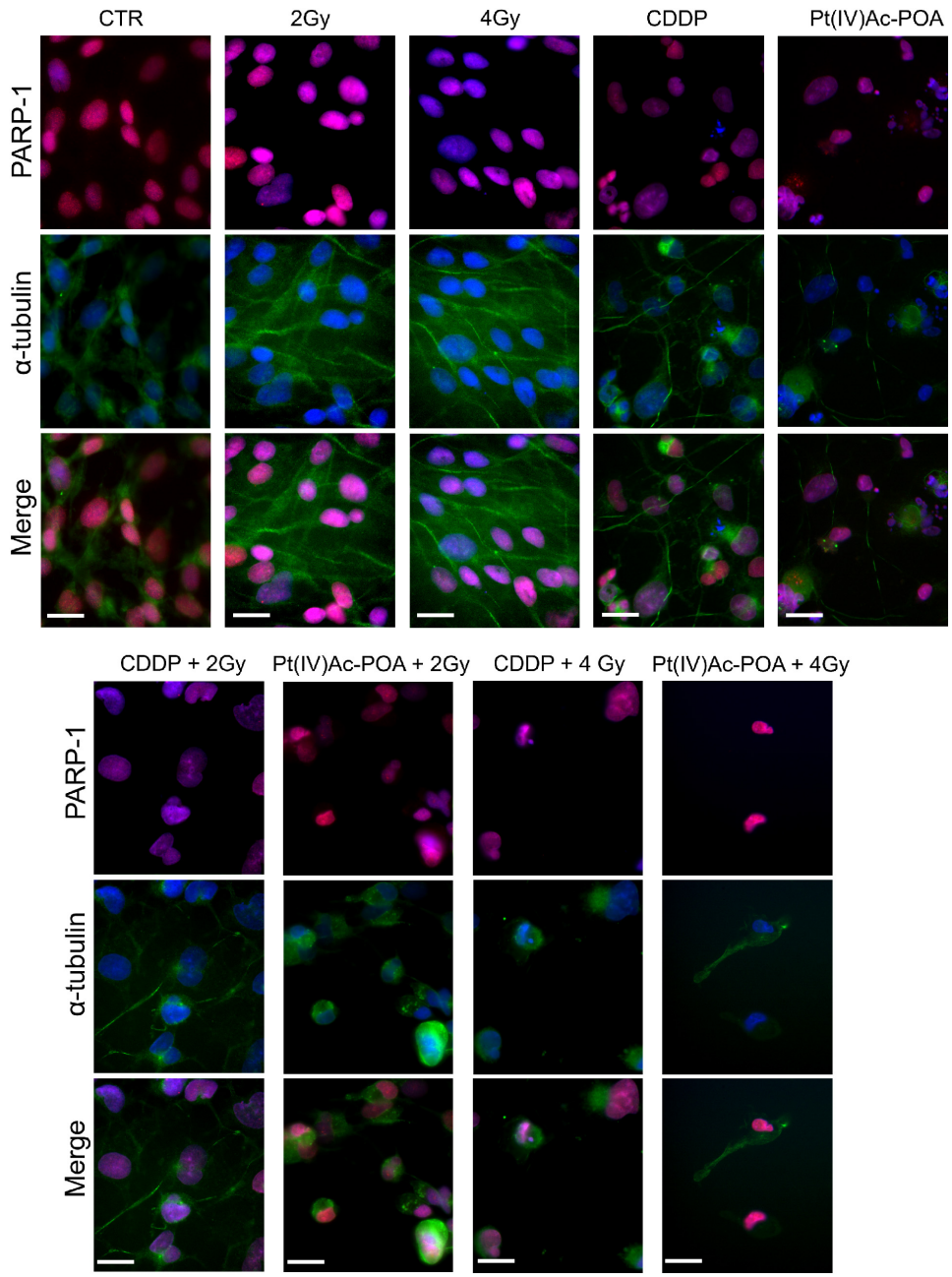


**FIGURE 12 |** Apoptotic pathway investigated using immunocytochemistry after treatment with chemotherapeutics alone and combined with carbon ion irradiation at different doses. Double immunofluorescence detection of poly[ADP-ribose] polymerase 1 (PARP-1, *red fluorescence*) and α-tubulin (*green fluorescence*) in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], and in combined exposure conditions [40 μM CDDP or 10 μM Pt(IV)Ac-POA 48-h CT + 2 or 4 Gy carbon ion radiation]. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm.

was also detected, resembling the physiological condition. After 40 μM CDDP 48-h CT alone or combined with 2 or 4 Gy of carbon ion irradiation, cytotoxic effects were detected, as demonstrated by the increased immunopositive cell frequency (41.27 ± 0.57%, 44.00 ± 0.66%, and 43.08 ± 0.73%, respectively) despite the strong reduction in the cell population. After 10 μM Pt(IV)Ac-POA 48-h CT, an enhancement in caspase-3-immunopositive cell frequency was also measured (61.03 ± 0.20%), associated with a reduction in cell number. After 10 μM Pt(IV)Ac-POA 48-h CT combined with 2 or 4 Gy of carbon ion irradiation, almost all cells were caspase-3-immunopositive (92.86 ± 0.40% and 95.00 ± 0.47%, respectively), and limited cell survival was detected. Notably, these data corroborated the notion that even a low-dose carbon

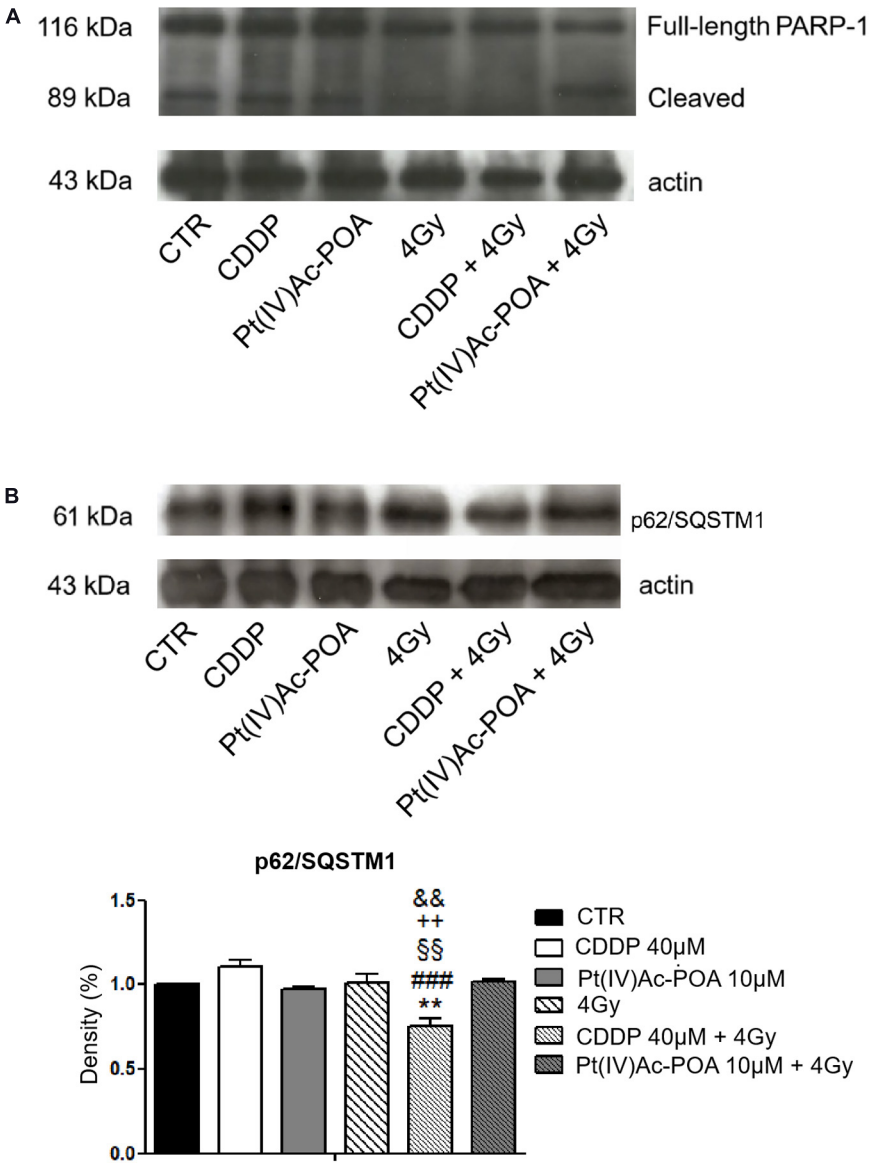


**FIGURE 13 | (A)** Western blotting analysis of full-length poly[ADP-ribose] polymerase 1 (PARP-1, 116 kDa) and cleaved PARP-1 (89 kDa). Representative Western blotting bands showing the expression levels of full-length PARP-1 (116 kDa) and cleaved PARP-1 in the controls, differently treated U251 cells, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA], after carbon ion irradiation (4 Gy dose) alone, and in combined exposure conditions [40 μM CDDP or 10 μM Pt(IV)Ac-POA 48-h CT + 4 Gy carbon ion irradiation]. The result was quantified from the average of three different samples. The density bands of full-length (116 kDa) and cleaved (89 kDa) PARP-1 were compared to the loading control and actin (43 kDa). **(B)** Western blotting study of p62/SQSTM1 as a typical marker of autophagy activation. Histograms representing density band quantification of p62/SQSTM1 (61 kDa) in the controls, differently treated U251 cells, i.e., after 48-h CT with 40 μM CDDP or 10 μM Pt(IV)Ac-POA, after carbon ion radiation (4 Gy dose) alone, and in combined exposure conditions [40 μM CDDP or 10 μM Pt(IV)Ac-POA 48-h CT + 4 Gy carbon ion radiation]. The means, obtained from three independent experiments, have been quantified and compared to the loading control and actin (43 kDa). Statistical significance calculated as follows: *control *vs*. CDDP + 4 Gy carbon ion radiation; $^{\#}$CDDP *vs*. CDDP + 4 Gy carbon ion radiation; $^{\S}$ Pt(IV)Ac-POA *vs*. CDDP + 4 Gy carbon ion radiation; $^{+}$4 Gy carbon ion radiation alone *vs*. CDDP + 4 Gy carbon ion radiation; $^{\&}$CDDP + 4 Gy carbon ion radiation *vs*. Pt(IV)Ac-POA + 4 Gy carbon ion radiation. $^{**}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\S\S}p < 0.01$; $^{++}p < 0.01$; $^{\&\&}p < 0.01$.

**TABLE 6 |** Protein (p62/SQSTM1) band density quantification after Western blotting experiments.

| % | CTR | CDDP | Pt(IV)Ac-POA | 4Gy | CDDP + 4Gy | Pt(IV)Ac-POA + 4Gy |
|---|---|---|---|---|---|---|
| **p62/SQSTM1** | 1.00 ± 0.00 | 1.11 ± 0.03 | 0.93 ± 0.02 | 1.01 ± 0.06 | 0.75 ± 0.04 | 1.02 ± 0.02 |

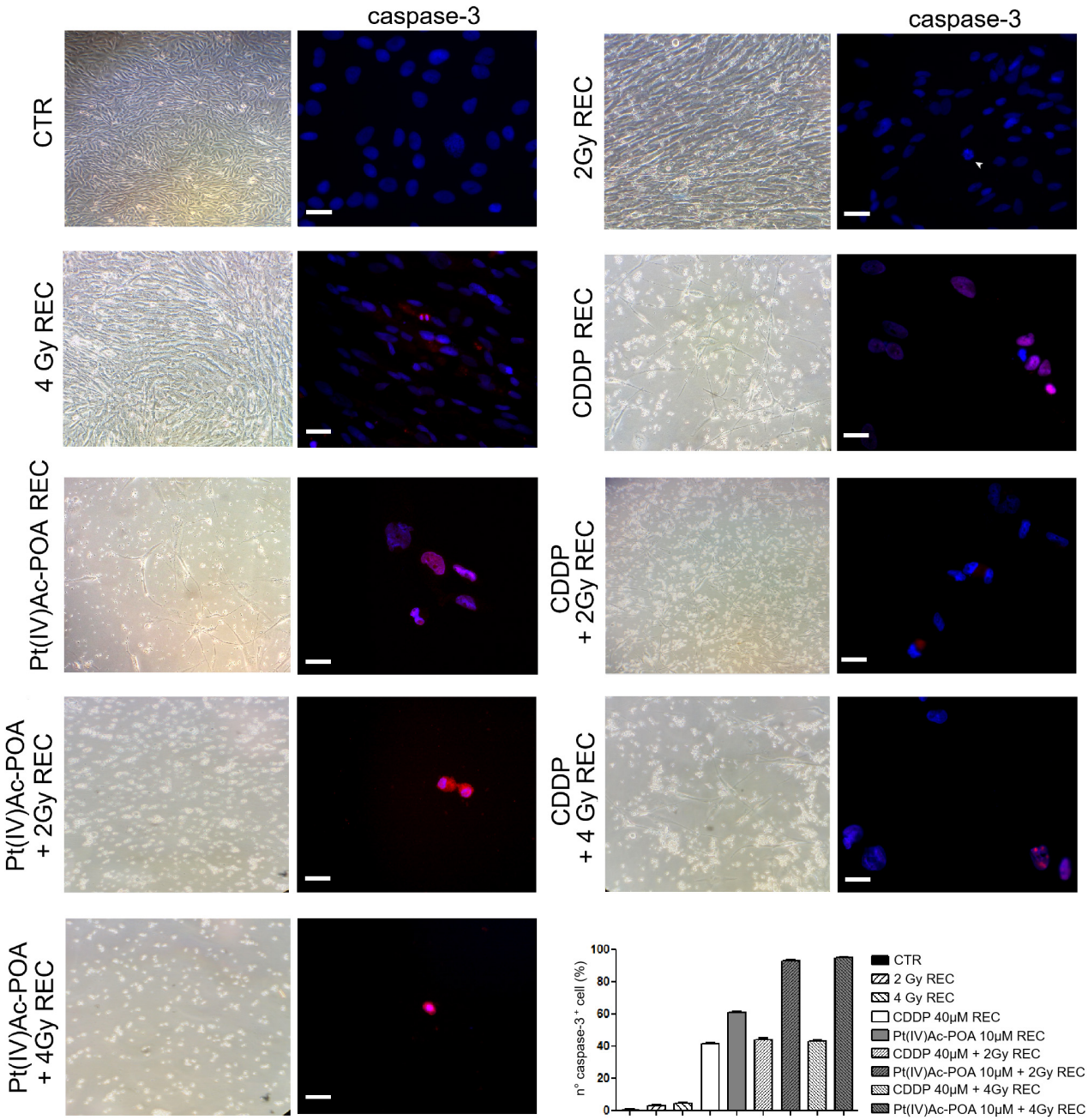


**FIGURE 14 |** Recovered (REC) conditions after combined experimental protocol (chemotherapeutics exposure + carbon ion radiation). The **left column** shows phase-contrast micrographs of U251 cells cultured in flask sterile on slide in different experimental conditions before being processed for immunofluorescence reactions. The **right column** displays caspase-3 immunolabeling (*red fluorescence*) in the controls (CTR) and all different REC conditions, i.e., after 48-h continuous treatment (CT) with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA] alone, after carbon ion irradiation (4 Gy dose) alone, or in combined exposure conditions [40 μM CDDP or 10 μM Pt(IV)Ac-POA 48-h CT + 2 or 4 Gy carbon ion radiation]. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm. Histograms (**lower right part**) represent the percentage values of caspase-3-immunopositive cells.

ion beam, i.e., 2 Gy, has a remarkable long-term effect on U251 cells. **Table 7** summarizes caspase-3-immunopositive cell quantification obtained in the REC condition.

With regard to PARP-1 in the REC conditions (**Figure 15**), any changes in immunofluorescence were detected comparing samples exposed to carbon ion radiation alone, both at 2 and 4 Gy, revealing that, at seven recovery days after irradiation, PARP-1 expression and localization at the nuclear level were comparable to the control condition. Diversely, the REC samples treated with either 40 μM

**TABLE 7 |** Summary of statistical significances.

| Bonferroni's Multiple Comparison Test | ρ value |
|---|---|
| CTR *vs.* 2 Gy *REC* | *ns* |
| CTR *vs.* 4 Gy *REC* | *** |
| CTR *vs.* 40 μM CDDP *REC* | *** |
| CTR *vs.* 10 μM Pt(IV)Ac-POA | *** |
| CTR *vs.* 40 μM CDDP + 2Gy *REC* | *** |
| CTR *vs.* 10 μM Pt(IV)Ac-POA + 2Gy *REC* | *** |
| CTR *vs.* 40 μM CDDP + 4Gy *REC* | *** |
| CTR *vs.* 10 μM Pt(IV)Ac-POA + 4Gy *REC* | *** |
| 2 Gy *REC vs.* 4 Gy *REC* | *ns* |
| 2 Gy *REC vs.* 40 μM CDDP *REC* | *** |
| 2 Gy *REC vs.* 10 μM Pt(IV)Ac-POA | *** |
| 2 Gy *REC vs.* 40 μM CDDP + 2Gy *REC* | *** |
| 2 Gy *REC vs.* 10 μM Pt(IV)Ac-POA + 2 Gy *REC* | *** |
| 2 Gy *REC vs.* 40 μM CDDP + 4 Gy *REC* | *** |
| 2 Gy *REC vs.* 10 μM Pt(IV)Ac-POA + 4 Gy *REC* | *** |
| 4 Gy *REC vs.* 40 μM CDDP *REC* | *** |
| 4 Gy *REC vs.* 10 μM Pt(IV)Ac-POA | *** |
| 4 Gy *REC vs.* 40 μM CDDP + 2 Gy *REC* | *** |
| 4 Gy *REC vs.* 10 μM Pt(IV)Ac-POA + 2 Gy *REC* | *** |
| 4 Gy *REC vs.* 40 μM CDDP + 4 Gy *REC* | *** |
| 4 Gy *REC vs.* Pt(IV)Ac-POA + 4 Gy *REC* | *** |
| 40 μM CDDP *REC vs.* 10 μM Pt(IV)Ac-POA | *** |
| 40 μM CDDP *REC vs.* 40 μM CDDP + 2 Gy *REC* | ** |
| 40 μM CDDP *REC vs.* 10 μM Pt(IV)Ac-POA + 2 Gy *REC* | *** |
| 40 μM CDDP *REC vs.* 40 μM CDDP + 4 Gy *REC* | *ns* |
| 40 μM CDDP *REC vs.* 10 μM Pt(IV)Ac-POA + 4Gy *REC* | *** |
| 10 μM Pt(IV)Ac-POA *REC vs.* 40 μM CDDP + 2 Gy | *** |
| 10 μM Pt(IV)Ac-POA *REC vs.* 10 μM Pt(IV)Ac-POA + 2 Gy | *** |
| 10 μM Pt(IV)Ac-POA *REC vs.* 40 μM CDDP + 4Gy | *** |
| 10 μM Pt(IV)Ac-POA *REC vs.* 10 μM Pt(IV)Ac-POA + 4 Gy | *** |
| 40 μM CDDP + 2 Gy *REC vs.* 10 μM Pt(IV)Ac-POA + 2 Gy *REC* | *** |
| 40 μM CDDP + 2 Gy *REC vs.* 40 μM CDDP + 4 Gy *REC* | *ns* |
| 40 μM CDDP + 2 Gy *REC vs.* Pt(IV)Ac-POA + 4 Gy *REC* | *** |
| 10 μM Pt(IV)Ac-POA + 2 Gy *REC vs.* 40 μM CDDP + 4 Gy *REC* | *** |
| 10 μM Pt(IV)Ac-POA + 2 Gy *REC vs.* 10 μM Pt(IV)Ac-POA + 4 Gy *REC* | *ns* |
| 40 μM CDDP + 4 Gy *REC vs.* 10 μM Pt(IV)Ac-POA + 4 Gy *REC* | *** |

*Different experimental conditions (CTR, control, 40 μM CDDP and 10 μM Pt(IV)Ac-POA 48 h-CT alone or combined with 2 or 4 Gy dose irradiation); ρ values: *ρ < 0.05; **ρ < 0.01; ***ρ < 0.001, ns, not significant.*

CDDP or 10 μM Pt(IV)Ac-POA still demonstrated apoptosis presence, as can be deduced observing the strongly altered tubulin cytoskeleton which indicates possible cellular damage. In particular, evaluating the long-term effects after 40 μM CDDP 48-h CT followed by exposure to 2 or 4 Gy carbon ion beam, some morphological alterations were perceived, nonetheless accompanied by a spotted-like PARP-1 immunofluorescent labeling detected, supporting the occurrence of early-onset cellular recovery. Interestingly, after 10 μM Pt(IV)Ac-POA 48-h CT followed by 2 or 4 Gy carbon ion beam exposure, the translocation of PARP-1 immunofluorescence signal from the nucleus to the cytoplasm was observed, triggering apoptosis in the few surviving cells.

## DISCUSSION

The present study was devoted to identifying a new valuable treatment useful in overcoming the limits associated with conventional cancer therapies currently used in clinical practice to treat CNS cancers. Among these limits, chemoresistance is one of the major obstacles in the treatment of nervous system (NS) tumors, as the standard protocols, as well as the treatment with TMZ, can only, in some cases, improve the patient's prognosis, but not lead to a complete resolution. Also, the high heterogeneity that characterizes these cancers, in particular GBM, represents a great challenge for the diagnosis and the development of tumor-specific therapies.

Hence, in this view, the present investigation aimed at exploring the effects of a new platinum-based compound properly synthesized to have greater efficacy than the CDDP standard treatment, paralleled by a lower systemic toxicity. Recent data confirmed that the recently synthesized platinum-based compounds possess suitable antitumor action (Grimaldi et al., 2019; Astesana et al., 2020) and lower cytotoxic effects on healthy cells (Piccolini et al., 2015), even though the problem of acquired chemoresistance remains a topic to be[ further clarified.

In this regard, we focused on a recently lab-manufactured platinum(IV) compound, namely Pt(IV)Ac-POA, which acts as a prodrug, thus reducing the possible side effect of cytotoxicity outside tumor cells (Johnstone et al., 2016; Gabano et al., 2017). Furthermore, the ability to associate biologically active axial ligands, i.e., HDACi, within the molecule positively allows obtaining a synergistic effect that would enhance the antitumor effect.

To date, the first studies of Pt(IV)Ac-POA provided promising data in several NS tumor cell lineages (Rangone et al., 2018; Ferrari et al., 2020).

Pt(IV)Ac-POA belongs to the platinum(IV) family and performs its cytotoxic action by acting as a prodrug. Indeed, it is inactive outside the tumor cells while being activated inside them through a reduction mechanism that leads to the splitting of both the CDDP molecule and the two axial ligands including the POA (Gabano et al., 2017). Due to the presence of POA, the Pt(IV)Ac-POA molecule, as a cisplatin/POA combination molecule, increases the ability to deliver at the same time huge amounts of cisplatin and POA in cells. The "synergistic cellular accumulation" of Pt(IV)Ac-POA is mainly due to the lipophilicity of the molecule assembly with respect to the hydrophilic cisplatin and the amphiphilic POA (in anionic form at physiologic pH) precursors which allow increasing cellular uptake. Moreover, POA is considered a very active HDACi, thus indirectly producing enhanced acetylation at the chromatin level, improving decondensation and, therefore, enhancing the DNA exposure to cisplatin action (Gabano et al., 2017; Novohradsky et al., 2017), with the double consequence of inducing chemosensitization and decreasing chemoresistance. Besides, HDACi, being an epigenetic agent, affects cell fate by altering the expressions of several genes. In particular, HDACi can modulate *inter alia* the expression and function of DNA repair proteins, increasing the persistence

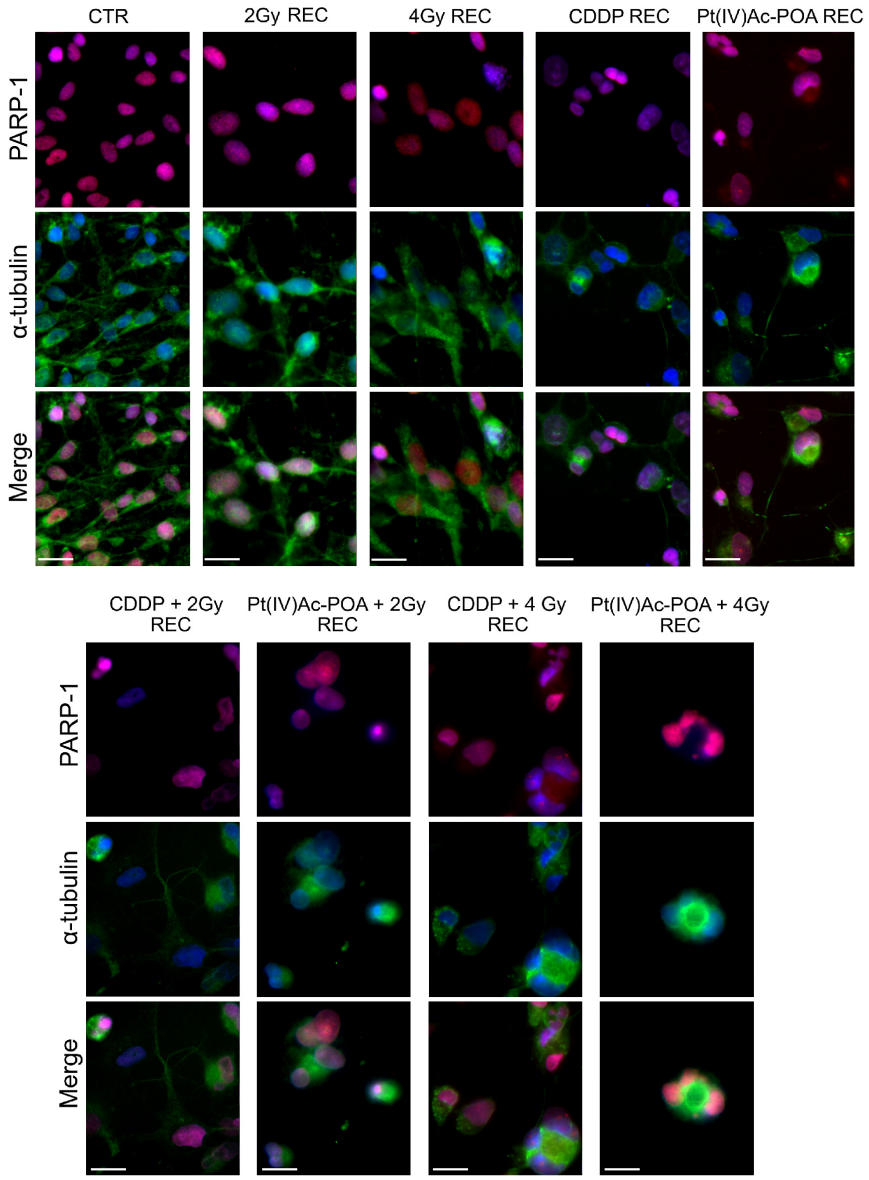


**FIGURE 15 |** Apoptotic pathway evaluation in the recovered (REC) condition after (i) carbon ion irradiation alone or (ii) treatment with chemotherapeutics alone or (iii) combined treatments: chemotherapeutics + carbon ion radiation at different doses. Double immunofluorescence detection of PARP-1 (*red fluorescence*) and α-tubulin (*green fluorescence*) in the controls (CTR) and all different REC conditions, i.e., after 48-h CT with 40 μM *cis*-dichlorodiammineplatinum (CDDP) or 10 μM (*OC*-6-44)-acetatodiamminedichlorido(2-(2-propynyl)octanoato)platinum(IV) [Pt(IV)Ac-POA] alone, after carbon ion irradiation (2 or 4 Gy dose) alone, or in combined exposure conditions [40 μM CDDP or 10 μM Pt(IV)Ac-POA 48-h CT + 2 or 4 Gy carbon ion radiation]. DNA counterstaining with Hoechst 33258 (*blue fluorescence*). *Scale bar*, 20 μm.

and efficacy of the Pt-DNA adducts (Gabano et al., 2014; Kenny et al., 2017).

In the present investigation, we verified the efficacy of Pt(IV)Ac-POA on human U251 MG cell line, which possesses an extremely variable cellular phenotype, thus reflecting the typical glioblastoma high heterogeneity and invasiveness. These latter features drastically influence the efficacy of the treatments in that no specific targets have been clearly identified that could facilitate the chemotherapeutics action. Different complementary techniques have been employed to analyze firstly the effects of Pt(IV)Ac-POA administered alone and then the potential synergistic effects of treatment with the anticancer agent followed by HT with carbon ion application, with the goal to assess the potential synergistic effects of combined treatments to improve therapy efficacy.

Taking into consideration the CDDP concentration applied in previous works on different cell lines (Grimaldi et al., 2016, 2019; Astesana et al., 2020), the first experimental step was carried out to select the effective Pt(IV)Ac-POA dose. Based on the MTS viability assay data, Pt(IV)Ac-POA was demonstrated to

induce cell death already at the concentration of 10 μM under the standard condition, i.e., 48-h CT, with a significant decrease (about 50%) in the number of living cells. Additionally, the clonogenic assay results provide further evidence that exposure to increasing Pt(IV)Ac-POA concentrations induced a dose-dependent impairment of U251 clonogenic ability, with the 10-μM dose able to cause a 70% colony inhibition after the chosen 10-day time window.

It has to be highlighted that, even after standard acute exposure, Pt(IV)Ac-POA was effective already at a concentration four times lower than that widely reported in *in vitro* investigations testing CDDP, describing the use of 40 μM CDDP on a variety of tumor cell lines, based on its ability to cause apoptosis (Çetin et al., 2017; Qian et al., 2018) and cell cycle and mitochondrial respiratory complex alterations (Kachadourian et al., 2007; Chiang et al., 2014). Interestingly, the data obtained by the clonogenic cell survival assay, after the 10-day time window, further demonstrated the U251 refractoriness to the CDDP treatment, after which a 50% surviving cell fraction was measured. Based on this data, it appeared clear that the CDDP-caused clonogenic inhibition is lower compared to that observed after Pt(IV)ac-POA exposure (about 70%), thus supporting a more effective long-lasting action of the prodrug on the final cell fate balance.

Cytofluorimetric results after PI staining demonstrated that Pt(IV)Ac-POA was able to induce programmed cell death through different mechanisms, even though to a lesser extent when compared with data previously observed in different cell lines (Gabano et al., 2017; Rangone et al., 2018; Ferrari et al., 2020). The presence of numerous apoptotic cells after treatment was confirmed by annexin V assay, while, at the ultrastructural level, alternative programmed cell death mechanisms, i.e., apoptosis, autophagy, and necroptosis, were identified by TEM analysis.

The encouraging aspect of 10 μM Pt(IV)Ac-POA 48-h CT was the detected lack of necrosis compared to the other concentrations used, indicating a possible lower pro-inflammatory outcome and adverse side effects for healthy cells.

Since GBM has an unfavorable prognosis mainly due to its high propensity for tumor recurrence, the "recovered condition" was used to mimic the renewal phase that follows chemotherapy treatment and therefore to observe the potential activation of resistance mechanisms in U251 cells.

Immunocytochemical staining, investigating specific representative markers, confirmed the activation of both intrinsic and extrinsic apoptotic pathways. Specifically, this technique allowed properly demonstrating the effects of 10 μM Pt(IV)Ac-POA 48-h CT and 40 μM CDDP 48-h CT, revealing that, after both treatments, cells displayed immunopositivity for both cleaved caspase-3 and caspase-8, showing an increase in the percentage of immunopositive cells compared to the control conditions. The most interesting finding was the progressive immunopositivity increase, observed in recovered cells after Pt(IV)Ac-POA exposure, showing a steady enhancement in the fluorescence signal for both caspases. This data seemed to suggest that Pt(IV)Ac-POA exposure, already at a dose of 10 μM, may have a long-lasting effect, inducing cell death over a long period after treatment. Concerning PARP-1 and RIP1, after both 10 μM Pt(IV)Ac-POA 48-h CT and 40 μM CDDP 48-h CT, an immunopositivity increase was detected. In particular, the translocation of the cleaved fragment p89 of PARP1 from the nucleus to the cell cytoplasm further confirms the activation of the intrinsic apoptotic pathway, although with a more marked effect after 10 μM Pt(IV)Ac-POA 48-h CT.

The cleavage of PARP-1 was also confirmed by the Western blotting data, by which the full length of PARP-1 and the fragment p89 were investigated. Notably, in recovered cells after 40 μM CDDP, the observed redistribution of the fluorescent PARP1 signal together with a particular *phagocytic-like* morphology may indicate a possible damage compensation played by cells, which may represent a survival mechanism after treatment.

Notably, the caspase-8 and RIP1 immunoreactivity of the Pt(IV)Ac-POA-treated cells clearly demonstrated the activation of the extrinsic apoptotic pathway, also suggesting that RIP1 translocation to the cell nucleus might represent a preliminary step of the necroptotic pathway, as observed in the treated samples by TEM.

The necroptosis triggering was also corroborated by the MLKL perinuclear localization within the fragmented nuclei in the cells treated with either CDDP or Pt(IV)Ac-POA, as previously supported by the morphological features detected by both TEM ultrastructural analysis as well as RIP1 immunofluorescence detection. This finding was particularly evident in the Pt(IV)Ac-POA REC condition, suggesting the persistence of a long-lasting activation of necroptosis after prodrug exposure. Moreover, further evidence of the involvement of different programmed cell death pathways after treatment with both platinum compounds was revealed by AIF translocation from the mitochondrial compartment to the damaged cell nuclei. This outcome, which indicated the activation of a caspase-independent pathway, was visible after both 40 μM CDDP 48-h CT and 10 μM Pt(IV)Ac-POA 48-h CT. AIF translocation was predominantly manifest after Pt(IV)Ac-POA 48-h CT and in REC samples, while it was no longer distinguishable in the CDDP REC condition. Pt(IV)Ac-POA's ability to induce different programmed cell death pathways, lessening necrosis induction, appears to be a valuable advantage, particularly based on the observation of the enduring effect, still evident in the REC condition.

Immunofluorescence staining also demonstrated the effects of Pt(IV)Ac-POA on intracellular organelles, revealing that the prodrug is able to act on cytoplasmic targets, as previously demonstrated for other platinum(II) compounds, i.e., CDDP and Pt(*O*,*O*-acac)(γ-acac)(DMS) (Bottone et al., 2008; Santin et al., 2012; Grimaldi et al., 2016). Specifically, already at a concentration of 10 μM, Pt(IV)Ac-POA (48-h CT) affected both the mitochondria and the Golgi apparatus, which lost their physiological organization, appearing degraded and accompanied by both actin and tubulin cytoskeletal alterations.

Notably, in recovered cells after Pt(IV)Ac-POA exposure, U251 cells were characterized by an even worsened condition, showing a highly degraded Golgi cistern arrangement and a clustered mitochondria, while in recovered cells formerly treated with CDDP, a tentative cell restoration was observed, with the

cytoplasmic organelles resuming their physiological structure and localization.

It has to be mentioned that mitochondrial alterations may lead to an improved ROS production and, therefore, to an increased oxidative stress status, therefore promoting better conditions to enable Pt(IV)Ac-POA activity (Muscella et al., 2011).

The present investigation also documented the autophagic pathway activation. Specifically, after 40 μM CDDP 48-h CT, the immunofluorescence result showed p62/SQSTM1-LC3B colocalization, which would be ascribable to the formation of autophagolysosomes and autophagosomes. Otherwise, after Pt(IV)Ac-POA 48-h CT, a lack of p62/SQSTM1-LC3B colocalization was detected, with the two markers displaying expression levels comparable to those observed in the controls. On the other hand, in the CDDP REC condition, an increased LC3B and p62/SQSTM1 immunoreactivity was measured, even though the two signals did not colocalize; in parallel, an enhancement in cell size was clearly observable. Differently to the effects observed after Pt(IV)Ac-POA 48-h CT, in the Pt(IV)Ac-POA REC conditions, the colocalization of p62/SQSTM1 and LC3B signals was detected. The p62/SQSTM1 expression levels measured by Western blotting analyses after 10 μM Pt(IV)Ac-POA REC supported the activation of the autophagic pathway, in which p62 may play a role.

Previous literature data demonstrated that autophagy and p62 are two interdependent parts of the protein control system, strictly interacting to maintain proteostasis, disclosing a frequent p62 upregulation and/or reduced degradation in cancer cells during tumor progression (Moscat et al., 2016; Islam et al., 2018). The p62 level homeostatic maintenance in cancers by autophagy-dependent or autophagy-independent mechanisms may contribute to the final outcome of the tumorigenic process, also having important implications for the design of forthcoming anticancer therapeutic protocols targeting autophagy or p62-regulated signaling pathways (Liu et al., 2016). The p62 levels, determined after Pt(IV)Ac-POA 48-h CT, could play a role in autophagy activation, representing a type II programmed death (Galluzzi et al., 2018) and not a cell survival mechanism (Belounis et al., 2016). This result differs from (i) the presently obtained results indicating a slight increase in p62 expression levels after both 40 μM CDDP 48-h CT as well as in recovered cells formerly exposed to CDDP, suggesting the possible occurrence of a cell survival mechanism, as also corroborated by the amelioration of the cell morphology, and (ii) previous data on cisplatin showing a chemoresistance phenomenon promoted by a strong decrease in p62 expression level (Chen et al., 2018; Lin et al., 2018).

In the second experimental phase, assessing the potential occurrence of a synergistic effect combining chemotherapeutics treatments with hadrontherapy, we firstly demonstrated that, after a 10-day time window, the clonogenicity of U251 was further decreased when exposed to combined treatments, i.e., 10 μM Pt(IV)Ac-POA + carbon ion irradiation at increasing doses (i.e., 1, 2, and 4 Gy). Specifically, a synergistic effect was detected when 10 μM Pt(IV)Ac-POA was combined with either 2 or 4 Gy radiation, with a measured reduction of colony formation of about 85%. Differently, the combined exposure to 10 μM Pt(IV)Ac-POA + 1 Gy induced a clonogenicity impairment analogous to that observed after treatment with the prodrug alone (26% *vs*. 30%). These results pertaining to the U251 clonogenicity impairment after the combined treatments provided an early evidence that exposure to a low-dose carbon ion beam, i.e., 2 Gy, was already effective to produce a long-lasting effect inducing U251 cell death.

Then, we hypothesized that the synergistic effect of chemotherapeutics plus hadrontherapy could effectively trigger and improve intrinsic apoptotic pathway activation (Di et al., 2013); therefore, caspase-3 activation and PARP-1 expression were assessed. With regard to active caspase-3, in order to compare the efficacy of the diverse combined treatments, a quantification of immunopositive cells was carried out. The measured percentages in 40 μM CDDP 48-h CT- and 10 μM Pt(IV)Ac-POA-treated cells and in the samples treated with chemotherapeutics and subsequently exposed to 2 or 4 Gy carbon ion radiation revealed a significantly increased activation of the intrinsic apoptotic pathway compared to the control condition.

Notably, the 10 μM Pt(IV)Ac-POA 48-h CT-induced cell death effect was significantly more pronounced compared to that observed after 40 μM CDDP 48-h CT exposure followed by carbon ion radiation, also highlighting that 4 Gy exposure had greater effects than the 2 Gy dose. Specifically, our data emphasize that, when preceded by Pt(IV)Ac-POA 48-h CT, the 4 Gy dose was able to induce a stronger nuclear degradation compared to the 2 Gy dose. Notably, in the recovered condition, i.e., cells exposed to 10 μM Pt(IV)Ac-POA 48-h CT followed by both 2 and 4 Gy carbon ion radiation, the efficacy of the combined treatment was particularly marked, even at the lowest dose of the high LET radiation tested.

Concerning PARP1, pivotally involved in DNA damage repair processes, it is known to act as a survival factor in case of limited damages to the DNA, while, when extensive DNA damage arises, it promotes cell death (Virág and Szabó, 2002), binding with high affinity to single- or double-strand breaking sites on the DNA (Gupte et al., 2017). Indeed, one of the immediate responses after DNA damage by ionizing radiation is the activation of poly(ADP-ribosyl)ation reaction, even if the role of PARP-1 in cluster DNA damage condition by high LET radiation is still under investigation (Atanu et al., 2016). In the present study, the localization of PARP-1 at the nuclear and cytoplasmic levels was evaluated, being the cytoplasmic translocation of cleaved PARP-1, i.e., the p89 fragment, a typical feature of apoptosis occurrence. Our findings demonstrated that the p89 fragment was detected only in selected conditions, i.e., at 40 μM CDDP 48-h CT + 4 Gy, 10 μM Pt(IV)Ac-POA 48-h CT + 2 Gy, and 10 μM Pt(IV)Ac-POA 48-h CT + 4 Gy.

It has to be recalled that, after 10 μM Pt(IV)Ac-POA 48-h CT, PARP1 was expressed at the nuclear level in cells during the early stages of apoptosis; differently, in late apoptosis, in which the nuclei were evidently fragmented, PARP1, or rather p89, moved to the cytoplasm. This latter phenomenon became particularly evident in the cells treated with 10 μM Pt(IV)Ac-POA 48-h CT and also when combining the 10 μM Pt(IV)Ac-POA 48-h CT with the following carbon

ion irradiation. Taken together, the present data stressed the strongest occurrence of PARP-1 translocation in irradiated samples. Western blotting analyses disclosed a significant reduction in the expression levels of cleaved-length PARP-1 both in cells exposed to the 4 Gy irradiation alone as well as in cells exposed to 40 μM CDDP 48-h CT followed by carbon ion radiation compared to the samples treated with 10 μM Pt(IV)Ac-POA followed by 4 Gy irradiation. These findings could support the key role of PARP-1 in chemo- and radioresistance phenomena.

Surprisingly, in the samples exposed to 10 μM Pt(IV)Ac-POA followed by 4 Gy carbon ion radiation, a reduction in the expression of full-length PARP-1 paralleled by the presence of cleaved PARP-1 was detected, corroborating the strong effect of the combined 10 μM Pt(IV)Ac-POA 48-h CT + 4 Gy treatment in reducing the expression of this protein essentially involved in DNA repair.

With regard to the autophagy role, as above reported, the slight reduction in p62/SQSTM1 expression after 10 μM Pt(IV)Ac-POA 48-h CT may confirm the activation of the autophagic pathway, further validated by the Western blotting data supporting the homeostatic imbalance in the activation of autophagy. After 40 μM CDDP 48-h CT, an increased expression of p62/SQSTM1 was above described. Interestingly, this trend persisted in the samples exposed to 40 μM CDDP 48-h CT followed by 4 Gy irradiation, with a significant decrease of p62 expression indicating that the protein may be probably degraded by a strong activation of autophagy. Notably, the combined exposure to 10 μM Pt(IV)Ac-POA 48-h CT followed by 4 Gy irradiation slightly enhanced p62, which nonetheless maintained its expression value similar to that determined in the controls. According to the literature (Liu et al., 2016; Moscat et al., 2016; Islam et al., 2018), this data suggested that the p62 levels, quantitatively closer to those measured in the physiological condition, determined after the combined treatment protocol could have a role in preventing tumor progression.

When investigating the REC conditions, based on the hypothesis that the combined treatment (chemotherapeutics exposure followed by carbon ion radiation) trigger and improve apoptosis activation (Di et al., 2013), our initial experiments focused on caspase-3 activation and PARP-1 expression.

The immunofluorescence analysis for both these proteins revealed a visible restoration of the cells exposed to carbon ion radiation only, both at the 2 and 4 Gy dose. Retrieval of physiological conditions following recovery was also observed in the samples exposed to Pt(IV)Ac-POA alone. Interestingly, REC cells, formerly treated with 40 μM CDDP 48-h CT and then exposed to carbon ion radiation, both at the 2 and 4 Gy dose, displayed a reduction of caspase-3 immunopositivity, paralleled by a progressive restoration of cell morphology associated with the absence of PARP1 translocation phenomenon.

Differently, REC samples previously treated with 10 μM Pt(IV)Ac-POA 48-h CT and then irradiated at 2 or 4 Gy still showed an evident caspase-3 immunopositivity, as well as significant morphological alterations. It has to be underlined that in the Pt(IV)Ac-POA REC condition after exposure to low irradiation, i.e., 2 Gy, a significant effect, which was not already detectable in the acute condition, was perceivable in the long-term experiment. This latter data supports the hypothesis that the low-dose carbon ion beam could have better long-lasting effects compared to the 4 Gy higher dose. Taken together, these initial results suggested that the p89 fragment translocation, governed by caspase-3 activity, could be particularly effective in glioma cells irradiated with a high LET carbon ion beam (Perez et al., 2019), in which the DNA damage repair and response (DRR) to double-strand breaks (DSB) is strongly increased (Kesari et al., 2011; Erasimus et al., 2016). Lastly, these findings demonstrated that 7 days after combined treatment, 10 μM Pt(IV)Ac-POA 48-h CT + 2 or 4 Gy carbon ion beam assured the most marked effect, which has to be correlated with the chemical nature of Pt(IV)Ac-POA bearing HDACi (Oertel et al., 2011; Barazzuol et al., 2015).

In summary, our present findings support at first the use of this manufactured prodrug as a potential alternative to the use of cisplatin and its analogs, considering that Pt(IV)Ac-POA is effective in human U251 GBM cells already at a concentration four times lower than that employed for standard *in vitro* treatment with CDDP (10 *vs.* 40 μM, respectively), being able to trigger cell death pathway activation. Pt(IV)Ac-POA peculiar molecular structural stability outside cancer cells and new subcellular targets are key elements essential to counteract drug resistance also improving chemotherapeutic efficacy, thus suggesting the possibility of incorporating this prodrug into the treatment regimen for GBM. Even more, interestingly, the present investigation provides emerging evidence of the efficacy of the combined protocol using the chemotherapeutic prodrug Pt(IV)Ac-POA followed by high LET, also able to synergistically cause a long-lasting clonogenicity reduction of glioblastoma cells, thus representing a promising approach in the neoadjuvant setting to primary and recurrent glioblastomas.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

MB, ER, and PR conceived and designed the experiments. BF, ER, EP, and AF performed the experiments and analyzed the data. MB, MR, FB, and CL contributed reagents, materials, and analysis tools. BF, EP, and FD searched and reviewed the literatures. BF and ER drafted the manuscript. ER, MB, AF, and PR critically revised the article. All the authors provided critical feedback, helped shape the research and analysis, read and agreed to the published version of the manuscript.

## FUNDING

This research was supported by the University of Pavia: Fondi Ricerca Giovani (FRG 2018). This research was also supported

by the Italian Ministry of Education, University and Research (MIUR): Dipartimenti di Eccellenza Program (2018–2022), Department of Biology and Biotechnology "*L. Spallanzani*", University of Pavia.

## ACKNOWLEDGMENTS

We thank Dr. Giuliano Mazzini (IGM-CNR, Pavia) for his excellent assistance in the flow cytometry analysis.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 18 May 2021
doi: 10.3389/fphar.2021.680021

# Targeting Neuroinflammation in Brain Cancer: Uncovering Mechanisms, Pharmacological Targets, and Neuropharmaceutical Developments

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OPEN ACCESS

**Edited by:**
*Maria Grazia Bottone, University of Pavia, Italy*

**Reviewed by:**
*Marco Peviani, University of Pavia, Italy*
*Marta Llansola, Principe Felipe Research Center (CIPF), Spain*

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**Specialty section:**
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology*

**Received:** *12 March 2021*
**Accepted:** *04 May 2021*
**Published:** *18 May 2021*

**Citation:**
*Alghamri MS, McClellan BL, Hartlage MS, Haase S, Faisal SM, Thalla R, Dabaja A, Banerjee K, Carney SV, Mujeeb AA, Olin MR, Moon JJ, Schwendeman A, Lowenstein PR and Castro MG (2021) Targeting Neuroinflammation in Brain Cancer: Uncovering Mechanisms, Pharmacological Targets, and Neuropharmaceutical Developments. Front. Pharmacol. 12:680021. doi: 10.3389/fphar.2021.680021*

Gliomas are one of the most lethal types of cancers accounting for ~80% of all central nervous system (CNS) primary malignancies. Among gliomas, glioblastomas (GBM) are the most aggressive, characterized by a median patient survival of fewer than 15 months. Recent molecular characterization studies uncovered the genetic signatures and methylation status of gliomas and correlate these with clinical prognosis. The most relevant molecular characteristics for the new glioma classification are *IDH* mutation, chromosome 1p/19q deletion, histone mutations, and other genetic parameters such as *ATRX* loss, *TP53,* and *TERT* mutations, as well as DNA methylation levels. Similar to other solid tumors, glioma progression is impacted by the complex interactions between the tumor cells and immune cells within the tumor microenvironment. The immune system's response to cancer can impact the glioma's survival, proliferation, and invasiveness. Salient characteristics of gliomas include enhanced vascularization, stimulation of a hypoxic tumor microenvironment, increased oxidative stress, and an immune suppressive milieu. These processes promote the neuro-inflammatory tumor microenvironment which can lead to the loss of blood-brain barrier (BBB) integrity. The consequences of a compromised BBB are deleteriously exposing the brain to potentially harmful concentrations of substances from the peripheral circulation, adversely affecting neuronal signaling, and abnormal immune cell infiltration; all of which can lead to disruption of brain homeostasis. In this review, we first describe the unique features of inflammation in CNS tumors. We then discuss the mechanisms of tumor-initiating neuro-inflammatory microenvironment and its impact on tumor invasion and progression. Finally, we also discuss potential pharmacological interventions that can be used to target neuro-inflammation in gliomas.

**Keywords: immunosuppression, inflammation, tumor microenvironment, glioma, immunotherapy**

## INTRODUCTION

Gliomas are the most commonly diagnosed malignant primary tumors that arise in the brain (Louis et al., 2016; Ceccarelli et al., 2016; Schwartzbaum et al., 2006). The traditional classification of gliomas is based on the histological features according to the microscopic similarity with the putative cell of origin along glial precursor cell lineages (i.e., astrocytes, oligodendrocytes, etc) and malignancy grade (Grade I-IV). Gliomas that lack features of aggressiveness and grow slower are classified as Low-Grade Gliomas (LGG) and correspond with WHO grades I and II, while gliomas that have hallmarks of aggressiveness and are more proliferative are classified as High-Grade Gliomas (HGG) and correspond with WHO grades III and IV. Although histopathologic classification is more established, it has the disadvantage of potential observer bias, particularly between grade III and IV tumors (van den Bent et al., 2010). In the last decade, the availability of glioma datasets has facilitated the correlation of genetic, transcriptional, and epigenetic signatures with clinical features (Noushmehr et al., 2010; Sturm et al., 2012; Verhaak et al., 2010).

The current stratification of gliomas is established by the WHO Classification of Central Nervous System Tumors, which was updated in 2016 (Louis et al., 2016; Wesseling and Capper, 2018). According to the new classification, gliomas are divided into diffusely infiltrating gliomas, which include grade II and III astrocytic tumors, grade II and III oligodendrogliomas, grade IV glioblastoma, and diffuse midline gliomas (Louis et al., 2016). This recent update is the first to include molecular parameters into consideration, besides the commonly used histopathological parameters. The more relevant molecular markers incorporated in glioma classification are *IDH* mutations, 1p19q deletion, MGMT promoter methylation, TERT promoter mutations, ATRX loss of function mutations, and p53 loss of function mutations and mutations in isocitrate dehydrogenase 1 and 2 genes (*IDH1*/2 m) (Louis et al., 2016).

*IDH1/2* m defines a distinct subgroup of glioma (GBM) and is clinically associated with favorable outcomes. *IDH*m have been identified in grade II and III astrocytomas, oligodendrogliomas, and oligoastrocytomas, and in secondary glioblastomas (which refers to gliomas with high-grade hallmarks such as nuclear atypia, and/or cellular pleomorphism as well as microvascular proliferation and/or necrosis), which result from tumor recurrence. *IDH1*m tumors are classified into two molecular subgroups: the first group carries a codeletion of the chromosomal bands 1p and 19q and TERT promoter mutation. Most of these gliomas are histologically defined as oligodendrogliomas. On the other hand, *IDH*m without 1p and 19q codeletion are mostly P53 and ATRX mutant, associated with hypermethylation phenotype (G-CIMP high) and astrocytic histology. The incorporation of *IDH*m status into diffuse glioma classification was in part prompted by its relevance in the clinical outcome of the tumors.

Diffuse gliomas with wild-type *IDH* (*IDH*-wt), even when they may be histologically defined as grade II or III, tend to behave like more aggressive glioblastomas. *IDH*-wt grade IV glioblastomas (GBM) are the most common malignant primary brain tumors. GBMs are densely cellular, pleomorphic tumors with mitotic activity and either microvascular proliferation or necrosis, or both. Histologic variants of this group include epithelioid glioblastoma (which normally occurs in children and young adults and carries BRAF V600 E mutations), along with giant cell GBM and gliosarcoma. The prognosis for all glioblastoma variants is poor, with survival commonly less than two years. Despite the clear differences between them, pediatric gliomas are still classified according to the histological resemblance to adult gliomas. As an exception, the WHO 2016 classification incorporated the entity of histone H3 K27 M diffuse midline glioma, which mainly occurs in pediatric patients and regardless of the histological grade, has a poor prognosis. Our molecular knowledge of pediatric gliomas has been refined over the last few years, and some molecular markers, such as H3 G34R/V, BRAF, NF1 mutations, and PDGFRα amplifications, are now becoming relevant for the decision of clinical interventions (Koschmann et al., 2016; Miklja et al., 2019; Haase et al., 2020).

Several aspects make it difficult to efficiently treat gliomas, i.e., the anatomical location, the presence of the blood-brain barrier (BBB), and the restricted immune reactivity within the CNS. The anatomical location, together with the infiltrative nature of high-grade glioma, makes the total resection of the tumor mass virtually impossible. The blood-brain barrier hampers the delivery of therapeutic compounds to the tumor site. Despite these limitations, intense research has opened up opportunities to explore tailored therapies for different glioma subtypes with particular molecular lesions that are currently under clinical trials (Haase et al., 2020).

Currently, the standard of care (SOC) for GBM is comprised of surgery to remove the tumor mass, followed by radiation therapy in combination with Temozolomide (TMZ) administration. The incorporation of TMZ in GBM treatment leads to a small progression-free survival (PFS) improvement (5 vs. 6.9 months), in addition to a significant benefit in 2-years overall survival (11 vs. 27%) (Brandes et al., 2008). The MGMT promoter methylation can predict the TMZ therapy efficacy, and in many glioma subtypes which do not have MGMT promoter methylation, TMZ does not improve patients' outcome. The extent of tumor resection is the most prominent treatment factor associated with survival (Brown et al., 2016; Molinaro et al., 2019).

## BLOOD-BRAIN BARRIER

The healthy Blood-Brain Barrier (BBB) is a selectively permeable barrier secured by endothelial cells linked by tight junctions, pericytes embedded in a basement membrane. Astrocytic end-feet is also anchored to the basement membrane (Varatharaj and Galea, 2017; Ros et al., 2018). Under homeostasis, these components protect the brain from toxic materials, regulate the transport of essential nutrients, and maintain a stable brain environment (Bergers and Benjamin, 2003; Ros et al., 2018). When BBB integrity is compromised, for example by glioma-associated inflammation, these cell layers change in

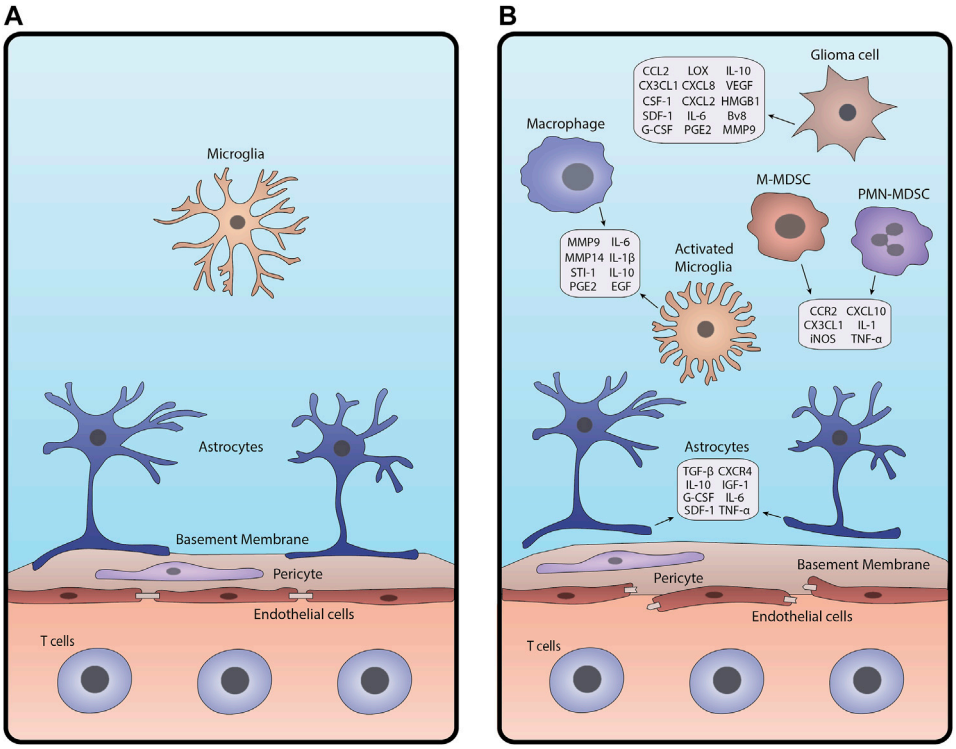


**FIGURE 1 |** Schematic of the BBB under **(A)** homeostasis and **(B)** glioma-associated inflammation. In inflamed conditions **(B)**, junctions between endothelial cells break apart, pericytes are disrupted, and astrocytic endfeet detach from the basement membrane. Glioma cells, macrophages and microglia, astrocytes, and MDSCs release cytokines and other factors that contribute to BBB breakdown and create an inflammatory tumor microenvironment.

both anatomy and function, altering the permeability of blood vessels in the brain (**Figure 1**; Erickson et al., 2012; Varatharaj and Galea, 2017).

Gliomas are characterized by the activation of numerous pathways that drive gliomagenesis. These include but are not limited to activation of hypoxia, abnormal angiogenesis, and tissue remodeling which disrupt the BBB and trigger an inflammatory response in the brain microenvironment (Bergers and Benjamin, 2003; Allavena et al., 2008; Ros et al., 2018). Response to hypoxic conditions is mediated by hypoxia-inducible factor-alpha (HIF-α), regulating the expression of angiogenic and inflammatory factors such as vascular endothelial growth factor (VEGF) (Murat et al., 2009; Belykh et al., 2020). VEGF disrupts the cellular barrier around existing blood vessels, pulling endothelial cells away to form new capillaries with fenestrations and fewer tight junctions (Dubois et al., 2014; Belykh et al., 2020). This results in an enhanced infiltration of cellular and plasma components into the brain, further exacerbating brain homeostasis.

While the intact BBB permits immune surveillance by effector immune cells, compromised BBB allows excessive immune cell infiltration magnifying inflammatory responses (Vries et al., 1998; Sonar and Lal, 2018). Tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) produced by inflamed immune cells such as tumor-associated macrophages (TAMs) promote the expression of inflammatory molecules in other cell types (Allavena et al., 2008; Sethi et al., 2008; Kore and Abraham, 2014). Moreover, reactive astrocytes, microglia, and endothelial cells can secrete mediators including transforming growth factor (TGF-β) and metalloproteinases (MMP2 and MMP9), augmenting the inflammatory tumor microenvironment (Sethi et al., 2008; Könnecke and Bechmann, 2013). Altered TGF-β signaling reduces the expression of cell adhesion molecules in pericytes and endothelial cells, weakening their attachments to the surrounding vessel (Joseph et al., 2013; Sonar and Lal, 2018). MMPs secreted by activated microglia cleave extracellular (ECM) components of the basal lamina, which alters the function of the basement membrane (Wolburg et al., 2012; Könnecke and Bechmann, 2013).

## GLIOMA TUMOR MICROENVIRONMENT

Previously, the CNS was thought to be an 'immune privileged' site. This idea was favored following the rejection of transferred foreign tissue into the brain parenchyma (Murphy and Sturm, 1923; Medawar, 1948; Galea et al., 2007). Further investigation demonstrated that this immune privilege status of the CNS is relative to other tissues and organs and it depends on the existence of neuroinflammation. This has led to the implementation of clinical trials aiming to develop immunotherapies against glioma. However, most of these trials have failed to show benefits in glioma patients due to the

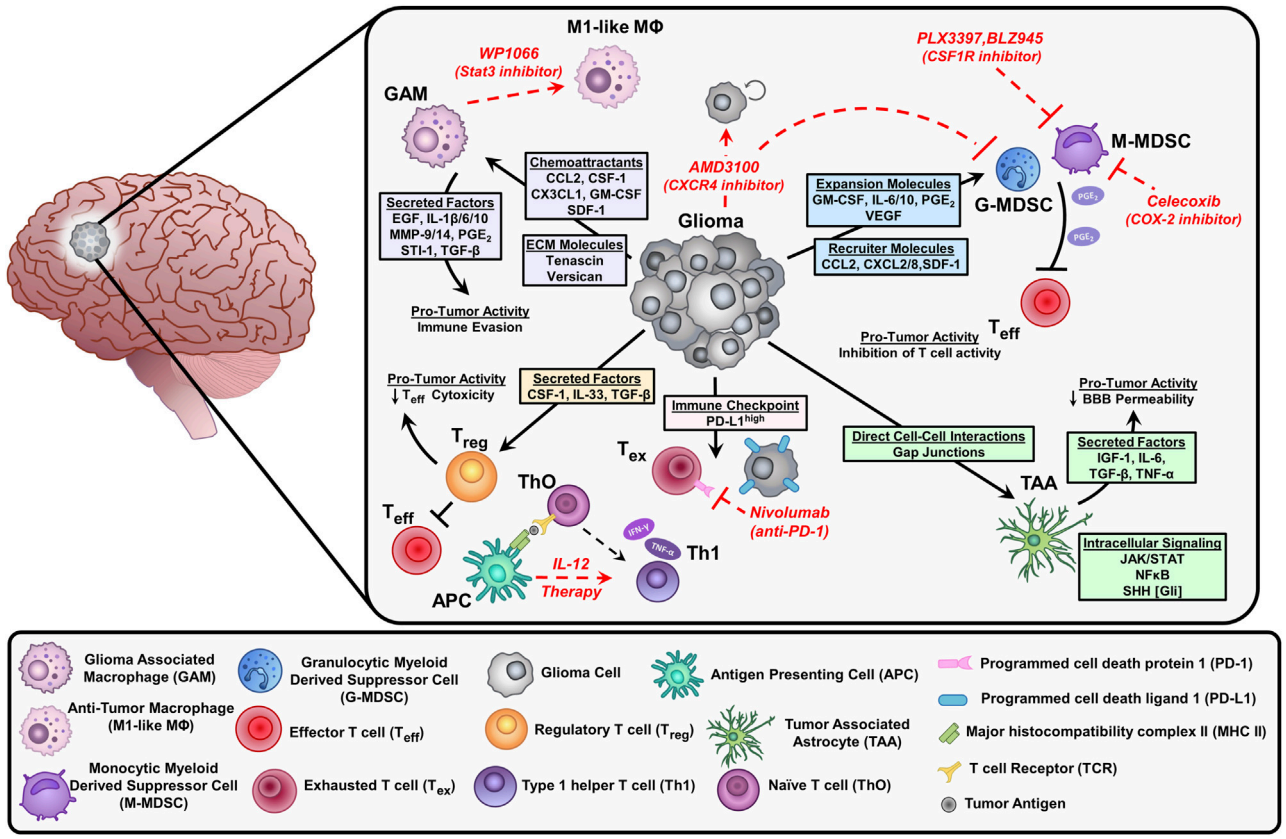


**FIGURE 2 |** Depiction of the immunosuppressive environment in glioblastoma and current therapeutic approaches (red) to stimulate anti-tumor responses or inhibit suppressive immune cell populations. Glioma cells release chemoattractants and cytokines that recruit immune cells into the tumor microenvironment (TME). These factors elicit pro-tumor activities that inhibit effector T cells (Teff) and contribute to tumor progression. Glioma cells can also directly interact with immune cells and astrocytes impacting the effectiveness of chemotherapy. Inhibition of Stat3 by WP1066 reduces glioma-associated macrophages (GAM) by blocking intracellular signals induced by IL-10 to promote an anti-tumor macrophage (M1-like MΦ) phenotype. The CXCR4 antagonist AMD3100 prevents the binding of stromal cell-derived factor (SDF-1), resulting in decreased tumor proliferation and blocks the migration of myeloid-derived suppressor cells (MDSCs) in the TME. The CSF1R inhibitors (PLX3397, BLZ945) suppress the infiltration of myeloid cells (macrophages and MDSCs) within the TME. MDSCs synthesize prostaglandin E2 (PGE2) via COX-2 which inhibits effector T cells by reducing Interferon-gamma (IFN-γ) release. The COX-2 inhibitor, celecoxib reduces MDSC immunosuppressive activity. Interleukin-12 (IL-12) is secreted by antigen-presenting cells (APC) like dendritic cells and macrophages, exogenous IL-12 therapy triggers a pro-inflammatory transition of naïve T cells (ThO) to type 1 helper T cells (Th1) leading to an increase in IFN-γ and tumor necrosis factor (TNF-α) secretion. Tumor cells express high levels of programmed cell death ligand (PD-L1) on their cell surface, which leads to exhausted T cells (Tex) by immune checkpoint signaling through their receptor programmed cell death protein 1 (PD-1). Inhibition of PD-1 with nivolumab reduces T cell exhaustion.

immunosuppressive nature of the glioma tumor microenvironment (TME) that hampers effective anti-glioma immune response. In addition to the infiltrating immune cells, glioma cells express immune suppressive molecules such as interleukin-6, interleukin-10, TGF-β, LDH5, galectin-1 (gal-1), and prostaglandin-E which reprogram the infiltrating immune cells to a pro-tumor phenotype (**Figure 2**; Van Meir, 1995; Huettner et al., 1997; Crane et al., 2012; Perng and Lim, 2015). Interleukin-10 not only inhibits the functions of DCs and macrophages but also has a pro-inflammatory and inhibitory effect against T-cell activation and proliferation (Moore et al., 2001). Gal-1 enhances tumor cell migration and induces T-cell apoptosis and the skewing of tumor-infiltrating macrophages to the immunosuppressive M2 type (Verschuere et al., 2011).

Myeloid cells are the most frequent immune cell type in the glioma TME, accounting for ~60% of all glioma infiltrating immune cells. In GBM, myeloid-derived suppressor cells (MDSCs) are major immunosuppressive cells that hamper glioma immune response (Kamran et al., 2017; Alghamri et al., 2020). Clinically, the frequency of MDSCs is associated with an unfavorable prognosis in glioma (Rahbar et al., 2016). Besides MDSCs, glioma-associated macrophages/microglia (GAMs) represent a major population of immune cells infiltrating gliomas (Pyonteck et al., 2013; Brown et al., 2018; Chen and Hambardzumyan, 2018). Several chemokines and cytokines, including CCL2 and CX3CL1, are responsible for GAMs recruitments and expansion in glioma (Okada et al., 2009; Held-Feindt et al., 2010; da Fonseca and Badie, 2013). Within the lymphoid cells, T cells and NK cells represent the major effector cells in glioma (Kmiecik et al., 2013; Kmiecik et al., 2014), albeit they represent a small percentage of the total

immune cells within the glioma TME (about 2% of immune-infiltrating cells). Tregs are also infiltrated in the glioma TME, they elicit a strong immunosuppressive response against anti-glioma T-cells (Chang et al., 2016). Multiple GBM-derived factors are responsible for Tregs recruitment including CCL22, CCL2, TGF-β, or indoleamine 2,3-dioxygenase 1 (IDO1) (Akasaki et al., 2004; Akhurst and Hata, 2012; Wainwright et al., 2012; Chang et al., 2016). Glioma-induced tissue hypoxia has also been shown to be responsible for the activation of regulatory T-cells (Saetta et al., 2011; Razavi et al., 2016). Collectively, recruitment and activation of immunosuppressive cells decrease the efficacy of anti-glioma immune response. Below, we will discuss in detail how some of these cells are recruited and activated to the glioma TME to support glioma invasion.

## Role of Myeloid Cells (Microglia/ Macrophages, MDSCs)

GAMs make up a high proportion of myeloid cells encountered in the glioblastoma microenvironment accounting for up to 30% of the tumor mass (Russo and Cappoli, 2018). Microglia are the brain's resident myeloid cells, which are not replenished by blood-derived monocytes under normal physiological conditions. Instead, they function as a self-sustaining population with an extended capacity to proliferate (Schetters et al., 2018). In contrast, peripheral macrophages are derived from hematopoietic stem cells arising in the bone marrow and migrate to the glioma microenvironment partially due to the breakdown of the blood-brain barrier (Russo and Cappoli, 2018). GAMs are recruited to the glioma microenvironment via the glioma cells' release of chemoattractant: CCL2, CX3CL1, colony-stimulating factor (CSF-1), stromal cell-derived factor 1 (SDF-1), granulocyte/macrophage-colony stimulating factor (GM-CSF), and lysine oxidase (LOX) (**Figure 2**; Zhang et al., 2012; Roesch et al., 2018; Gutmann and Kettenmann, 2019; Chen et al., 2019). Under pathological conditions, microglia and peripheral macrophages/monocytes activate, proliferate, and contribute to the disruption of immunological homeostasis (Gutmann and Kettenmann, 2019; Sankowski et al., 2019). In the glioma microenvironment, macrophages are impacted to elicit pro-tumoral effects through the activation of immunosuppressive pathways enhancing glioma progression (Hambardzumyan et al., 2016; Roesch et al., 2018). For example, glioma cells produce let7, tenascin-C (TNC), and versican resulting in increased production of cytokines and matrix metalloproteases (MMP9 and MMP14) by GAMs (Hambardzumyan et al., 2016; Gutmann and Kettenmann, 2019). In response to these signals, GAMs release multiple cytokines such as transforming growth factor $\beta$ (TGF-β), stress-inducible protein 1 (STI-1), prostaglandin E2 ($PGE_2$), IL-6, IL-1β, IL-10, and epidermal growth factor (EGF), which promote glioma cell proliferation and inhibit T cells function (Hambardzumyan et al., 2016; Gutmann and Kettenmann, 2019). GAMs' depletion and/or inhibition by chlodronate and microglial inhibitory factor (MIF/TKP) drastically reduced tumor growth, further suggesting GAMs as a potential therapeutic target (Markovic et al., 2009; Zhai et al., 2011).

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that express high levels of immunosuppressive molecules and inhibit anti-tumor immunity (Grabowski et al., 2021). These cells can derive from monocytic (M-MDSCs) or granulocytic (PMN-MDSCs) origin (Grabowski et al., 2021). M-MDSCs have been shown to have greater immunosuppressive capability and are more common in the blood of GBM patients; whereas, PMN-MDSCs make up a greater portion of MDSCs in the glioma microenvironment (Mi et al., 2020). Tumor-derived cytokines are the major drivers of MDSCs expansion in the glioma microenvironment. These can be divided into two classes: MDSCs recruiters (such as CCL2, CXCL8, SDF-1, and CXCL2) and MDSCs expanders (such as IL-6, PGE2, IL-10, VEGF, and GM-CSF) (Mi et al., 2020; Miyazaki et al., 2020). These cytokines result in the recruitment and expansion of MDSCs infiltrating the glioma microenvironment. There, MDSCs suppress mainly T cell and NK cell functions (Gieryng et al., 2017). This inhibition is triggered by multiple mechanisms including induction of oxidative stress, inhibition of T cell migration, expression of T cell inhibitory ligands, and depletion of critical T cell metabolites (Mi et al., 2020; Grabowski et al., 2021). The establishment of MDSCs as a major immunosuppressive population identifies them as a target for anti-glioma therapy.

Several immunotherapies are being evaluated in clinical trials to target the immunosuppressive and pro-tumoral myeloid cells. The large majority of these immunotherapies target the pro-tumoral myeloid cell recruitment to the glioma. Representatively, the chemoattractant molecules responsible for the myeloid cell migration to the glioma, for example, CSF-1R, avβ3/5 integrins, and CXCR4 are being targeted in multiple trials (Russo and Cappoli, 2018; Roesch et al., 2018). PLX3397 (ClinicalTrials.gov NCT identifiers: CNCT01349036 and NCT01790503) and BLZ945 (ClinicalTrials.gov NCT identifier: NCT02829723) are CSF-1R inhibitors currently being evaluated for their efficacy as glioblastoma treatments (Butowski et al., 2015; Colman et al., 2018). Unfortunately, patients treated with PLX3397 alone showed no significant improvement, nor did PLX3397 improve the results when combined with the current standard of care (SOC), temozolomide chemoradiotherapy. The trial utilizing BLZ945, alone and in combination with the PD-1 checkpoint receptor inhibitor PDR001, as a treatment of glioblastoma, is still ongoing. An inhibitor of avβ3 and avβ5 integrins, cilengitide, in combination with SOC has been evaluated in clinical trials (ClinicalTrials.gov NCT identifier: NCT00689221) and shown no survival benefits compared to SOC alone. CXCR4 inhibitor, AMD3100, is in clinical trials for treating GBM, and one completed trial of AMD3100 in combination with SOC (ClinicalTrials.gov NCT identifier: NCT01977677) showed an improvement in tumor control (Thomas et al., 2019). In addition to inhibiting the recruitment of myeloid cells, some immunotherapy agents are also being evaluated in trials to target the functions of GAMs and MDSCs (Roesch et al., 2018; Mi et al., 2020). For stimulating GAM anti-tumoral activity, the small molecule inhibitor of STAT3, WP1066, is being evaluated in clinical trials (ClinicalTrials.gov NCT identifiers: NCT01904123 and NCT04334863). Research has shown that WP1066 causes

GAM upregulation of costimulatory molecules CD80 and CD86, but no results have been released for the WP1066 clinical trials (Hussain et al., 2007). Studies have shown that COX-2 inhibition suppresses MDSC activation, however COX-2 inhibition, via celecoxib, failed to show any favorable outcome in clinical settings (ClinicalTrials.gov NCT identifier: NCT00112502) (Penas-Prado et al., 2015; Mi et al., 2020).

## Role of T-Cells

T cells are the largest group of lymphocytes infiltrating the glioma microenvironment and are key factors for effective anti-glioma immunity (Yang et al., 2010; Mohme and Neidert, 2020). The number of CD8 T cells infiltrating the glioma microenvironment is correlated with positive outcomes in clinical settings (Yang et al., 2010; Gieryng et al., 2017). The first step for initiation and development of anti-glioma $CD8^+$ T cells, i.e., T cell priming, takes place in lymphoid tissues (cervical draining lymph nodes). The sequence of events begins with antigen loading on DCs and culminates with $CD8^+$ T cell-mediated tumor cytolysis (Dranoff, 2004; Farhood et al., 2019). DCs cross-present the tumor-associated antigen via MHC class I molecules to $CD8^+$ T cells to trigger the formation of cytotoxic anti-glioma $CD8^+$ T cells (CTLs) (Joffre et al., 2012; Spranger and Gajewski, 2018). The activated CTLs use two main pathways to induce target cell apoptosis, i.e., granule exocytosis and Fas ligand (FasL)-mediated apoptosis (Voskoboinik et al., 2006; Anel et al., 1994; de Saint Basile et al., 2010; Hassin et al., 2011). The former is mediated by releasing granular proteins such as granzymes (Gzs) and perforin from the CTLs. The second mechanism is triggered by the FasL pathway which induces apoptosis through activation of caspases. This prompts the release of cytochrome *c* and other essential enzymes in the target cells. Other effector molecules being releases by the CTLs, such as interferon-$\gamma$ (IFN-$\gamma$) and tumor necrosis factor $\alpha$ (TNF-α), augment their cytotoxicity toward cancer cells [(Schoenborn and Wilson, 2007; Farhood et al., 2019)].

However, studies found that the majority of $CD8^+$ T cells infiltrating the glioma microenvironment are in an exhausted, hypo-responsive state (Woroniecka et al., 2018). Exhaustion is a hypo-responsive T-cell state resulting from chronic antigenic exposure under sub-optimal conditions. This state represents a specific transcriptional program that is often characterized by up-regulation of various co-inhibitory receptors including PD-1, LAG-3, and TIM-3, on the T cells (Koyama et al., 2016; Kadiyala et al., 2020; Mohme and Neidert, 2020). These exhausted $CD8^+$ T cells ($CD8^+$ $T_{ex}$ cells) have lower secretion of their effector cytokines, but they still provide some, albeit reduced antitumor immunity (Woroniecka et al., 2018).

On the other hand, $CD4^+$ T cells primarily mediate anti-tumor immunity by providing help for $CD8^+$ CTL and antibody responses, as well as via secretion of effector cytokines such as interferon-$\gamma$ (IFN$\gamma$) and tumor necrosis factor-α (TNFα). $CD4^+$ T cells are highly versatile, polyfunctional cells that can differentiate into one of several diverse functional subtypes (Tay et al., 2021). The most extensively studies subtype in glioma immunology is Tregs. While exhausted CD8 T cells are merely non-functional, Tregs acquire immunosuppressive function and have pro-tumor functions within the glioma microenvironment (Andaloussi and Lesniak, 2006; Mu et al., 2017). Tregs suppress the anti-tumor immune response directly via expression of immunosuppression inducing ligands, such as PD-L1 and CTLA-4, or indirectly via the release of anti-inflammatory cytokines, including IL-10 and TGF-β (Grabowski et al., 2021). Therefore, Tregs represent an attractive target to enhance glioma immunotherapy.

A variety of therapeutic approaches targeting the T cell compartment in gliomas are being evaluated for clinical testing. Monoclonal antibody-based therapies targeting the immune checkpoint molecules mainly PD-1, PD-L1, and CTLA-4 are being used to block the direct cell-to-cell contact-mediated inhibition of $CD8^+$ T cells (Domingues et al., 2016; Wang et al., 2019). So far, clinical trials using the immune checkpoint inhibitors separately have shown inconsistent results in improving patient survival (Wang et al., 2019). For example, CheckMate 143 (ClinicalTrials.gov NCT identifier: NCT02017717) investigated the use of nivolumab, an anti-PD-1 monoclonal antibody, on patients with recurrent GBM, in a phase III clinical trial, but the trial was ended due to failure to improve overall survival compared to bevacizumab, an anti-VEGF therapy (Mitchell and Dey, 2019). However, this study prompted further investigation by showing the efficacy of nivolumab on the overall survival of a subset of recurrent GBM patients (Mitchell and Dey, 2019). A later phase II clinical trial (ClinicalTrials.gov NCT identifier: NCT02550249) demonstrated the safety of presurgical (neoadjuvant) nivolumab in a set of primary and recurrent GBM patients. Although no obvious clinical benefit was observed in recurrent GBM patients, 2 of the 3 primary patients treated with neoadjuvant nivolumab showed long-term survival (Schalper et al., 2019). Potential reasons for this lack of efficacy are low infiltration of $CD8^+$ T cells in the tumor and several immune checkpoint molecules being expressed simultaneously. Several combination therapies are being tested to counteract these reasons for low efficacy encountered when using single-treatment modalities. These combination therapies utilize multiple checkpoint inhibitors along with DC-based vaccines to stimulate more $CD8^+$ T cell recruitment (Wang et al., 2019; Miyazaki et al., 2020). ClinicalTrials.gov NCT identifier: NCT02529072 is an example of a recently tested DC-based vaccine combined with the nivolumab on recurrent gliomas. This study was terminated early due to adverse clinical effects, inefficacy of the combination and nivolumab alone treatments, and the failure of nivolumab alone to improve GBM survival (Peters et al., 2019). The combination immune checkpoint inhibitor therapies haven't shown consistent efficacy yet, but more research has the potential to optimize the immune checkpoint inhibitor combinations to enhance clinical outcomes (Wang et al., 2019).

## Role of Astrocytes

The brain microenvironment consists of multiple cell types, including the most abundant glial cell, astrocytes. Astrocytes constitute the majority of brain cells ~50% of all brain cells and, therefore, they are expected to play a major role in GBM [(O'Brien et al., 2013; Gieryng et al., 2017)]. GBM cells activate tumor-associated astrocytes (TAAs) to enhance GBM invasion

within healthy brain tissue (Guan et al., 2018; Henrik Heiland et al., 2019). TAAs are also involved in tumor progression and resistance to radiation and chemotherapy (Yang et al., 2014). Astrocytes themselves can become tumorigenic, which can be triggered by aberrant signaling in essential genes related to astrocyte development. For example, the nuclear factor kappa-B (NF-κB)- pathway has been shown to be involved in the transformation of normal astrocytes into TAAs which enhance GBM evasion (**Figure 2**; Kim et al., 2014). Recently, STAT-3 was found to play a major role in the induction of reactive astrocytes that contribute to the metastatic melanoma to the brain (Priego et al., 2018). Moreover, astrocytes secrete factors that maintain the tight junctions of the blood-brain barrier (BBB) (Abbott et al., 2006; Alvarez et al., 2011; Heithoff et al., 2021), which in turn regulates the success or failure of metastatic cells extravasating the brain. Tumor cells within the brain make direct contact with astrocytes through gap junctions (Soroceanu et al., 2001; Horng et al., 2017), leading to increased chemoresistance (Chen et al., 2015). Astrocytes also promote the release of degradative enzymes, cytokines, chemokines, and growth factors, thereby promoting tumor cell proliferation, survival, and invasion (Brandao et al., 2019).

Distinct astrocytic phenotype exists in the tumor environment, which leads to a large release of anti-inflammatory cytokines such as TGFβ, IL10, and G-CSF through JAK/STAT pathway activation (Henrik Heiland et al., 2019). Inhibition of the JAK/STAT pathway shifts the balance of pro- and anti-inflammatory cytokines toward a pro-inflammatory environment. The complex interaction of astrocytes and microglial cells can further contribute to the immunosuppressive glioma TME, suggesting that tumor-associated astrocytes contribute to anti-inflammatory responses (Henrik Heiland et al., 2019). Studies show that astrocytes with activated sonic hedgehog (SHH) signaling pathways are localized mainly in the glioma perivascular niche (Becher et al., 2008). Moreover, dysregulation of SHH signaling leads to enhanced proliferation of precursor cells likely to be involved in initiating the formation of gliomas (Clement et al., 2007; Komada, 2012). Moreover, the dysregulation of CXCL12/CXCR4 signaling induces aberrant astrocyte proliferation, which could also contribute to glioma progression (Bonavia et al., 2003). In response to CNS injury, reactive astrocytes can secrete IL-6, tumor growth factor-β (TGF-β), insulin growth factor-1 (IGF-1), and tumor necrosis factor-α (TNF-α), (Seike et al., 2011; Placone et al., 2016). Furthermore, GDF-15 overexpressed in reactive astrocytes caused an enhanced proliferation of GBM cells *in vitro* while GDF-15 deficient cells exhibit decreased *in vivo* tumor growth (Roth et al., 2010; Zamanian et al., 2012). Astrocytes can also promote the invasion of CD133 + GBM stem Cells in the brain microenvironment (Rath et al., 2013). Overall, these processes directly or indirectly cause astrocytes transformation and promote gliomagenesis.

## Role of Cytokines

Cytokines are multifunctional molecules that control neo-angiogenesis, proliferation, invasion, and immune cell infiltration within the TME. Dysregulation of tumor-associated cytokine results in failure by the immune system to recognize tumor cells and thereby suppresses effective cell-mediated immunity (Zhu et al., 2012). Brain cells, tumor cells, and immune cells cross-talk through the complex cytokine network in the tumor microenvironment which promotes GBM progression (Iwami et al., 2011).

Glioma cells express multiple immune-suppressive cytokines such as transforming growth factor-beta (TGF-β), IL-10, IL-4, IL-6, and IL-13, all of which interfere directly or indirectly with anti-glioma immune response (Debinski et al., 1999; Gomez and Kruse, 2006; Perng and Lim, 2015; Brown et al., 2016; Harshyne et al., 2016). IL-33 has been shown to drive tumor progression, reduce overall survival, and orchestrate the GBM microenvironment to overcome resistance to immunotherapy (De Boeck et al., 2020). Both pro-inflammatory cytokines interleukin-6 (IL-6) and colony-stimulating factor-1 (CSF-1) trigger an immunosuppressive environment in GBM by inhibiting T-cell functions (Hao et al., 2002; Bender et al., 2010).

Despite the fact that most glioma-derived cytokines promote an inflammatory TME and are associated with glioma progression, some cytokines have been shown to have antitumor activity and have a therapeutic value. For example, IL-12 is endogenously secreted by antigen-presenting cells (APCs) which elicit adaptive cell-mediated immune responses (Heufler et al., 1996). However, despite the encouraging results of glioma eradication in mouse models, clinical studies using recombinant IL-12 in patients with advanced cancer were discontinued due to poor tolerability (Atkins et al., 1997; Leonard et al., 1997).

Another novel strategy used to target recurrent glioma is based on the differential expression of the IL-13 receptor by GBMs (IL13Rα2) that differs from the physiological IL4R/IL13R receptor expressed by normal cells. Adenovirus vector encoding mutated IL-13 (binds specifically to IL13Rα2) fused to active portion *Pseudomonas* exotoxin (Ad.mhIL-4. TRE.mhIL-13-PE) showed effective anti-GBM cytotoxicity, with minimal neurotoxicity (Kunwar, 2003; Hsieh and Lesniak, 2005; Mineharu et al., 2012). A similar strategy using a recombinant form of IL-13 fused to *Pseudomonas* Exotoxin A (IL13-PE38QQR) was introduced into GBM cells by convection-enhanced delivery (CED) method (Kunwar, 2003; Hsieh and Lesniak, 2005). This strategy is in multiple phase I/II clinical trials for the treatment of patients with recurrent malignant gliomas. Results from these trials indicated that local delivery of IL13-PE38QQR is safe and associated with an increase in median survival (Kunwar, 2003; Hsieh and Lesniak, 2005). Weiss et al. have recently developed fusion molecules that directly deliver cytokines to the tumors after systemic administration to promote immune responses. They combined immunostimulatory cytokines, such as IL2, IL12, or TNF, with an L19 antibody against GBM-specific fibronectin (Weiss et al., 2020). The resulting fusion immune-cytokines can be administered intravenously and accumulate in tumors, showing encouraging results in mouse models and a pilot study in human patients [NCT03779230]. Uncovering the modulatory role of glioma-derived suppressor factors and understanding the complex crosstalk between glioma cells and their microenvironment will assist in developing therapeutic strategies for glioma eradication.

## Role of Oxidative Stress

Chronic inflammation is the leading cause of oxidative stress in astrocytes and microglia.(Salazar-Ramiro et al., 2016). Excessive ROS production can induce damage at different cellular processes, such as metabolism, signaling molecules, and most importantly at the genomic level (including mitochondrial and chromosomal DNA).(Redza-Dutordoir and Averill-Bates, 2016). Changes in cellular ROS is one of the hallmarks that favor GBM cells' survival and proliferation and provide further protection from apoptosis (Salazar-Ramiro et al., 2016). Genetic lesions commonly found in glioma are the leading cause of oxidative stress and genetic instability. GBMs exhibit epidermal growth factor receptor (EGFR) amplification, phosphatase and tensin homolog (PTEN) mutation, and loss of chromosome 10 all of which shift the balance toward higher ROS production within the tumor microenvironment (Reardon et al., 2015). For instance, constant activation of EGFR enhances the formation of intracellular ROS in neoplastic cells (Miller et al., 2007). This increase in ROS further enhances the tyrosine autophosphorylation by EGFR (Weng et al., 2018). Similarly, ROS can oxidize the PTEN protein and induce the disulfide bond formation between Cys71 and Cys124 within the N-terminal phosphatase domain resulting in the inactivation of the PTEN tumor suppressor activity (Lee et al., 2002; Kwon et al., 2004). Also, several studies suggested that TP53 mutation drives tumor progression by sustaining an oncogenic oxidant intracellular environment through altering the signaling pathways and of redox-related enzymes. For example, a mutation in TP53 deactivates mitochondrial-super oxide dismutase-2 (SOD2), and glutathione peroxidase (GPx), causing the accumulation of $O_2^-$ and $H_2O_2$, respectively (Hussain et al., 2004; Macedo et al., 2012; Cordani et al., 2020). All these processes induce aberration in the interstitial microenvironment and can drive glioma progression through excessive ROS production by tumor-infiltrating immune cells (Li et al., 2013). ROS-induced DNA damage triggers the release of a classical DAMP i.e. high-mobility group 1 (HMGB1) molecule which is released after oxidation of cysteine residues promoting genomic instability in GBM cells (Kang et al., 2013). TLRs can bind to the released HMGB1 and signal via the NF-κB, pathway causing enhanced production of pro-inflammatory cytokines, exacerbating ROS production in GBM cells [(Galdiero et al., 2013; Gieryng et al., 2017)].

The presence of ROS within the glioma TME can increase the immunosuppressive phenotype of immune cells infiltrating the TME, which interferes with effective immunotherapy. This immunosuppressive effect can be direct or indirect. Examples of direct ROS-mediated immunosuppression include the accumulation of Tregs (Kachikwu et al., 2011), M2-macrophages (Tsai et al., 2007), as well as interfering with dendritic cell activation and maturation (Kim et al., 2007). The indirect effect of ROS-mediated immunosuppressive TME includes the upregulation of PD-L1 by the tumor cells (Bailly, 2020; Raninga et al., 2020) as well enhanced expression of cytokines and chemokines that will recruit immunosuppressive myeloid cells (Bianchi et al., 2014; Eckert et al., 2018).

Studies also revealed that lower amount of nitric oxide (NO) produced through inducible nitric oxide synthase (iNOS/NOS2) in various tumors, including gliomas, can promote angiogenesis, invasion, and cellular proliferation (Fahey and Girotti, 2019; Maccallini et al., 2020). iNOS is a key regulator of glioma transformation downstream of the EGFRvIII/STAT3 signaling pathway. STAT3 directly binds to the promoter of the iNOS and thereby stimulates its expression (Jahani-Asl and Bonni, 2013). Moreover, iNOS-derived NO in glioma cells elicits resistance to various therapies including 5-aminolevulinic acid (ALA)-based photodynamic therapy (PDT) and endows gliomas of greater proliferation and aggressiveness (Fahey and Girotti, 2019; Maccallini et al., 2020). Several lines of evidence have showed that decreased proliferation of glioma cells could be mediated by selective inhibition of iNOS such as 1,400 W (N-(3-(aminomethyl) benzyl) acetamidine) (Fedorov et al., 2003) or mercapto-ethyl guanidine (MEG) (Southan et al., 1996) which inhibit the iNOS activity.

Generally, the CNS is composed of high fatty acids content which contributes to its high metabolic activity, making it particularly sensitive to oxidative damages by ROS (Panov et al., 2014). Neurons and astrocytes are equipped with antioxidant systems such as glutathione (GSSG-GSH) that defend these cells from oxidative damage. However, due to the high expression of SOD and catalase enzymes in astrocytes, there is an accelerated conversion of superoxide to hydrogen peroxide in these cells (Dokic et al., 2012). This makes astrocytes particularly sensitive to damage induced by ROS, leading to genetic instability and exacerbating neuroinflammation. The negative regulation of SOD-1 results in increased ROS production and enhanced phosphorylation of the signaling molecules Akt (Ghosh et al., 2010). This triggers rearrangement of the actin cytoskeleton downstream PI3K/Akt pathways; favoring the invasion and migration of GBM cells (Qian et al., 2005; Ghosh et al., 2010). Tumor cells exploit this inflammatory environment to invade, proliferate and sustain angiogenesis, by producing vascular endothelial growth factor (VEGF), Bv8, and MMP9 (Cao et al., 2013).

# IMMUNOTHERAPY IN GLIOMA

The concept of tumor immunotherapy involves activation of the immune system to eradicate the tumor by stimulating effector components or blocking immune suppressors. Glioblastoma is a "cold tumor" in that it harbors a low mutational load with low infiltrating cytotoxic T-cells, making it difficult to mount an effective immune response. Moreover, glioma is highly infiltrated by immunosuppressive immune cells such as regulatory T cells, and myeloid cells that actively promote inflammatory microenvironment and suppress the effector anti-tumor immune response. Despite all these challenges, Immunotherapy for glioma is a promising area of active investigation (Dunn-Pirio and Vlahovic, 2017; Kamran et al., 2018). Various preclinical studies have demonstrated the success of immunotherapy-based approaches in animal models and many phase I and II clinical trials have shown immunotherapy to be safe and in some cases lead to improved progression-free survival (PFS) (Phuphanich et al., 2013; Bloch

**TABLE 1 |** Selected ongoing clinical trials of immunotherapy in glioma.

| Target | NCT number | Drug | Disease | Status | References |
|---|---|---|---|---|---|
| Stat3 | NCT01904123 | WP1066 | Recurrent GBM | Recruiting | Hussain et al. (2007) |
| Cox2 | NCT00112502 | Celecoxib | GBM | Completed | Penas-Prado et al., (2015), Mi et al. (2020) |
| Stat3 | NCT04334863 | WP1066 | Brain metastases | Recruiting | Hussain et al. (2007) |
| Cxcr4 | NCT01977677 | AMD3100 | Adult glioblastoma | Completed | Thomas et al. (2019) |
| avβ3 and avβ5 integrins | NCT00689221 | Cilengitide | Glioblastoma | Completed | Russo and Cappoli, (2018), Roesch et al. (2018) |
| CSF1R | NCT01790503 | PLX3397 | GBM | Completed | Butowski et al. (2015), Colman et al. (2018) |
| CSF1R | NCT02829723 | BLZ945, PDR001 | Solid tumors | Recruiting | Butowski et al. (2015), Colman et al. (2018) |
| PD-1 | NCT02017717 | Nivolumab | Recurrent GBM | Active, not recruiting | Domingues et al. (2016), Woroniecka et al. (2018), Wang et al. (2019) |
| PD-1 | NCT02550249 | Nivolumab | GBM | Completed | Domingues et al. (2016), Woroniecka et al. (2018), Wang et al. (2019) |
| PD-1, CD8 T cells | NCT02529072 | Nivolumab + DC vaccine | Astrocytoma | Completed | Peters et al. (2019) |
| TNF | NCT03779230 | L19TNF | Glioma | Recruiting | Weiss et al. (2020) |
| IL-13Rα2 | NCT02208362 | Genetically modified T cells | Refractory malignant glioma | Recruiting | Kunwar. (2003), Hsieh and Lesniak, (2005) |
| EGFRv III | NCT01454596 | CAR T cell | Malignant glioma | Completed | Migliorini et al. (2018) |
| HERs | NCT01109095 | CAR T cell | GBM | Completed | Migliorini et al. (2018), Wang et al. (2020) |
| Tumor cells | NCT03576612 | Ad-TK + valacyclovir | High-grade gliomas | Active, not recruiting | |
| Tumor cells | NCT02414165 | Cytosine deaminase+ 5FU | GBM | Terminated | Cloughesy et al. (2020) |
| Antigen presenting cells | NCT02026271 | Ad-RTS-hIL-12 + veledimex | GBM | Active, not recruiting | Chiocca et al. (2019), Garcia-Fabiani et al. (2020) |
| Antigen presenting cells | NCT03330197 | Ad-RTS-hIL-12 + veledimex | Pediatric brain tumor | Recruiting | Chiocca et al. (2019), Garcia-Fabiani et al. (2020) |
| Antigen presenting cells | NCT04006119 | Ad-RTS-hIL-12 + veledimex + PD1 | Glioblastoma | Active, not recruiting | Chiocca et al. (2019), Garcia-Fabiani et al. (2020) |
| Dendritic cells | NCT01811992 | Ad-hCMV-TK + Ad-hCMV-Flt3L | Malignant glioma | Active, not recruiting | Curtin et al. (2009), Mineharu et al. (2011), Lowenstein et al. (2019) |
| Dendritic cells | NCT00045968 | Dendritic cell immunotherapy | GBM | Unknown | Vik-Mo et al. (2013), Hunn et al. (2015), Akasaki et al. (2016) |
| Dendritic cells | NCT00639639 | Autologous dendritic cells | Brain tumor | Active, not recruiting | Batich et al. (2017) |
| Tumor-associated antigens | NCT04013672 | SurVaxM (peptide vaccine) | Recurrent GBM | Active, not recruiting | Platten et al. (2018), Hollingsworth and Jansen, (2019) |
| Tumor-associated antigens | NCT03149003 | DSP-7888 (peptide vaccine) | Glioblastoma | Active, not recruiting | Johanns and Dunn, (2017), Platten et al. (2018), Hollingsworth and Jansen, (2019) |
| Tumor-associated antigens | NCT02078648 | SL-701 (peptide vaccine) | Adult GBM | Completed | Johanns and Dunn, (2017), Platten et al. (2018) |
| IDH1 | NCT02454634 | IDH1 peptide vaccine | Glioma | Completed | Platten et al. (2021) |
| Tumor cells | NCT02661282 | Cytomegalovirus-specific cytotoxic T-lymphocytes | Glioblastoma | Active, not recruiting | Woo et al. (2012), Kim et al. (2017), Chheda et al. (2018) |
| CD200 | NCT00648739 | Samalizumab | Glioblastoma | Recruiting | Wright et al. (2000), Gorczynski et al. (2000), Wright et al. (2003) |

et al., 2014; Mitchell et al., 2015). Below, we provide an overview of the immunological approaches which yielded promising results in the preclinical setting and are currently being tested in the clinic. A list of these trials are summarized in **Table 1**.

## CAR-T Cells

Adoptive transfer of chimeric antigen receptor (CAR) T cells is a promising immunotherapy glioma treatment (Migliorini et al., 2018; Akhavan et al., 2019). CAR T cells are T cells that have been removed from the patients and modified to have tumor antigen-binding receptor specificity before adoptively transferring them back to the patient (Akhavan et al., 2019). This therapy increases the ability of T cells to recognize and target cancerous cells. The current CAR T cells in clinical trials target IL-13Rα2, epidermal growth factor receptor variant III (EGFRvIII), and human epidermal growth factor receptor 2 (HER2) (Migliorini et al., 2018). These targets were selected due to their expression by glioma cells and negligible expression by normal brain cells since CAR T cell targeting of normal brain cells could result in lethal levels of toxicity (Akhavan et al., 2019). The clinical trials, ClinicalTrials.gov NCT identifiers: NCT02208362, NCT01454596, NCT01109095, targeting IL-13Rα2, EGFRvIII, and HER2, respectively, provide useful insights on the immunotherapeutic ability of CAR T cells against gliomas (Migliorini et al., 2018). These trials showed the CAR T cells': low toxicity, moderate ability to traffic to the glioma microenvironment, limited ability to target glioma cells, and inconsistent results in increasing overall survival (Migliorini et al., 2018; Akhavan et al., 2019; Wang et al., 2020). For example, HER2-specific CAR T cells, in the NCT01109095

trial, were used to treat 17 patients with progressive GBM (Ahmed et al., 2017). Of these, 8 patients had clinical benefits, and the overall survival of the cohort was 11.1 months post T cell infusion (Ahmed et al., 2017). Recently, several therapies have been designed to improve the efficacy of CAR T cells by combining them with checkpoint inhibitors, such as PD-1, CTLA4, and PD-L1, but the clinical trials are still ongoing and have not released results (Akhavan et al., 2019).

## HSC Transplantation

Hematopoietic stem-cell transplantation (HSCT) refers to a procedure in which hematopoietic stem cells are infused to restore bone marrow function in cancer patients who receive harmful doses of cytotoxic drugs or radiation therapy. Studies showed HSCs infusion is associated with enhanced recruitment and migration of tumor-specific T-cells within the tumor bed (Flores et al., 2015). This effect is mediated by HSCs-derived cytokines such as CCL3 (MIP-1α) that facilitates the subsequent recruitment of activated tumor-specific T cells causing enhanced anti-glioma immune response (Flores et al., 2015; Bryukhovetskiy et al., 2016). HSCs may be obtained from the transplant recipient (autologous HSCT) or a donor (allogeneic HSCT) and harvested from bone marrow, peripheral blood, or umbilical cord blood shortly after delivery of neonates. An early study compared survival outcomes of 27 children with recurrent malignant astrocytomas who underwent myeloablative chemotherapy and autologous HSCT with a matched historical cohort (n = 56) that received standard chemotherapy following tumor recurrence (Finlay et al., 2008). The results of this study suggest myeloablative chemotherapy with autologous HSCT can produce long-term survival among children with recurrent malignant astrocytoma. Bouffet et al. reported on a series of 22 children and young adults with high-grade gliomas treated with autologous HSCT, the response rate was 29% with one complete and three partial responses (Bouffet et al., 1997). However, the authors concluded that overall survival using this procedure was no better than that reported with conventional treatments (Bouffet et al., 1997). . In conclusion, HSC therapy may extend glioma patients' survival particularly for those patients being treated with high dose chemotherapy regimens. However, the dose of chemotherapeutics used in this treatment regimen should be carefully balanced to avoid intolerance and toxic side effects (Brandes et al., 2001; Egan et al., 2016).

## Gene Therapies and Virotherapies

Gene therapy is a therapeutic approach that involves the insertion of the genetic material into a tissue or organ to treat a variety of diseases, including solid cancers, such as brain tumors. These genetic elements include whole genes, oligonucleotides, or regulatory elements which are usually inserted into the target cells either by gene delivery vectors or liposomal-nanoparticle formulations. (Asad et al., 2017; Kamran et al., 2018). In glioma, gene therapy is an attractive approach due to the fact that its local administration can overcome the challenges needed to bypass the BBB, and therefore it may have lower systemic side effects (Okura et al., 2014). Albeit, its clinical application still presents many challenges (Lowenstein et al., 2009).

Among prevalent gene therapy approaches for cancer, suicide gene therapy is one of the most studied approaches for the treatment of glioma. Currently, there are around 20 Phase-I/II clinical trials testing the effectiveness of adenoviral vectors mediated suicide gene therapies in multiple subtypes of glioma. An ongoing Phase-I trial evaluates the safety and tolerability of Ad-TK in combination with systemic administration of the prodrug valacyclovir in combination with SOC and Nivolumab in patients with HGG (NCT03576612). The trial is still ongoing with no results posted.

Another example of a clinically applied conditional cytotoxic/suicidal approach is the delivery of the cytosine deaminase (CD) in cancer cells. CD is expressed mainly by yeast and bacteria but is absent in mammalian cells (Takahashi et al., 2014). GBM cells expressing CD will be susceptible to 5-fluorocytosine, due to it is the ability to convert it to its active form Fluorouracil (5-FU) (Okura et al., 2014; Takahashi et al., 2014). The initial Phase-I clinical trial of Toca 511, a replication-competent RV encoding CD, showed safety and good tolerability, with effective tumoricidal responses in patients with recurrent high-grade glioma (Cloughesy et al., 2018). However, a recent phase III clinical trial (NCT02414165) did not show improved overall survival compared to the SOC (Cloughesy et al., 2020).

Another attractive approach is immune stimulatory gene therapy which entails the expression of immune activators in glioma cells to enhance the anti-tumor immune response.

Two independent Phase-I trials (NCT02026271, NCT03330197) investigating the tolerability of Ad-RTS-hIL-12 in combination with veledimex (VDX) showed promising results (Chiocca et al., 2019). In a separate Phase-I trial, the safety of Ad-RTS-Hil-12/VDX is being tested in combination with Nivolumab (NCT03636477) (Garcia-Fabiani et al., 2020). In addition, a Phase-II trial is studying the inducible hIL-12 in combination with PD-1 antibody Libtayo (NCT04006119) (Garcia-Fabiani et al., 2020).

In an effort to overcome the shortcomings of monotherapies, combination therapies have been developed. We have pioneered the combination of Ad-Flt3L and Ad-TK (Castro et al., 2014; Kamran et al., 2018). In this therapeutic approach, adenoviral vectors encoding for herpes simplex type 1-thymidine kinase (TK) and FMS-like Tyrosine kinase 3 ligand (Flt3L) are delivered into the brain where glioma cell death is induced upon systemic ganciclovir treatment (Ali et al., 2005). This triggers the release of tumor-associated antigens and damage-associated molecular pattern molecules (DAMPs) into the tumor microenvironment, triggering an antitumor immune response (Kamran et al., 2018; Altshuler et al., 2020). Our results indicate that the release of DAMPs such as HMGB1 from Ad-TK infected tumor cells is required for the efficacy of Ad-TK + Ad-Flt3L mediated immunotherapy (Candolfi et al., 2009; Curtin et al., 2009). Flt3L increases the migration and infiltration of DCs into the TME. This glioma infiltrating DCs can phagocytose antigens that are released during TK-induced glioma cell death (Curtin et al., 2009; Candolfi et al., 2012). Moreover, HMGB1 released from dying tumor cells activates DCs through stimulation of TLR2 pathway. These activated DCs then transport the tumor antigens to the draining lymph nodes to generate T-cell mediated cytotoxic

immune responses against tumor cells (Curtin et al., 2009). This strategy effectively eradicated the tumor in multiple glioma models and granted long-term survival in tumor-bearing mice (Mineharu et al., 2011). Results from Phase-I dose-escalating trial (NCT01811992) showed well-tolerability and safety when giving a combination of AdVs expressing TK and Flt3L. There was also higher infiltrating DCs and lymphoid cells within the TME patients receiving the gene therapy (Lowenstein et al., 2019).

## Dendritic Cells Vaccines

Dendritic Cells (DCs) are professional antigen-presenting cells (APC) that are able to engulf antigenic proteins, process peptides, and traffic to the draining lymph nodes (dLNs) where they present antigens to naive T-cells (Palucka and Banchereau, 1999). This process leads to induction and activation of effector T-cell, the goal standard for anti-tumor immune response. Administration tumor antigen pulsed-DCs has been validated in multiple solid tumors such as melanoma, prostate cancer, and renal cell carcinoma (Schadendorf et al., 2006; Su et al., 2003; Anguille et al., 2014). In glioma, DCs therapy represents an attractive approach especially because of the paucity of professional DCs in GBM (Eagles et al., 2018; Srivastava et al., 2019). Preclinical models have shown promising results for DCs treatment in combination with PD-1 blockade (Antonios et al., 2016). In addition, phase I clinical trial revealed that the combination of TMZ with DCs therapy is safe and tolerable in GBM patients (Vik-Mo et al., 2013; Hunn et al., 2015; Akasaki et al., 2016). Moreover, autologous DCs pulsed with autologous tumor lysate (DCVax) are currently being investigated in phase III clinical trials for newly diagnosed GBM (NCT00045968). Beside the direct administration of autologous DCs to glioma patients, the activation and recruitment of endogenous DCs *in vivo* have been shown to be safe and effective in glioma patients. This is illustrated by infusion of TLR ligands, or by intratumoral injection of Flt3L which causes robust DCs expansion and activation (Hou et al., 2008; Belderbos et al., 2019; Jones et al., 2010). Another trial tests the efficacy of DCs loaded with glioma-associated protein (pp65) along with the lysosomal associated protein (LAMP) (CMV-pp65-LAMP mRNA-loaded DCs) in combination with the TMZ and GM-CSF. This study showed increased progression-free survival (PFS) and OS, and upregulation in IFN$\gamma$ levels (Batich et al., 2017) (NCT00639639). These trials showed that DCs therapy is safe, and well-tolerated in glioma patients and elicited a minimal inflammatory response. Future trials will further reveal the effectiveness of this treatment on patients with recurrent GBM and its impact on overall survival.

## Peptide Vaccines

Peptide vaccines are developed based on peptides derived from tumor-associated antigens (TAAgs) and used to induce effective anti-tumor T-cell response (Lee et al., 2020). Some of the TAAgs tested in glioma, survivin, WT-1 protein, EphrinA2, and IL13RA2, have been investigated as potential peptide vaccines (NCT04013672, NCT03149003, NCT02078648); however, therapeutic efficacy has been limited (Platten et al., 2018). The low efficacy of TAAgs vaccines could be attributed to the tolerance mechanisms to self-antigens, which also implies the risks for off-target toxicity (Hollingsworth and Jansen, 2019). Alternatively, neoantigens that are exclusively expressed in tumor cells can be employed in peptide vaccines. The major challenge for glioma lies in the epitope selection: it harbors only 30–50 non-synonymous mutations per megabase in glioblastomas, and roughly 1–3% of the mutations result in immunogenic neoantigens (Schumacher and Schreiber, 2015; Johanns and Dunn, 2017). Despite the low mutational burden, some shared neoepitopes, such as EGFRvIII and IDH1R132H, are identified, and corresponding vaccines have been extensively evaluated in clinical trials (Weller et al., 2017; Platten et al., 2018; Reardon et al., 2020). Recently, a peptide vaccine targetting the most common mutation in glioma *IDH1*m (R132H) has been developed and showed promising results in Phase-I clinical trial (NCT02454634) (Platten et al., 2021). Overall, neoantigen vaccines demonstrate good safety and immunogenicity, but the response rate and survival benefits are marginal. It has been recognized that neoantigens are usually unique, and the subclonal expression would lead to spontaneous antigen loss, opening avenues for immune escape and tumor progression (Lim et al., 2018).

## High-Density Lipoprotein Nanoparticle Vaccines

The tantalizing results and heterogeneity of neoantigen expression emphasize the need for the development of personalized vaccination. Currently, neoantigen identification is based on the whole-exome sequencing (WES), followed by computational predications of the antigenicity through HLA binding (Kreiter et al., 2015), which can be further confirmed by the transcriptome analysis with mass spectrometry (Yadav et al., 2014). In particular, Kuai, et al. have developed a sHDL nanodisc composed of phospholipids and apolipoprotein A1-mimetic peptides for neoantigen vaccination (Kuai et al., 2017). After coupling with Cytosine-phosphorothioate-guanine CpG oligonucleotide, a TLR-9 agonist, nanodiscs promoted co-delivery of Ag/CpG to lymphoid organs, eliciting strong neoantigen-specific anti-tumor $CD8^+$ T-cell responses in murine models of melanoma and colon carcinoma. Taking one step further, Scheetz et al. developed the personalized peptide vaccine against gliomas with the sHDL nanodisc platform (Scheetz et al., 2020). Three neoantigens of GL261 tumor cells were screened by *in silico* MHC-I affinity prediction algorithms and subsequent immunogenicity verification in tumor-bearing mice. Selected peptides were loaded to nanodiscs together with CpG. Immunizing mice with the vaccine plus anti-PD-L1 therapy improved the survival rate to approximately 90% in the subcutaneous GL261 tumor model, and it showed 33% complete tumor regression in the orthotopic model, which represented a significant survival advantage compared with soluble neoantigens plus anti-PD-L1 treatments. Nanodisc vaccination elicited robust IFN-$\gamma^+$ T cell response against all three epitopes, and the addition of anti-PD-L1 further augmented the T cell responses. TME analyses on CNS tumors showed an increased frequency of $CD8^+$ T cells with lower levels of PD-1 expression. Meanwhile, compared with the PBS group, lower Tregs frequency and higher M1:M2-like

macrophage ratio was observed in tumor tissues, suggesting a reversed immunosuppressive milieu. In parallel, the therapeutic efficacy of nanodiscs with either $mIDH1_{123–132}$ or $mIDH1_{126–141}$ neoantigens was evaluated in a genetically engineered mIDH1 murine glioma model. The results showed that nanodiscs vaccination significantly suppressed tumor growth and extended the survival rate. All survived mice showed resistance to tumor recurrence upon rechallenge with mIDH1 neurospheres in the contralateral hemisphere.

Aside from TAAs and neoantigens, cancer stem cells (CSCs) and other non-neoplastic compartments may serve as potential targets for gliomas. Nanodiscs delivering CSC-derived ALDH1-A1 and -A3 peptides have been shown to induce potent anti-tumor activity and prolong animal survival in multiple mouse models (Hassani Najafabadi et al., 2020). Overall, sHDL nanodisc serves as a versatile platform with potent anti-tumor efficacy.

## Other Immunotherapies

Immune checkpoint inhibitors targeting the program death receptors and its ligand (PD-1/PD-L1) and T lymphocyte-associated antigen-4 (CTLA-4) have significantly enhanced immunotherapy efficacy for selected solid tumors (Ferris et al., 2010; Ferris et al., 2016; Bouffet et al., 2016). However, CNS tumors at best have a moderate response to current immunotherapies (Kurtulus et al., 2015; Bouffet et al., 2016). Recently, there has been increasing interest in alternative immunotherapy strategies including the synergy between PD1, TIM3, and LAG3 blockade and CAR T cells therapy (NCT02661282) for the treatment of gliomas [(Woo et al., 2012; Ahmed et al., 2017; Kim et al., 2017; Chheda et al., 2018)]. Recently, there have been advances with an alternative immune checkpoint using a novel immune checkpoint inhibitor peptide ligand. The CD200 immune checkpoint is modulated by an inhibitory receptor (CD200R1) and multiple activation receptors (CD200AR) (Wright et al., 2000; Gorczynski et al., 2000; Wright et al., 2003; Gorczynski et al., 2004; Wong et al., 2012; Damiani et al., 2015). Rigorous studies by several groups have provided evidence that targeting the CD200 checkpoint enhances immunotherapy efficacy (Kretz-Rommel et al., 2007; Copland et al., 2007; Gorczynski et al., 2011; Rygiel et al., 2012; Gorczynski et al., 2013). To this end, a monoclonal antibody against the CD200 protein (Samalizumab) (Adler, 2010) was evaluated in a clinical trial in 2008 for relapsed refractory B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma (NCT00648739) (Adler, 2010). Alexion reported that 95% of the patients with B-CLL had up to 98% reduction in $CD200^{+}CD4^{+}$ T cells. The loss of these immune cells created an immunocompromized state and may be the reason why only 36% of the patients had a 10% reduction in bulk disease. Investigators have taken a new approach for improving immunotherapies by targeting the CD200ARs on antigen-presenting cells with a peptide ligand. They reported that the peptide ligand activates the immune system enhancing cytokine/chemokine production, Co-stimulatory molecules CD80/CD86 and MHC-II enhancing an antigen response while downregulating the inhibitory CD200R1 and PD-1 (Moertel et al., 2014; Xiong et al., 2016; Xiong et al., 2019). They reported that treatment with autologous tumor lysate given concomitantly with the peptide ligand significantly enhanced survival in a spontaneous canine high-grade glioma trial with pet-owned dogs (Olin et al., 2019) and recently initiated a phase I adult glioblastoma recurrent trial (NCT04642937) (**Table 1**).

# PROSPECTS AND CONCLUSION

In this review, we discuss multiple mechanisms by which gliomas induce the development of an immunosuppressive TME and evade immune surveillance. Various components within the tumor microenvironment such as secreted immune-modulating factors, hypoxia, and oxidative stress result in activation of the surrounding astrocytes and microglia to an inflammatory state. This suppressive microenvironment is further enhanced by infiltrating immunosuppressive myeloid cells, immunosuppressive macrophages, exhausted T cells, and Tregs which contribute to a tumor-supportive TME, promoting glioma cell growth and invasion. We also discuss recent advances in immunotherapeutic strategies that if used in combination could provide promising novel approaches that could elicit clinical benefit. These include adding immune checkpoint blockade to immune-mediated gene therapies, virotherapy, CAR-T cells, dendritic cells' vaccines, or nanoparticle-mediated vaccination technologies.

Despite the advances in treatment regimen in multiple solid tumors in recent years, the SOC for glioma has not changed since 2005 with the inclusion of TMZ. The prognosis for GBM remains dismal with no major improvements in the median or overall survival. Major challenges need to be overcome in gliomas, such as tumor heterogeneity, the immunosuppressive TME (MDSCs, TAMS, Tregs), the low mutational burden, and the antigen heterogeneity in order to elicit effective immunotherapies. Further studies are crucial to delineate the complex mechanisms and interactions between tumor cells, immune cells, tumor stroma, resident brain cells, and the brain and tumor vasculature.

Over the past 2 decades, a pressing need to deeply profile the glioma TME has led investigators to integrate data obtained from traditional approaches with those obtained with new, single-cell technologies, including high parameter mass cytometry, single-cell sequencing, and high-resolution imaging. These advances have enabled us to investigate in detail the complexity of the TME and to interrogate in-depth previously unexplored tumor-infiltrating cell types. This expanded our understanding of the multifaceted tumor ecosystems and allowed us to profile cellular heterogeneity, dynamicity and plasticity, and complex cell-cell interactions.

Current open clinical trials testing immunotherapy in GBM could provide promising avenues for gene therapy, CAR-T cells as well as vaccine therapies in various combinatorial treatment strategies. These combinatorial approaches which have proven beneficial in the preclinical setting could prove particularly valuable in the clinical arena, when targeting multiple immune checkpoints or when adding cytokine therapy, likely to result in better outcomes. Furthermore, recent results from trials of neoadjuvant immune checkpoint blockade provide a new cause for optimism as we work to decode mechanisms for

treatment resistance. On the other hand, an increased understanding of the possible indicators of side effects of immunotherapies will lead to improved clinical care and make the application of immunotherapies far safer and well-tolerated for glioma patients. Despite formidable challenges, with the advent of single-cell transcriptomic analysis (Wang et al., 2019; Gibellini et al., 2020; Mathewson et al., 2021), high parameter mass cytometry (Hartmann et al., 2019; Wang et al., 2019; Gohil et al., 2021), and high-resolution Hyperion imaging technologies (Carvajal-Hausdorf et al., 2019; Xie et al., 2020), the glioma immunotherapy field is undergoing unprecedented growth in the discovery of novel therapeutic targets and interventions, that we envisage will lead to effective novel treatments for this devastating brain cancer in the near future.

## AUTHOR CONTRIBUTIONS

MSA, BM, MSH, SH, SF, RT, AD, KB, MO, JM, AS, AM, PL, and MC wrote the manuscript, with overall guidance, revisions, and edits from PL and MC. MH and SC prepared Figures. MSA, PL, MC reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

## FUNDING

This work was supported by the National Institutes of Health/ National Institute of Neurological Disorders andamp; Stroke (NIH/NINDS) Grants R21-NS091555, R37NS094804, and R01NS074387 to MC; R01NS076991, R01NS082311, and R01NS096756 to PL; Rogel Cancer Center Scholar Award and Forbes Senior Research Scholar Award to MC; National institutes of Health/National Institute of Biomedical Imaging and Bioengineering (NIH/NIBIB) Grant R01-EB022563 to JM, PL, and MC; University of Michigan M-Cube; the Department of Neurosurgery; the University of Michigan Rogel Comprehensive Cancer Center; the Pediatric Brain Tumor Foundation (BTF), Leah's Happy Hearts Foundation, The Chad Tough Foundation (Ian's Friends Foundation (IFF)), and the Biosciences Initiative in Brain Cancer to MC and PL. NIH/NCI T32- CA009676 Post-Doctoral Fellowship to MSA.

## ACKNOWLEDGMENTS

We thank Karin Muraszko for her academic leadership and Marta Edwards, Brandye Hill, and Katherine Wood for administrative and technical assistance.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 25 May 2021
doi: 10.3389/fnins.2021.662064

# CAR T Cell-Based Immunotherapy for the Treatment of Glioblastoma

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Glioblastoma multiforme (GBM) is the most common and aggressive malignant primary brain tumor in adults. Current treatment options typically consist of surgery followed by chemotherapy or more frequently radiotherapy, however, median patient survival remains at just over 1 year. Therefore, the need for novel curative therapies for GBM is vital. Characterization of GBM cells has contributed to identify several molecules as targets for immunotherapy-based treatments such as EGFR/EGFRvIII, IL13Rα2, B7-H3, and CSPG4. Cytotoxic T lymphocytes collected from a patient can be genetically modified to express a chimeric antigen receptor (CAR) specific for an identified tumor antigen (TA). These CAR T cells can then be re-administered to the patient to identify and eliminate cancer cells. The impressive clinical responses to TA-specific CAR T cell-based therapies in patients with hematological malignancies have generated a lot of interest in the application of this strategy with solid tumors including GBM. Several clinical trials are evaluating TA-specific CAR T cells to treat GBM. Unfortunately, the efficacy of CAR T cells against solid tumors has been limited due to several factors. These include the immunosuppressive tumor microenvironment, inadequate trafficking and infiltration of CAR T cells and their lack of persistence and activity. In particular, GBM has specific limitations to overcome including acquired resistance to therapy, limited diffusion across the blood brain barrier and risks of central nervous system toxicity. Here we review current CAR T cell-based approaches for the treatment of GBM and summarize the mechanisms being explored in pre-clinical, as well as clinical studies to improve their anti-tumor activity.

**Keywords: CAR T cell, clinical trial, glioblastoma, immunotherapy, preclinical, tumor antigen**

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***Edited by:***
*Elisa Roda,*
*Clinical Scientific Institutes Maugeri (ICS Maugeri), Italy*

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***Specialty section:***
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

***Received:*** *31 January 2021*
***Accepted:*** *14 April 2021*
***Published:*** *25 May 2021*

***Citation:***
*Maggs L, Cattaneo G, Dal AE, Moghaddam AS and Ferrone S (2021) CAR T Cell-Based Immunotherapy for the Treatment of Glioblastoma. Front. Neurosci. 15:662064. doi: 10.3389/fnins.2021.662064*

## INTRODUCTION

Gliomas are the most common type of primary brain cancer (Weller et al., 2015; Ostrom et al., 2019). They originate from brain cells including astrocytes, oligodendrocytes, and ependymal cells, which support neural cells. Gliomas can be divided into six types based on their histological characteristics; among them glioblastoma multiforme (GBM), classified as a grade IV glioma, is the most common malignant primary brain tumor. GBM, which originates from astrocytes, the most abundant cell type in the central nervous system (CNS), is highly aggressive and has an extremely unfavorable prognosis. Current treatment options typically consist of surgery followed by chemotherapy or radiotherapy with a dismal 2 years patient survival rate of less than 30% (Ostrom et al., 2019). While several studies are being performed in attempts to optimize

combinatorial strategies incorporating radiotherapy and/or chemotherapy, the disappointing clinical results generated by these therapies emphasize the urgent need to develop novel and more effective treatments.

Several immunotherapeutic strategies have been tested in clinical trials in patients with GBM; surprisingly the impact on clinical outcomes has been limited. Recently there has been a growing interest in the application of immunotherapy which utilizes as an effector mechanism a patient's own T cells transduced with a tumor antigen (TA)-specific chimeric antigen receptor (CAR). This approach has generated impressive clinical responses in patients with hematological malignancies (Boyiadzis et al., 2018), but has had thus far limited, if any success in patients with solid tumors, including GBM. The latter results most likely reflect the limited specificity of the target TA, the immune escape mechanisms facilitated by the hostile conditions of the tumor microenvironment (TME) and the limited ability of CAR T cells to infiltrate the tumor site as well as their insufficient activity and persistence. This information is being used for the rational design of strategies to enhance the efficacy of CAR T cell-based immunotherapy of patients with solid cancers. The exciting potential of these strategies is indicated by the large number of investigators who are testing them both in preclinical models and in clinical trials.

In this review, we will briefly discuss the current range of immunotherapeutic strategies being explored to treat GBM before focusing specifically on CAR T cell therapy. TA being utilized as targets in CAR T cell based preclinical and clinical studies will be reviewed followed by a discussion of the variables which limit CAR T cell therapy against GBM (**Figure 1**). Finally, we will describe the novel strategies and combinatorial therapies which are being tested in pre-clinical studies to enhance the antitumor activity of CAR T cells with the expectation that they may overcome these limitations.

## IMMUNOTHERAPY BASED STRATEGIES FOR THE TREATMENT OF GBM

Current immunotherapeutic strategies for the treatment of GBM are mainly focused on cognate T cell-based therapies, such as the use of vaccines and immune checkpoint inhibitors (ICI), or on TA-specific monoclonal antibodies (mAb), either alone or conjugated to an antitumor drug, toxin or radioisotope (Gan et al., 2017; Choi et al., 2019a; Medikonda et al., 2020). Each strategy has advantages and limitations, which in combination with the specific challenges imposed by GBM, have thus far failed to provide significant improvements in patient outcomes (**Table 1**).

### T Cell-Based Immunotherapeutic Strategies: Vaccines

Cancer vaccines are constructed using isolated cancer peptides, synthesized peptides derived from TA amino acid sequences or dendritic cells (DCs) pulsed with purified cancer TAs or an autologous or allogeneic tumor cell lysate. Several ongoing vaccine-based clinical trials are in early Phase I/II trials. Two therapeutic vaccines reached the Phase III stage of clinical testing in newly diagnosed GBM patients: one utilized autologous tumor cell lysate-pulsed dendritic cell vaccine (DCVax) (NCT00045968) (Liau et al., 2018), and the other one utilized Rindopepimut, an EGFR variant III (EGFRvIII)-specific peptide conjugated to keyhole limpet haemocyanin (NCT01480479) (Weller et al., 2017; Lim et al., 2018). Unfortunately, both displayed minimal clinical benefit. These disappointing clinical results are likely to reflect the many limitations of T cell-based therapeutic strategies based on vaccination. They include: (i) the extent of immune response is generally insufficient to achieve clinical benefits, (ii) the immune response to vaccination is highly variable among immunized patients, (iii) the defective TA derived peptide presentation to cognate T cells caused by abnormalities in HLA class I antigen processing machinery (APM) component expression and/or function frequently present in GBM cells provides them with an escape mechanism from immune surveillance and from the TA-specific immune response mounted by the immunized host and, (iv) the costly and time-consuming preparation of cancer vaccines imposes strictures on their production (Oh et al., 2015; Lim et al., 2018).

### T Cell-Based Immunotherapeutic Strategies: Immune Checkpoint Inhibitors (ICI)

Immune checkpoint specific mAbs have been used to unleash T cells which recognize cancer cells through blockade of inhibitory signaling pathways (Abril-Rodriguez and Ribas, 2017). They have induced impressive clinical responses in several cancer types (Hodi et al., 2010; Ansell et al., 2015; Borghaei et al., 2015). ICIs targeting cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death 1 (PD-1) or indoleamine 2,3-dioxygenase 1 (IDO) have yielded encouraging preclinical results in mouse models of glioma (Zeng et al., 2013; Wainwright et al., 2014). However, they have failed to demonstrate survival benefit for GBM patients in a clinical setting. The first large-scale clinical trial designed to evaluate the safety and efficacy of the anti-PD-1 Nivolumab in patients with recurrent GBM (CheckMate-143: NCT02017717) did not prolong patients' overall survival (OS) (Filley et al., 2017; Mantica et al., 2018).

Factors limiting the efficacy of T cell-based therapeutic strategies include the low mutational burden of GBM, the limited trafficking of immune cells within the tumor and the highly suppressive nature of the tumor microenvironment (TME) (Desai et al., 2019). Another important mechanism that plays a role in the limited clinical impact of T cell-based immunotherapies is represented by defects in HLA class I and APM component expression and function in GBM (Facoetti et al., 2005; Thuring et al., 2015). Downregulation, loss or lack of function of HLA class-I APM components have a negative impact on T cell-mediated recognition and lysis of GBM cells.

### Oncolytic Virus-Based Immunotherapy

Several viruses designed to directly kill cancer cells via oncolysis and to stimulate an immune response to the released TAs have been tested in many types of solid cancer (Lawler et al., 2017)

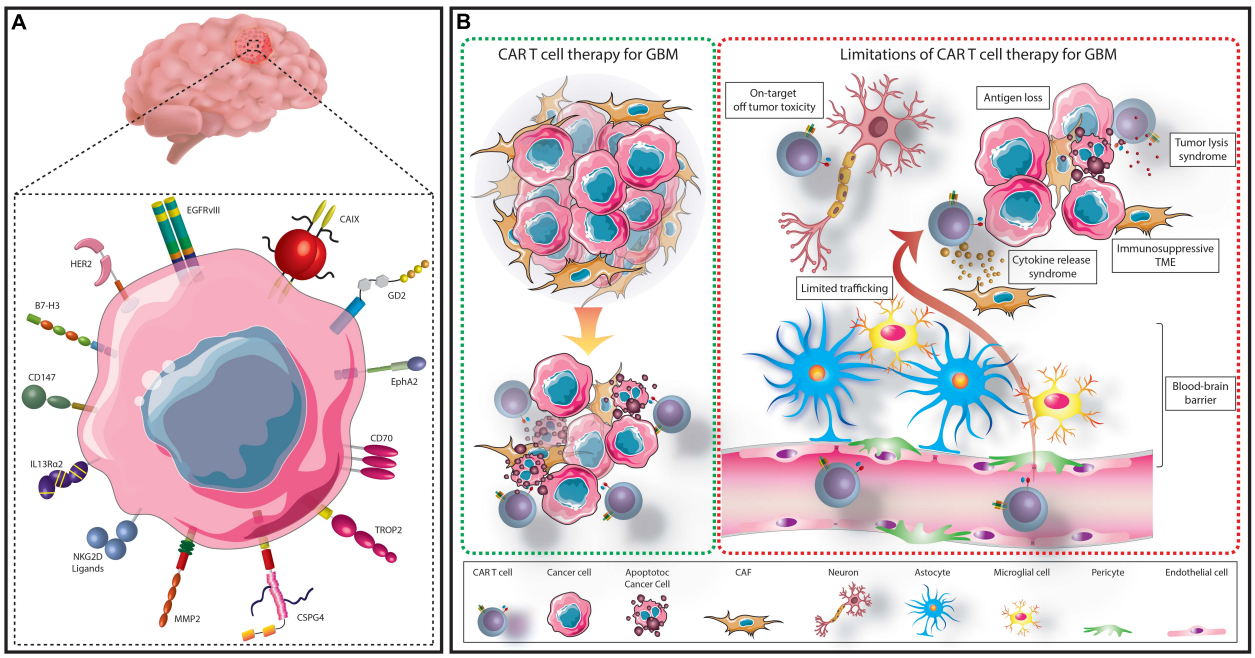


**FIGURE 1 |** Summary of CAR T cell treatment for GBM. GBM cells express several targetable TAs which have either been assessed in clinical or preclinical CAR T cell studies **(A)**. TA-specific CAR T cells can be administered to patients to recognize and eliminate TA expressing GBM cells **(B)**. However, several limitations hinder this process. These include; the immunosuppressive TME, limited access across the BBB, on-target, off-tumor toxicity, cytokine release syndrome, tumor lysis syndrome, and selective antigen loss.

including GBM (Iorgulescu et al., 2018; Martikainen and Essand, 2019). Phase I/II clinical trials have shown the feasibility and safety of this approach but have yielded limited clinical benefit in the majority of patients (Cloughesy et al., 2018; Desjardins et al., 2018; Lang et al., 2018). In some GBM patients oncolytic viruses can significantly improve survival (Gesundheit et al., 2020). However, further investigation into the molecular mechanisms involved are needed to improve this therapeutic strategy. Attempts to enhance the effect of oncolytic viruses through addition of a therapeutic payload such as a cytokine, chemotherapeutic agent or ICI are also under investigation (Xu B. et al., 2018; Martikainen and Essand, 2019; Passaro et al., 2019).

## Antibody Based Immunotherapies: mAbs Targeting Molecules Crucial for Cell Survival or TAs

The anti-VEGF mAb Bevacizumab has been used to treat patients with GBM for over a decade with limited survival advantage in the recurrent setting (Diaz et al., 2017; Wick et al., 2017). MAbs targeting EGFRvIII have been administered to patients with newly diagnosed GBM, however, they were unable to significantly inhibit the signaling pathway and improve survival (Westphal et al., 2015). Likewise mAbs targeting EGFR signaling linked to toxins such as *Pseudomonas aeruginosa* exotoxin A (PE) and diphtheria toxin (DT) or to radioisotopes including Iodine-125 and Rhenium-188 as cytotoxic payloads have also yielded relatively disappointing results in patients (Gan et al., 2017). Antibody drug conjugates (ADCs) such as ABT-414 (an anti-EGFR mAb conjugated to tubulin assembly inhibitor monomethyl auristatin F) and AMG 595 (an anti-EGFRvIII mAb conjugated to microtubule-assembly inhibitor maytansinoid DM1) have been tested in clinical trials (NCT01800695; NCT01475006) (Hamblett et al., 2015; Lassman et al., 2015). Results with these agents have been more promising and have encouraged targeting of alternative antigens expressed on GBM cells. An ongoing Phase I/II clinical trial (NCT01631552) is targeting TROP2 in patients with epithelial cancers or other types of cancer, including GBM; this clinical trial utilizes an ADC (IMMU-132) which contains SN-38, the active metabolite of the chemotherapeutic agent irinotecan, linked to the anti-TROP2 antibody hRS7. This ADC construct has shown encouraging results in the treatment of metastatic breast cancer (Zaman et al., 2019). Furthermore, preclinical data obtained both *in vitro* and *in vivo* have proven the efficacy of ADC and radiolabeled antibody approaches targeting Ephrin type-A receptor 3 (EphA3) expressed by GBM cells (Offenhäuser et al., 2018). Similarly the use of an anti-integrin α10 antibody conjugated to the cytotoxin saporin (anti-α10-SAP) exerted antitumor effects against GBM cell lines both *in vitro* and in an orthotopic xenograft mouse model of GBM (Munksgaard Thorén et al., 2019). These results altogether indicate the potential of ADC antitumor activity for the treatment of GBM. However, low efficacy, difficulties with drug delivery, toxicity, acquired resistance and inadequate antitumor activity *in vivo* have markedly reduced the enthusiasm for this type of antibody based therapy (Gan et al., 2017), which in general no longer appears to be a primary

**TABLE 1 |** Immunotherapeutic strategies tested in patients with GBM.

| Immunotherapy | Strengths | Weaknesses | Applied to GBM patients | References |
|---|---|---|---|---|
| Antibodies | High specificity and affinity<br>Low cost | Low efficacy<br>Limited ability to cross BBB<br>Off target toxicity | Bevacizumab (anti-VEGF)<br>Nimotuzumab (anti-EGFR)<br>Cetuximab (anti-EGFR) | Neyns et al., 2009; Westphal et al., 2015; Lombardi et al., 2017 |
| Adoptive effector cell transfer | Ex vivo stimulation and expansion of tumor cell targeting immune cells<br>Safe (auto) | Poor survival and proliferation<br>Poor trafficking to tumor sites<br>Risk of GvHD (allo) | Allogeneic T cells<br>Autologous T cells<br>Autologous lymphokine-activated killer cells<br>Autologous tumor infiltrating lymphocytes | Quattrocchi et al., 1999; Weenink et al., 2020 |
| CAR T cells | Specificity<br>HLA independent<br>Long term proliferation and survival ('Living drug') | Costly and time-consuming manufacture<br>Potential toxicity<br>Acquired resistance | EGFRvIII-specific CAR T cells<br>HER2-specific CAR T cells<br>IL13Rα2-specific CAR T cells | Brown et al., 2015; Ahmed et al., 2017; Goff et al., 2019 |
| Checkpoint Inhibitors | Easily applicable<br>Proven response in certain subsets of patients | Reliant on cognate T cells<br>Reliant on HLA class I APM function in cancer cells<br>Variable response<br>Lack of markers to identify potentially responding patients | Pembrolizumab (anti-PD-1)<br>Nivolumab (anti-PD-1)<br>Nivolumab and ipilimumab (anti-CTLA-4)<br>Durvalumab (anti-PD-L1)<br>Avelumab (anti-PD-L1) | Cloughesy et al., 2019; Schalper et al., 2019; Reardon et al., 2020 |
| Oncolytic Viruses | Specificity<br>Low toxicity | Low anti-tumor efficacy<br>Dependent on enhancement of host immune system response<br>Dependent on cancer cell susceptibility to the induced effector mechanism | HSV-1 M032<br>DNX-2401<br>AdV-tK<br>Reolysin<br>G207 | Markert et al., 2000; Forsyth et al., 2008; Patel et al., 2016; Kieran et al., 2019; Philbrick and Adamson, 2019 |
| Vaccines | Specificity<br>Safe<br>Patient specific<br>Ability to target multiple TAs | Variable immune response<br>Reliant on cognate T cell efficacy (HLA restricted)<br>Identification of immunogenic source required<br>Can be costly | Peptide vaccines:<br>Autologous peptides<br>EGFRvIII protein<br>Multiple tumor antigens<br>Survivin<br>Dendritic cell vaccines:<br>Tumor lysate<br>Glioma stem like cell antigens | Weller et al., 2017; Liau et al., 2018; Weenink et al., 2020 |

interest within the field of immunotherapy of hematological and solid malignancies.

## CAR T Cell Therapy to Treat GBM

The development of cellular engineering technology has resulted in the ability to genetically modify T cells to express a TA-specific CAR. Impressive clinical responses produced by CD19-specific CAR T cells in the field of hematological malignancies (Porter et al., 2015; Schuster et al., 2017; Boyiadzis et al., 2018) have led to FDA approval of three CAR T cell-based therapies (Beyar-Katz and Gill, 2020; Holstein and Lunning, 2020). These remarkable clinical responses have stimulated interest in developing and applying CAR T cell based therapeutic strategies for the treatment of solid tumors including GBM (Choi et al., 2019a).

Chimeric antigen receptors are recombinant receptors typically composed of an extracellular antigen-recognition moiety, mostly (although not exclusively) derived from an antibody that is linked, via spacer/hinge and transmembrane domains, to intracellular signaling domains. The latter include costimulatory domains and T-cell activation moieties, which have been optimized in successive generations of CAR T cells to enhance the signaling activity. T cells grafted with CARs acquire the ability to specifically recognize cancer cells and lyse them. As the recognition and effector mechanism of CAR T cells is HLA class I independent, this type of therapy is not negatively impacted by the downregulation or defective presentation of TAs due to structural and/or functional abnormalities in HLA class I APM components, which often occur in GBM (Facoetti et al., 2005; Thuring et al., 2015). It is noteworthy that T cell receptors can only recognize short peptide sequences. In contrast CARs have the flexibility to be able to recognize TAs in several forms such as carbohydrates, glycolipids and proteins (Abbott et al., 2020) which do not have to be processed for their recognition. However, a limitation of CARs is that with rare exceptions (Maus et al., 2012) they require that the target TA is expressed on the cell membrane, while T cell receptors recognize mostly intracellular moieties which are transported to the cell membrane by MHC class I antigens.

Another major limitation in the antitumor activity of CAR T cell-based immunotherapy is the selectivity and heterogeneity of the targeted TA expression. An ideal target is expected to be homogenously expressed on all differentiated and cancer initiating cells (CICs) within a primary tumor and in metastases. CICs is a practical term used to indicate cells which are in an early stage of differentiation within a lineage, including cancer stem cells (Zhou et al., 2009). These cells are resistant to conventional chemo- and radio-therapy (Maccalli et al., 2018; Steinbichler et al., 2018). Growing experimental

evidence argues in favor of the possibility that elimination of CICs is a crucial requirement for a therapy to be curative because these cells appear to play a major role in disease recurrence and metastatic spread. In addition, the TA should be undetectable or have such a minimal expression on normal tissues that it will not mediate elimination of normal cells by the CAR T cells which recognize it. Toxicity and cytokine release syndrome caused by the targeting of normal cells are potentially significant side effects of CAR T cell therapy and highlight the need for Phase I trials to test their safety and feasibility.

Immunohistochemical (IHC) analysis of GBM tumors has helped identify several molecules as potential targets for CAR T cell-based immunotherapy treatments; many of them have been used in clinical trials (**Tables 2**, **3**). In the next section we will discuss the target TAs currently in clinical and preclinical studies. We will describe first the CARs which have been tested in clinical trials, dividing them into those for which clinical trials have been completed and those for which clinical trials are ongoing. Then we will describe the CARs which are being tested in preclinical models of GBM.

## Targets of CAR T Cell Therapy for Which Clinical Trials Have Been Completed

### EGFRvIII, IL13Ra2, and HER2

A deletion-mutation form of EGFR, termed EGRFvIII, is expressed in around 30% of GBM tumors (Wikstrand et al., 1997). The changes caused by this mutation to the structure of the extracellular domain provide unique epitopes which can be targeted by mAbs specific for the mutated form of EGFR, limiting the likelihood of on-target/off-tumor toxicity (Johns et al., 2004; Yang et al., 2017). In preclinical models EGFRvIII-specific CAR T cells have displayed effective tumor control (Yang et al., 2017; Chen et al., 2019); in contrast, administration of CAR T cells targeting this mutated molecule in patients with GBM has been met with limited success. O'Rourke et al. (2017) reported antigen loss and adaptive resistance in patients with recurrent GBM after intravenous injection of EGFRvIII-specific CAR T cells. A phase I/II trial (NCT01454596) utilizing CAR T cells with an anti-EGFRvIII human 139-scFv and CD28 and 4-1BB costimulatory domains in patients with malignant gliomas expressing EGFRvIII demonstrated no adverse events associated with the administration of up to $1 \times 10^{10}$ CAR T cells. However, one patient receiving $3 \times 10^{10}$ CAR T cells experienced serious adverse events including dyspnea and hypoxia, and another patient, who received $6 \times 10^{10}$ CAR T cells, died 4 h post-administration after developing acute dyspnea and severe hypotension (Goff et al., 2019). These adverse events are thought to be due to a dose dependent congestion of the pulmonary vasculature by the activated T cells, likely a limitation of the route of administration utilized (Goff et al., 2019). These effects are unlikely to reflect targeting of wild-type EGFR due to cross-reactivity of the antibody used to generate the EGFRvIII-specific CAR, although this possibility cannot be ruled out. A major limitation of EGFRvIII as a target TA is its heterogeneous expression in glioma tumors, which most likely will lead to the generation of escape variants resistant to CAR T cell therapy (Rutkowska et al., 2019). These potential limitations, taken together with the low success of the first clinical trials, have markedly decreased enthusiasm for the use of this TA as a target for CAR T cells.

### IL13Rα2

IL-13 is a cytokine released by T helper cells to regulate inflammation and immune response. Its binding to its receptor IL13Rα1 activates signaling via the JAK/STAT pathway. IL-13 can also bind to the high affinity decoy receptor IL13Rα2 which does not possess a functional cytoplasmic domain and therefore does not trigger an intracellular signaling pathway (Thaci et al., 2014). IL13Rα2 is expressed in the majority of both adult and pediatric GBM tumors but is not expressed at significant levels in normal brain or most normal tissues, with the exception of the testis (Debinski and Gibo, 2000; Kawakami et al., 2004; Jarboe et al., 2007; Brown et al., 2013; Thaci et al., 2014). Therefore it has been used as a target of CAR T cells possessing a mutated form of IL-13 in the CAR construct (Thaci et al., 2014). Administration of IL13Rα2-specific CAR T cells has been shown to be feasible and safe with encouraging clinical responses reported in a first in-human pilot study (Brown et al., 2015). Three patients with recurrent GBM received up to twelve intracranial infusions with a maximum dose of $1 \times 10^8$ CAR T cells. Delivery of the IL13Rα2 CAR T cells was well-tolerated with evidence of an antitumor response of short duration in two of the three treated patients.

### HER2

HER2 is an epidermal growth factor receptor which is expressed in normal epidermal cells. It is overexpressed on several types of cancer cells including around 80% of GBM tumors (Mineo et al., 2007). HER2 expression in normal tissues was associated with fatal toxicity in a colon cancer patient who intravenously received $1 \times 10^{10}$ CAR T cells expressing a trastuzumab-based antigen recognition exodomain and a dual CD28.41BB.ζ signaling endodomain (Morgan et al., 2010). Subsequent analysis of the treated patient's organs showed findings consistent with cytokine storm syndrome as well as a high accumulation of CAR T cells in normal lung and abdominal/mediastinal lymph nodes, on which HER2 is expressed, although at low level. This troubling case highlighted the need to select target TAs with limited expression on normal cells. A recent study has shown that third generation HER2-specific CAR T cells can efficiently eliminate GBM cells *in vitro* and that the activity of the administered CAR T cells is increased by their combination with PD-1 blockade (Shen et al., 2019). Successful administration of up to $1 \times 10^8$ HER2-specific CAR T cells, constructed with a CD28.ζ endodomain, was achieved in GBM patients without dose-limiting toxic effects (Ahmed et al., 2017). The CAR T cells, which were generated from virus (cytomegalovirus, Epstein-Barr virus, or adenovirus) specific T cells in order to potentially provide a co-stimulatory effect through latent virus antigen recognition,

**TABLE 2 |** Completed CAR T cell-based clinical trials in patients with GBM.

| Molecular target | Clinical trial identifier and title | Study phase | CAR T cell dosage (+ combination) | Sponsor/site (+ collaborators) | Enrolment | Response |
|---|---|---|---|---|---|---|
| **EGFRvIII** | **NCT02209376** Autologous T Cells Redirected to EGFRVIII-With a Chimeric Antigen Receptor in Patients With EGFRVIII+ Glioblastoma | 1 | Intravenous single dose of $1.75 \times 10^8$–$5 \times 10^8$ CAR T cells | University of Pennsylvania (University of California) | 11 | **Median overall survival** ∼8 months, nil benefit Terminated (to pursue combination therapies) (O'Rourke et al., 2017) |
| | **NCT01454596** CAR T Cell Receptor Immunotherapy Targeting EGFRvIII for Patients With Malignant Gliomas Expressing EGFRvIII | 1/2 | Two intravenous doses of $6.3 \times 10^6$ to $2.6 \times 10^{10}$ CAR T cells per infusion, 2 h apart | National Cancer Institute | 18 | **Median overall survival** 6.9 months **Median Progression-free survival** 1.3 months, nil benefit (Goff et al., 2019) |
| **HER2** | **NCT01109095** CMV-specific Cytotoxic T Lymphocytes Expressing CAR Targeting HER2 in Patients With GBM (HERT-GBM) | 1 | One or more intravenous infusion of $1 \times 10^6/m^2$ – $1 \times 10^8/m^2$ CAR T cells | Baylor College of Medicine (The Methodist Hospital System, Center for Cell and Gene Therapy) | 16 | **Median overall survival** 24.5 months **Median progression-free survival** 3.5 months, 1 (6%) patient had partial response, 7 (44%) had a stable disease (Ahmed et al., 2017) |
| **IL13Rα2** | **NCT00730613** Cellular Adoptive Immunotherapy Using Genetically Modified T-Lymphocytes in Treating Patients With Recurrent or Refractory High-Grade Malignant Glioma | 1 | Intravenous infusions of up to $10^8$ CAR T cells on days 1, 3, and 5 for 2 weeks. Treatment repeated after 3 weeks. | City of Hope Medical Center (National Cancer Institute) | 3 | **Mean survival after relapse** 11 months, positive response (Brown et al., 2015) |
| | **NCT01082926** Phase I Study of Cellular Immunotherapy for Recurrent/Refractory Malignant Glioma Using Intratumoral Infusions of GRm13Z40-2, An Allogeneic CD8+ Cytolitic T-Cell Line Genetically Modified to Express the IL 13-Zetakine and HyTK and to be Resistant to Glucocorticoids, in Combination With Interleukin-2 | 1 | Intratumoral injections of $1 \times 10^8$ CAR T cells and aldesleukin (IL-2) twice per week for 2 weeks. | City of Hope Medical Center | 6 | **Median overall survival** 19.7 months (Keu et al., 2017) |

were well tolerated, persisted for up to 1 year in the blood of patients and produced clinical benefit in eight out of the 17 treated patients.

The high frequency of HER2 in GBM tumors, its involvement in tumor development and progression (Hynes and MacDonald, 2009), and the ability of HER2-specific CAR T cells to eliminate both differentiated GBM cells and GBM CICs (Ahmed et al., 2010) make it an attractive target TA. The major concern when targeting HER2 are potential side effects due to HER2 expression in various normal tissues and especially in vital organs (Press et al., 1990; Morgan et al., 2010), although this has rarely been an issue with HER2-specifc CAR T cell administration in humans thus far.

# TARGETS OF CAR T CELL THERAPY IN ON-GOING CLINICAL TRIALS

## B7-H3

B7-Homolog 3 (B7-H3), also known as CD276, is a member of the B7 family of immune checkpoint molecules including PD-L1 (B7-H1) and CD80 (B7-1) (Collins et al., 2005). It is highly expressed in some hematological malignancies and in most, if not all types of solid cancer including head and neck squamous cell cancer (HNSCC), triple negative breast cancer (TNBC), intrahepatic cholangiocarcinoma (ICC), pancreatic ductal adenocarcinoma (PDAC), and chondrosarcoma (Kontos et al., 2020). Importantly it is not detectable by IHC with

**TABLE 3 |** Ongoing CAR T cell-based clinical trials in patients with GBM.

| Molecular target | Clinical trial identifier and title | Study phase | CAR T cell dosage (+ combination) | Sponsor/Site (+ collaborators) | Estimated enrolment | Estimated primary completion date |
|---|---|---|---|---|---|---|
| **B7-H3** | **NCT04385173** Pilot Study of B7-H3 CAR-T in Treating Patients With Recurrent and Refractory Glioblastoma | 1 | Three intratumoral or intracerebroventricular injections of CAR T cells at two doses in between temozolomide cycles. | Second Affiliated Hospital, School of Medicine, Zhejiang University [BoYuan RunSheng Pharma (Hangzhou) Co., Ltd. (China)] | 12 | May, 2022 |
| | **NCT04077866** B7-H3 CAR-T for Recurrent or Refractory Glioblastoma | 1/2 | Three intratumoral or intracerebroventricular injections of CAR T cells at two doses in between temozolomide cycles. | Second Affiliated Hospital of Zhejiang [Ningbo Yinzhou People's Hospital, Huizhou Municipal Central Hospital, BoYuan RunSheng Pharma (Hangzhou) Co., Ltd. (China)] | 40 | June, 2024 |
| **CD147** | **NCT04045847** CD147-CART Cells in Patients With Recurrent Malignant Glioma | 1 | Intracavity injection of CAR T cells, once per week for three weeks. | Xijing Hospital | 31 | October, 2020 |
| **GD2** | **NCT04099797** C7R-GD2.CART Cells for Patients With GD2-expressing Brain Tumors (GAIL-B) | 1 | Intravenous injection of between $1x10^7$ – $1x10^8$ CAR T cells with or without lymphodepletion chemotherapy. | Baylor College of Medicine (Center for Cell and Gene Therapy, Baylor College of Medicine) | 34 | February, 2023 |
| **EGFRvIII** | **NCT03726515** CART-EGFRvIII + Pembrolizumab in GBM | 1 | CART-EGFRvIII + pembrolizumab. | University of Pennsylvania | 7 | December, 2020 |
| | **NCT03283631** Intracerebral EGFR-vIII CAR-T Cells for Recurrent GBM (INTERCEPT) | 1 | Starting dose of $2.5 \times 10^8$ per CAR T cells per intracerebral infusion, with doses escalated in successive cohorts. | Duke University (National Cancer Institute, Duke Cancer Institute) | 24 | December, 2021 |
| **IL13Ra2** | **NCT02208362** Genetically Modified T-cells in Treating Patients With Recurrent or Refractory Malignant Glioma | 1 | IL13Rα2-specific, hinge-optimized, 41BB/truncated CD19-expressing CAR T cells by intratumoral, intracavitary, or intraventricular catheter. Weekly for three weeks with additional infusions if eligible. | City of Hope Medical Center (National Cancer Institute, Food and Drug Administration) | 92 | January, 2021 |
| | **NCT04003649** IL13Ralpha2-Targeted Chimeric Antigen Receptor (CAR) T Cells With or Without Nivolumab and Ipilimumab in Treating Patients With Recurrent or Refractory Glioblastoma | 1 | Intravenous administration of nivolumab and ipilimumab followed by intracranial intraventricular/intracranital intratumoral infusion of CAR T cells. Up to four cycles. | City of Hope Medical Center (National Cancer Institute) | 60 | December, 2022 |
| **MMP2 (Chlorotoxin)** | **NCT04214392** Chimeric Antigen Receptor (CAR) T Cells With a Chlorotoxin Tumor-Targeting Domain for the Treatment of MPP2+ Recurrent or Progressive Glioblastoma | 1 | Three weekly cycles of one or two CAR T cell infusions. | City of Hope Medical Center (National Cancer Institute) | 36 | February, 2023 |
| **Variable** | **NCT03423992** Personalized Chimeric Antigen Receptor T Cell Immunotherapy for Patients With Recurrent Malignant Gliomas | 1 | CAR T cells expressing receptors specific for EGFRvIII, IL13Rα2, Her-2, CD133, EphA2 or GD2, with or without anti- PD-L1 mAb. | Xuanwu Hospital [Beijing Mario Biotech Company, Hebei Senlang Blotech Company, Beijing HuiNengAn Biotech Company (China)] | 100 | January, 2021 |

mono- and poly-clonal antibodies in normal tissues, with the exception of salivary glands, gastric epithelial cells and adrenal glands; in the latter normal organs its expression level is low, as suggested by the weak staining intensity. Interestingly B7-H3 is also expressed in tumor-associated vessels and fibroblasts suggesting that B7-H3 CAR T cells may eliminate cancer cells not only by direct targeting, but also through stroma disruption and neo-angiogenesis inhibition (Picarda et al., 2016; Seaman et al., 2017). The described features make B7-H3 a highly attractive target of antibody-based immunotherapy. The efficacy of B7-H3 CAR T cell-based therapy has been explored in a wide range of cancer types including neuroblastoma, pancreatic ductal adenocarcinoma and ovarian cancer (Du et al., 2019; Majzner et al., 2019). Tang et al. (2019) have recently assessed the antitumor effect of B7-H3 CAR T cells in intracranial GBM mouse models, and concluded that they could induce a significant tumor regression and prolong survival of tumor bearing mice compared to vehicle-transduced T cells. A randomized, parallel-arm, phase I/II study (NCT04077866) has been designed to assess the effect of B7-H3 CAR T cell administration between cycles of Temozolomide treatment for patients with refractory or recurrent GBM.

A potential concern, however, is that B7-H3 mRNA is present practically in all normal tissues, although it is not translated due to inhibition by microRNAs (Xu et al., 2009). This inhibition can easily be modulated by conditions such as inflammation, resulting in B7-H3 expression in normal tissues, which then may be targeted by B7-H3 CAR T cells.

## CD147

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN), is a 57-kilodalton (kDa) type-I transmembrane protein belonging to the immunoglobulin superfamily of adhesion molecules. It induces fibroblasts to secrete metalloproteinases-1, -2, -3, -9, -14, and -15, which degrade the extracellular matrix (ECM), promoting tumor growth, invasion and metastasis (Xiong et al., 2014). CD147 expression has been found to be significantly higher in glioma than in normal brain tissues; its expression level is inversely correlated with prognosis in patients with GBM (Yang et al., 2013; Li et al., 2017). An open label early phase I clinical trial has started to recruit patients with recurrent GBM to test the safety, tolerance and efficacy of treatment with CD147-specific CAR T cells (NCT04045847). While CD147 is overexpressed on malignant cells, it is still expressed at a low level on various normal tissues such as epithelial and endothelial cells, and brain and heart tissue (Riethdorf et al., 2006; Liao et al., 2011; Tseng et al., 2020). Therefore, there is a concern that CD147-specific CAR T cells may cause on-target off-tumor side effects.

## GD2

Gangliosides are commonly expressed on normal tissues. Interestingly disialoganglioside GD2 is not, but is highly expressed on several tumor types including melanoma, retinoblastoma and neuroblastoma (Nazha et al., 2020). GD2 is expressed on GBM cell lines and patient samples making it an attractive target TA (Golinelli et al., 2018). Furthermore, GD2 has been reported to be a CIC marker in breast cancer (Battula et al., 2012), however, this finding is questionable for GBM as Woo et al. (2015) found that patient-derived GBM cells with or without GD2 had similar *in vitro* neurosphere formation capacity.

GD2 targeting CAR-T cells have successfully demonstrated potent cytotoxicity against neuroblastoma cell lines *in vitro* as well as against cell lines grafted subcutaneously in NOD/SCID mice models (Prapa et al., 2015). Additionally, GD2-specific CAR T cells are able to effectively eliminate patient-derived diffuse midline glioma orthotopic xenograft models (Mount et al., 2018). In an alternative approach, the anti-tumor activity of TNF-related apoptosis-inducing ligand (TRAIL) expressing mesenchymal stromal/stem cells (MSCs) have been used to eliminate cancer cells (Grisendi et al., 2015). The addition of a truncated GD2-specific CAR to the MSCs enhanced specific targeting of GD2-positive GBM cells *in vitro* (Golinelli et al., 2018).

GD2-specific CAR T cells have been safely administered to patients with neuroblastoma, albeit with limited clinical benefit (Louis et al., 2011; Heczey et al., 2017). Together this information has led to the initiation of a clinical trial targeting high grade gliomas including GBM (NCT04099797). In this trial GD2-specific CAR T cells were also transduced to express a constitutively active IL-7 cytokine receptor to enhance their antitumor activity (Shum et al., 2017).

## MMP2

Chlorotoxin (CLTX) is a small, naturally derived 36–amino acid long peptide found in the venom of the death stalker scorpion Leiurus quinquestriatus (DeBin et al., 1993). Interestingly CLTX binds selectively to primary brain tumors, but displays barely detectable binding to normal brain tissue as well as many other normal human tissues tested including skin, kidney and lung (Lyons et al., 2002). The specific surface receptor for CLTX on GBM cells has not been identified, however, the expression of matrix metalloproteinase 2 (MMP2), chloride channel CLCN3, and phospholipid protein annexin A2 (ANXA2) all appear to be required for CLTX binding to GBM cells (Soroceanu et al., 1998; Deshane et al., 2003; McFerrin and Sontheimer, 2006; Tatenhorst et al., 2006). CLTX bioconjugate administration for imaging and therapeutic purposes has been well tolerated in patients with no dose limiting toxicity observed (Cohen et al., 2018). CLTX-directed CAR T cells generated to target GBM cells showed potent antitumor activity in orthotopic xenograft tumor models (Wang et al., 2020). GBM cells in which MMP2 expression was knocked down using short hairpin RNA (shRNA) resulted in significantly lower CLTX CAR T cell activation and cytotoxicity (Wang et al., 2020). These results imply that membrane associated MMP2 is required for effective CLTX CAR T cell targeting of GBM cells. In light of this information a phase I study (NCT04214392) for the treatment of MMP2 positive recurrent or progressive GBM with T cells expressing CLTX CARs has been initiated.

## NKG2D Ligands

The human NKG2D receptor is expressed by the majority of natural killer (NK) cells of the innate immune system as well as by NKT, γδ T cells, CD8+ T cells, and some autoreactive or

immunosuppressive CD4+ T cells (Duan et al., 2019). NKG2D ligands, which include MHC class I related chains (MICA/B) and six UL16-binding proteins (ULBPs), are often upregulated on stressed, transformed and pathogen-infected cells; therefore, they play a crucial role in their detection and elimination by effector immune cells (Bauer et al., 1999; Raulet et al., 2013). NKG2D ligands are expressed on GBM cell lines, patient samples and importantly on GBM stem-like cells (Flüh et al., 2018; Yang et al., 2019). Treatment with chemotherapy or radiotherapy can upregulate NKG2D ligand expression on GBM cells, emphasizing the potential of combinatorial therapeutic strategies (Weiss et al., 2018a). CAR T cells expressing full length murine NKG2D in combination with radiotherapy have been reported to significantly prolong overall survival of immunocompetent mice with intracranial grafts of mouse glioma cells (Weiss et al., 2018b). Furthermore, Yang et al. (2019) have demonstrated effective eradication of human differentiated GBM cells and GBM CICs *in vitro* and in subcutaneous tumor models. However, toxicity in humans cannot be excluded given the expression of NKG2D ligands on normal tissues under distress. This information should have been provided by a phase I clinical trial to test the safety and clinical response to NKG2D-based CAR T cells in solid tumors including GBM (NCT04270461). However, this trial has been withdrawn for administrative reasons.

## TARGETS OF CAR T CELL THERAPY IN PRECLINICAL STUDIES

### CAIX

Carbonic anhydrases (CA) are a group of enzymes which catalyze the reversible hydration of carbon dioxide, a process which is important for several cellular functions including maintenance of pH balance (Supuran, 2016). CAIX is induced under hypoxic conditions and is therefore often overexpressed in many solid tumors including GBM (Proescholdt et al., 2005), for which it is a prognostic marker of poor patient survival outcome (Proescholdt et al., 2012). CAIX-specific CAR T cells have been tested against GBM cells *in vitro* and following direct intratumoral injection in an *in vivo* xenograft mouse model (Cui et al., 2019). The CAIX-specific CAR T cells effectively eliminated GBM cells and prolonged survival of tumor bearing mice. However, the regulation of CAIX expression level on cancer cells by the degree of hypoxia in the TME is likely to cause marked changes in its expression on cancer cells. Therefore, there may be a significant degree of intra- and inter-tumor heterogeneity in CAIX expression in a patient. This expression pattern is likely to facilitate the generation of cancer cell escape variants, which may represent a major obstacle to the successful application of CAIX-specific CAR T cell-based therapy.

### CD70

CD70 is a type II transmembrane protein binding only to CD27, a glycosylated transmembrane protein of the tumor necrosis factor (TNF) receptor family. Besides being expressed on activated B and T cells and mature DCs, CD70 is expressed on certain hematological and solid cancers including GBM. Constitutive CD70 expression may provide GBM cells with an immune escape mechanism by promoting T cell death (Chahlavi et al., 2005). Jin et al. (2018) have recently reported that both human and mouse CD70-specific CAR T cells could recognize and eliminate CD70+ GBM tumors *in vitro* and in xenograft and syngeneic models, with no toxicity reported. A major strength of this TA as a target is its restricted expression mostly to malignant tumors, both primary and recurrent lesions; Jin et al. (2018) also showed that CD70 gene expression was not detected in 52 types of normal tissues. While there could be a concern related to targeting of CD70+ T cells and DCs, CD70-specific CAR T cells do not seem to affect these activated immune cells. Preclinical studies utilizing CD70-specific CAR T cells in glioma and head and neck cancer (Park et al., 2018) have shown encouraging results and provide enthusiasm for the targeting of this TA.

### CSPG4

Chondroitin Sulfate Proteoglycan 4 (CSPG4), also known as neuron-glial antigen 2 (NG2) and high molecular weight-melanoma associated antigen (HMW-MAA), was initially identified on melanoma cells, but has since been shown to be expressed by a wide variety of cancer types, as well as on CICs in many of the cancer types analyzed (Campoli et al., 2010). It is thought to play a role in cell proliferation and migration *in vitro* as well as in metastatic spread *in vivo*; its expression level is inversely correlated with patient survival in glioma (Tsidulko et al., 2017) as well as in other solid cancers such as HNSCC and chordoma (Wang et al., 2010b; Warta et al., 2014; Schoenfeld et al., 2016). CSPG4 expression, assessed via commercially available antibodies, has been reported in the Protein Atlas to have a broad distribution in normal tissues. However, IHC staining with our mAbs which recognize distinct CSPG4 epitopes has not detected them in any normal tissue with the exception of activated pericytes in the TME (Campoli et al., 2010). Furthermore, negative reverse protein assay results derived from the analysis of 94 normal tissues (Beard et al., 2014), lack of toxicity in CSPG4-specific mAb treated animal models (Mittelman et al., 1992; Wang et al., 2010a; Riccardo et al., 2014) and inability of CSPG4-specific CAR T cells to recognize and lyse normal cells *in vitro* (Geldres et al., 2014) convincingly argue against the possibility that CSPG4 expression on normal tissues is an issue when targeting this molecule as a TA. This conclusion is supported by the following additional lines of evidence: no toxicity was detected in melanoma patients who developed CSPG4-specific antibodies after immunization with anti-idiotypic antibodies which mimic CSPG4 (Mittelman et al., 1992). Furthermore Pellegatta et al. (2018) recently demonstrated that CSPG4 is highly expressed with limited heterogeneity in GBM tissue and tumor associated vessels, and is not detected in healthy brain parenchyma. Intracranial delivery of CSPG4 CAR T cells was able to control tumor progression in orthotopic GBM neurosphere xenograft models (Pellegatta et al., 2018). The encouraging preclinical results, the lack of expression in normal brain tissue and the involvement in several processes of tumorigenesis make CSPG4 a very attractive target TA. Furthermore, its expression on CICs will enable CAR T cells to recognize and eliminate this particularly hostile cell subpopulation.

## EphA2

Erythropoietin-producing hepatocellular carcinoma A2 (EphA2) belongs to the Eph family of receptor tyrosine kinases (RTKs); it plays a role in a wide variety of functions in malignant cells, such as tumorigenesis, invasion, angiogenesis and metastasis (Wykosky and Debinski, 2008; Baharuddin et al., 2018). Therefore, targeting EphA2 could prevent tumor progression and recurrence. EphA2 is overexpressed in GBM with no detection in normal brain tissues (Hatano et al., 2005; Wykosky et al., 2005). It has also been found to be expressed on GBM CICs (Binda et al., 2012). EphA2 targeting CAR T cells effectively eliminate differentiated GBM cells and GBM cancer stem-like cells *in vitro*, and significantly prolong survival of orthotopic xenograft SCID mouse models (Chow et al., 2013). In a subsequent study Yi et al. (2018) generated an optimized version of the anti-EphA2 CAR construct utilizing a short spacer region. This CAR displayed greater anti-glioma activity compared to the original CAR since survival of glioma bearing mice was prolonged to a similar extent using a 20-fold lower dose (Yi et al., 2018).

## TROP2

Trophoblast cell surface antigen 2 (TROP2) is a 36 kDa transmembrane glycoprotein, highly expressed on several types of solid cancer (Cubas et al., 2009; Zeng et al., 2016). It is also considered a stem cell marker (Lenárt et al., 2020). Hou J. et al. (2019) demonstrated high TROP2 expression on GBM cells from surgically removed patient tumors compared to low levels on normal brain cells. Its expression level was found to inversely correlate with survival in GMB patients (Hou J. et al., 2019). TROP2 is thought to modulate cell proliferation and promote metastasis via activation of JAK/STAT3 pathway (Hou J. et al., 2019). Furthermore, in GBM TROP2 is associated with promotion of blood vessel formation through VEGF upregulation (Hou J. et al., 2019). Therefore, targeting TROP2 may also help to inhibit cancer growth through abrogation of neoangiogenesis.

In a recent study, Bedoya et al. (2019) have shown that TROP2-specific CAR T cells can target TROP2 expressing breast, pancreas and prostate cancer cells highlighting the potential of this TA to mediate the targeting of different types of solid cancer. GBM cell recognition and elimination by TROP2-specific CAR T cells is under investigation. Although the latter results suggest that TROP2 may be a promising target, its wide expression on various healthy tissues (Li et al., 2020) raises concerns about the induction of on-target off-tumor toxicity, which could potentially greatly limit its clinical application.

# LIMITATIONS OF CAR T CELL THERAPIES TO TREAT GBM

Whilst CAR T cells targeting CD19+ hematological malignancies have shown remarkable clinical responses resulting in FDA approval of tisagenlecleucel (KYMRIAH, Novartis), axicabtagene ciloleucel (YESCARTA, Kite Pharmaceuticals) and brexucabtagene autoleucel (TECARTUS, Kite Pharmaceuticals) CAR T cell therapies for clinical use (Beyar-Katz and Gill, 2020), this success has not yet been paralleled in solid tumors. Several challenges face the application of CAR T cell therapy in solid tumors; they include: (i) insufficient trafficking of CAR T cells to the tumor site, (ii) defective recognition of the targeted TA, (iii) expression of the targeted TA in normal tissues, leading to killing of normal cells, (iv) limited persistence and low proliferation of effector immune cells in the TME, (v) unregulated strength and timing of effector functions resulting in adverse effects such as those caused by cytokine release syndrome and, (vi) immunosuppressive TME which provides multiple escape mechanisms to tumor cells (Lim and June, 2017). These variables and their role have been extensively reviewed elsewhere (June and Michel Sadelain, 2018; June et al., 2018; Schmidts and Maus, 2018; Martinez and Moon, 2019). Here we focus on issues specifically affecting CAR T cell treatment of GBM (Chuntova et al., 2021) such as acquired resistance, penetration of blood brain barrier (BBB) and toxicity of central nervous system (CNS).

## Acquired Resistance

Gliomas are characterized by genetic, epigenetic, and environmental intratumoral heterogeneity (Nicholson and Fine, 2021). Intratumoral TA heterogeneity and antigen/epitope loss following treatment are potential causes of failure of CAR T cell therapies in GBM. EGFRvIII TA escape has previously been observed following the use of an EGFRvIII targeted peptide vaccine in GBM patients; 82% of the patients who had disease recurrence had lost the targeted TA (Sampson et al., 2010). Similarly EGFRvIII loss/downregulation has been reported following EGFRvIII-specific CAR T cell administration (O'Rourke et al., 2017). While peripheral blood engraftment and persistence of CAR T cells could be detected in patients with disease controlled in the short term, surgically resected tumors had EGFRvIII loss or downregulation. A similar observation has been made following IL13Rα2 CAR T cell administration (Krenciute et al., 2017). The resulting resistance is thought to be due to the survival and growth of differentiated GBM cells and/or GBM CICs which escape CAR T cell-mediated killing because of the lack of expression of the targeted TA and progress the disease with an altered phenotype. Therefore, selection of a TA with high homogenous expression and high expression stability, or development of methods to enhance the ability of CAR T cells to eliminate cancer cells which do not express the targeted TA are necessary to prevent this cancer escape mechanism.

## Blood Brain Barrier

The BBB is a physiological barrier between the blood vessels carrying oxygen and nutrients and the brain tissues they supply. The structure of the BBB is complex, but primarily consists of specialized endothelial cells very tightly joined to each other in contact with pericytes and astrocytes. This morphology helps isolate brain from disease-causing pathogens and toxins and was historically thought to help make the brain an 'immune privileged organ' (Carson et al., 2006). This view has changed due to the recognition that peripheral immune cells can cross the intact BBB, allowing brain to interact with immune system. However, BBB does limit leukocyte migration into CNS and therefore regulates the rate of T cell recruitment (Engelhardt, 2010). Cell

adhesion molecules on endothelial cells as well as specific antigen expression by APCs are thought to be necessary to recruit antigen-specific CD8+ T cells across BBB (Galea et al., 2007; Engelhardt, 2010). However, T cell recruitment in the presence of cancer is often reduced (Sackstein et al., 2017). This reduction may provide cancer cells with an immune escape mechanism.

Glioblastoma multiforme was previously suggested to uniformly damage BBB; therefore, permeability of drugs, antibodies and immune cells should not be an obstacle. However, it has been recently demonstrated that BBB may be intact in spite of GBM even with significant tumor burden (Sarkaria et al., 2018). Therefore, the mode of delivery of CAR T cells to treat GBM should be reevaluated.

Systemic intravenous administration of CAR T cells results in limited CAR T cell infiltration of tumors located in the CNS (Mulazzani et al., 2019). Intravenous administration of EGFRvIII-specific CAR T cells to treat GBM displayed some infiltration of CAR T cells into tumor, although this was not consistent in all patients (O'Rourke et al., 2017). To overcome this limitation, direct administration of CAR T cells to the tumor site has been assessed as a mode of delivery, negating the need for cells to migrate across the BBB. This can either be achieved by intratumoral/intracavitary injection into the tumor or the resected tumor site, or by intracerebral/intraventricular injection into the brain tissue or cerebral ventricle. Locoregional administration of CAR T cells has been found to improve T cell tumor infiltration and tumor control in several preclinical models of brain tumors, compared to intravenous delivery (Priceman et al., 2018; Mulazzani et al., 2019; Theruvath et al., 2020). Intracerebral CD19-specific CAR T cell injection to mice with CNS lymphoma was found to result in the migration of CAR T cells to the 'healthy' contralateral brain hemisphere, albeit at lower numbers (Mulazzani et al., 2019). Similarly in a murine GBM model intraventricular administration of IL13Rα2-specific CAR T cells was found to convey greater control of the contralaterally grafted tumor (Brown et al., 2018). This finding is of great clinical relevance as multifocal GBM accounts for nearly a quarter of primary GBMs and therefore targeting of all tumor sites is crucial to cure a patient (Syed et al., 2018). Furthermore, in a recent case report a multifocal GBM patient initially received IL13Rα2 CAR T cells via intracavitary infusion to the resected tumor site, which was able to control locoregional progression, but not that of non-resected tumors in the contralateral temporal lobe or the development of new tumors (Brown et al., 2016). Interestingly, once the administration method was changed to intraventricular infusions into the opposite lateral ventricle, all intracranial and spinal tumors were reduced in size. Therefore, intraventricular infusion of CAR T cells appears to be the most effective delivery method to administer CAR T cells to all tumor sites, as demonstrated by its current use in most ongoing clinical trials (**Table 3**).

## Toxicity Associated With CAR T Cell Treatment in GBM

Cytokine release syndrome (CRS) is the most common adverse event associated with CAR T cell therapy usually seen within one or 2 weeks after the initial infusion (Lee et al., 2014; Bonifant et al., 2016). Substantial activation of CAR T cells can cause the release of an excessive amount of inflammatory cytokines which can subsequently result in fever, tachycardia, hypotension and in some cases even death due to multiple organ failure (Schuster et al., 2017).

A major concern in the administration of CAR T cells to the brain is the potential for neurotoxicity, which can occur alone or together with CRS. The mechanism(s) underlying CAR T cell mediated neurotoxicity has not been elucidated yet; however, CNS endothelial cell activation is thought to play a role (Mackall and Miklos, 2017; Wang and Han, 2018). Gust et al. (2017) showed that CD19-specific CAR T cell administration for the treatment of B cell acute lymphoblastic leukemia increased permeability of BBB as a result of endothelial cell activation. The latter, in turn, leads to a cytokine influx into CNS causing a range of side effects such as seizures and cerebral edema, resulting in several fatalities.

Therefore, CAR T cell administration directly to brain raises some concerns about the risks of CRS and neurotoxicity occurring in this sensitive organ. CAR T cell treatment of GBM has resulted so far in only one such fatality of a patient who received the highest number of EGFRvIII CAR T cells in a dose escalation phase I trial (Goff et al., 2019). Aside from this episode most trials of GBM CAR T cell therapy have shown relatively few adverse events.

To decrease CAR T cell toxicity, strategies to inhibit excessive cytokine release are under investigation. These include (i) administration of high dose steroids, and/or of a mAb (tocilizumab) targeting the IL-6 receptor, (ii) selection of optimal CAR T cells in terms of binding avidity and/or antitumor activity, (iii) administration of lower numbers of CAR T cells per infusion, and (iv) utilization of CAR T cells with a suicide gene or switchable signaling components.

## Need to Eliminate CICs

Cancer initiating cells are a subpopulation of cells within a tumor, which according to the cancer stem cell theory (Reya et al., 2001) play a crucial role in cancer initiation and metastatic spread due to their characteristics of self-renewal and multi-lineage differentiation. In GBM CICs are defined by the expression of intracellular markers usually associated to stem cells such as MYC, NANOG, and SOX2 and of cell surface markers including CD133 (Lathia et al., 2015). However, a single diagnostic marker has not been identified yet and indeed GBM CICs may represent a more plastic state of cancer cells (Dirkse et al., 2019). Alternatively, but not exclusively GBM CICs can be detected by their ability to form neurospheres *in vitro* (Lathia et al., 2015) and to induce tumors when injected in low numbers to immunodeficient mice (Lathia et al., 2015). CICs have been suggested to be partially responsible for treatment failure and disease recurrence due to their self-renewal ability and treatment resistance. Ability of GBM sphere generation *in vitro* using dissociated patient tumor samples as well as the number of CD133+/Ki67+ cells in the lesion are both prognostic markers of tumor progression and poor patient survival outcome (Pallini et al., 2008). These findings emphasize the need to develop and

implement therapeutic strategies that efficiently eliminate the CIC subpopulation.

CD133-specific CAR T cells have been shown to successfully eliminate GBM CICs in an orthotopic *in vivo* model (Zhu et al., 2015). CAR T cells targeting B7-H3, CSPG4 and HER2 have also been shown to eliminate both differentiated GBM cells and GBM CICs in preclinical models of GBM (Ahmed et al., 2010; Pellegatta et al., 2018; Nehama et al., 2019). Taken together these results provide evidence of the efficacy of CAR-engineered T cells in targeting both differentiated GBM cells and GBM CICs.

## STRATEGIES TO ENHANCE EFFICACY OF CAR-BASED IMMUNOTHERAPY AGAINST GBM

In many clinical studies CAR T cells used as a monotherapy have been found not to be sufficient to induce sustained clinical responses in various types of solid cancers (Hou B. et al., 2019). In GBM the reported clinical trials have displayed limited clinical efficacy with one trial utilizing EFGRvIII-specific CAR T cells terminated in order to pursue combination therapy with Pembrolizumab (NCT03726515). One might argue that the lack of clinical response to therapy with CAR T cells alone is partially due to recruitment of patients at later stages of disease with high tumor burden and a history of unsuccessful therapies. These disappointing clinical results highlight the need to develop novel strategies to increase CAR T cell antitumor activity and persistence (Xu J. et al., 2018).

### Enhancing Functional Properties of CAR T Cell Constructs

Successive generations of CAR T cells have enhanced the strength and potency of their antitumor activity through the addition of co-stimulatory domains and functionality moieties. Current CAR T cells in clinical trials are utilizing these approaches such as the chlorotoxin (EQ)-CD28-CD3zeta/truncated CD19-expressing CAR T cells and IL13Rα2-specific hinge-optimized 4-1BB/truncated CD19-expressing CAR T cells. Interestingly both of these CAR T cells incorporate a truncated version of CD19 which has no functional activity, but can be utilized as a marker to identify transduced CAR T cells.

One mechanism to enhance antitumor efficacy is to further engineer the CAR construct to induce or constitutively secrete active cytokines in order to increase CAR T cell activity and persistence. IL13Rα2 CAR T cells engineered to additionally express IL-15 displayed greater anti-glioma activity and improved persistence and significantly prolonged survival of mice than control IL13Rα2 CAR T cells in orthotopic glioma xenograft models (Krenciute et al., 2017). Several mechanisms designed to disrupt immunosuppressive immune checkpoint molecule signaling between CAR T cells and malignant cells have been tested (Chen et al., 2016). CAR T cells engineered to secrete a PD-L1 antibody (Suarez et al., 2016), CAR T cells with PD-1 and Lag3 genes knocked out using CRISPR/Cas9 technology (Ren et al., 2017; Zhang et al., 2017), and CAR T cells designed with a PD-1 ectodomain linked to the transmembrane and cytoplasmic domains of CD28 in order to convert an immunosuppressive signal into a co-stimulatory one (Prosser et al., 2012; Liu et al., 2016) have all been explored in several solid cancer types. In preclinical GBM models, use of EGFRvIII-specific CAR T cells in which PD-1 signaling had been disrupted through a CRISPR-Cas9 approach, resulted in significantly prolonged survival of the orthotopically engrafted mice (Choi et al., 2019c).

Similarly, CAR T cells can be engineered to express chemokine receptors to enhance intra tumoral T cell trafficking. CXCR1 and CXCR2 modified CD70-specific CAR T cells were shown to have improved T cell trafficking and antitumor efficacy through IL-8 mediated chemotaxis in *in vivo* models of GBM (Jin et al., 2019). The higher CAR T cell antitumor activity resulted in improved tumor regression and survival of mice compared to those treated with unmodified CD70-specific CAR T cells. Additional approaches to enhance CAR T cell antitumor activity include incorporation of the hypoxia transcription factor HIF-1α subdomain in a CAR construct. This results in the activation of CAR T cells only under hypoxic conditions such as in the TME (Juillerat et al., 2017). This strategy may reduce on target/off tumor toxicity. Cancer cells often secrete adenosine which can inhibit T cell activity within the TME (Ohta, 2016). Blockade of the A2A adenosine receptor using pharmacological antagonists or target specific shRNA can increase CAR T cell efficacy in orthotopic models of breast cancer (Beavis et al., 2017). However, blockade of the A2A adenosine receptor may be counterproductive in GBM as the FDA-approved A2A adenosine receptor agonist lexiscan has been shown to increase BBB permeability in *in vitro* and *in vivo* models (Kim and Bynoe, 2015).

Tumor antigen expression on normal tissues often hinders the usage of CAR T cells due to the concern of unexpected side effects and toxicity that will be seen in healthy tissues as a result of the treatment. To overcome this challenge CARs designed using scFvs with altered affinity allows differential recognition of the targeted TA which are highly expressed on cancer cells but have a lower expression level on normal tissues. For example CARs targeting HER2 or EGFR with reduced affinity demonstrated effective elimination of cancer cells with no damage to normal cells both *in vitro* and in ovarian and prostate murine models, and therefore may be useful in GBM treatment (Liu et al., 2015).

### Targeting Multiple TAs

As TA expression is frequently heterogeneous on GBM tissues, as on other types of solid cancer tissues, malignant cells lacking the targeted TA may escape CAR T cell recognition and elimination. Recurrences of the disease have been observed due to outgrowth of cancer cells that do not express the targeted TA after CAR T cell treatment (Krenciute et al., 2017; O'Rourke et al., 2017). One strategy to overcome this escape mechanism relies on targeting multiple TAs at the same time (Grada et al., 2013). For instance, CAR T cells which target multiple ligands such as NKG2D and ErbB have been investigated for their efficacy against solid tumors both *in vitro* and *in vivo*, and have displayed effective antitumor results (Davies et al., 2012; Sentman and Meehan, 2014). Another strategy is to incorporate multiple antibody

scFvs in the same CAR T cell construct. Bi-specific CAR T cells expressing both HER2 and IL13Rα2 displayed increased tumor elimination compared to singular TA-specific CAR T cells in a murine model of GBM (Hegde et al., 2016). Similarly, CAR T cells specific for both CD70 and B7-H3 have shown effective preclinical antitumor function against a range of solid tumors (Yang et al., 2020). Furthermore, tri-specific CAR T cells targeting HER2, IL13Ra2, and EphA2 provide an even more comprehensive coverage of TAs and have been shown to significantly prolong survival of mice bearing GBM patient derived xenografts (Bielamowicz et al., 2018). An alternative approach involves the use of EGFRvIII-specific CAR T cells engineered to secrete bi-specific T-cell engagers (BiTE) (Choi et al., 2019b). These bi-specific mAbs can link T cells to wild type EGFR, overcoming the resistance of EGFRvIII heterogenous GBM to EGFRvIII-specific CAR T cells. BiTE-armored CAR T cells successfully eliminated cancer cells and prolonged survival of mice orthotopically grafted with either GBM cell lines or patient derived glioma neurospheres (Choi et al., 2019b). In order to improve the antitumor effect of bi-specific CAR T cells their signaling requirements may have to be optimized (Han et al., 2019). Activation of bi-specific CAR T cells could be triggered by only one of the targeted TAs or may require the expression of both TAs on target cells. Alternatively, inhibition of bi-specific CAR T cells could be triggered by recognition of a selected target antigen expressed on non-malignant cells to minimize on-target/off-tumor toxicity.

## CAR NK Cells as Effectors for the Treatment of GBM

An alternative therapeutic strategy to CAR T cells is to incorporate a CAR construct into other types of effector immune cells such as NK cells (Burger et al., 2019). In particular NK cells, usually considered a component of the innate immune system, offer additional advantages over T cells and CAR effector cells in that they can (i) recognize multiple non-CAR specific oncogenic antigens, (ii) be administered as an allogeneic transplant with low risk of graft versus host disease, (iii) regulate adoptive immune responses though DC editing, and (iv) do not induce CRS. However, major limitations of CAR NK cells are the relatively low yield of NK cells available from an individual donor's peripheral blood compared to T cells, as well as the low transduction efficiency of CAR constructs in these cells. Strategies to expand NK cell populations isolated from the blood of a donor, as well as use of NK cell lines to create an 'off the shelf' CAR NK cell product are highly promising approaches to overcome these limitations.

Preclinical studies have demonstrated the potent antitumor activity of CAR NK cells recognizing several TAs including EGFR and EGFRvIII. The NK cell line NK-92 has been utilized most prominently to successfully target these TAs on GBM cells in *in vitro* culture assays and in orthotopic *in vivo* models (Han et al., 2015). Notably, strategies to improve CAR NK cell antitumor activity have also been assessed including additional co-expression of the chemokine receptor CXCR4 to promote homing to the tumor site (Müller et al., 2015) and dual EGFR and EGFRvIII targeting CAR NK cells (Genßler et al., 2016). Both of these strategies demonstrated improved tumor control in NSG mice harboring orthotopic GBM xenografts. HER2-specific CAR NK cells have displayed antitumor activity against GBM cells *in vitro* and in a GBM xenograft mouse model (Zhang et al., 2016).

This convincing preclinical data has led to implementation of the CAR2BRAIN phase I clinical trial (NCT03383978) treating recurrent or refractory HER2-positive GBM patients with HER2-specific CAR NK cells derived from the NK-92 cell line (Burger et al., 2016). As murine models indicate that intravenously administered NK-92 cell line derived CAR NK cells cannot cross the BBB without ultrasound disruption (Alkins et al., 2013), intracranial injection of cells into the wall of the resection cavity has been selected as the route of administration. No results are available yet. Several other clinical trials using NK-92 derived CAR NK cells are ongoing in China to treat various cancer types, demonstrating the feasibility and overall safety of this approach (Tang et al., 2018; Zhang et al., 2019). A major limitation of using NK cell lines compared to primary effector cells, however, is the need to irradiate them prior to infusion in order to abrogate the possibility of secondary malignancy formation. As a result, NK cells cannot engraft or expand *in vivo* and therefore have a limited activity timespan. The results of clinical trials are needed to assess the efficacy of CAR NK cell treatment of GBM.

## Universal CAR T Cells

The future of CAR T cell engineering lies in overcoming reliance on selective TAs and allowing them the flexibility to target several TAs, and the capacity to be turned on or off in a timely and effective manner to avoid toxicity. Several mechanisms to reach this goal are in development. T cells with CARs specific for antibodies such as FITC-tagged mAbs can effectively target cancer cells *in vitro* and *in vivo* using FITC-conjugated mAbs, but display minimal functionality in the presence of unlabeled mAbs (Tamada et al., 2012). Similarly, T cell constructs expressing CD16 (CD16-CR T cells) and CD32 (CD32-CR T cells) to target cancer cells through the addition of TA-specific mAbs and induction of antibody dependent cellular cytotoxicity have been explored (Caratelli et al., 2017). More recently, split universal and programmable (SUPRA) engineered CAR T cells, which link an intracellular signaling domain to a leucine zipper extracellular domain have been generated. Following addition of zipFv adaptor molecules containing a leucine zipper that will bind to the extracellular domain of the CAR, and a ligand binding domain specific for a selected TA, the CAR T cell is then able to recognize and eliminate target cells (Cho et al., 2018).

The advantages of this type of CAR T cells are the broad cancer applicability, capacity to overcome TA loss and ability to regulate functional effects. They therefore are attractive candidates to be tested in GBM.

# COMBINATORIAL THERAPY TO ENHANCE CAR T CELL EFFICACY

Whilst enhancement of a CAR construct can improve the antitumor activity of the CAR effector cell it may not be sufficient to overcome the limitations of the immunosuppressive TME.

Combining CAR T cells with other therapeutic strategies can produce an additive or a synergistic effect enabling functional CAR T cells to recognize and eliminate cancer cells which they otherwise were unable to. Whilst there are many therapeutic strategies being investigated in combination with CAR T cells such as epigenetic drugs and kinase inhibitors against various cancer types, here we review those currently being explored in GBM.

## Combination With Chemo-/Radio-Therapy

As standard therapeutic options for GBM, chemotherapy and radiotherapy are theoretically easily applicable therapies that could be used in combinatorial strategies with CAR T cells. It is thought that both chemotherapeutic agents and radiotherapy may sensitize cancer cells to elimination by CAR T cells through several mechanisms.

Radiotherapy can induce changes in the TME that may help boost CAR T cell efficacy (Flynn et al., 2017; Minn et al., 2019). Firstly, radiation can upregulate the expression of many types of TAs on malignant cells. This phenotypic change can result in enhanced malignant cell recognition and elimination by cognate cytotoxic $CD8^+$ T cells (Reits et al., 2006; Zhang et al., 2007; Jin et al., 2018). Secondly, radiation can increase infiltration of a tumor by immune cells because of the release of proinflammatory cytokines such as IFN-γ (Lugade et al., 2008) as well as chemokine ligands which can recruit T cells to the TME (Lugade et al., 2008; Matsumura et al., 2008). Furthermore, radiation may structurally alter the TME, disrupting the established vasculature as well as increasing the permeability of the BBB (Portella and Scala, 2019; Arvanitis et al., 2020). Radiation also induces tumor necrosis and apoptosis which can trigger the release of danger signals. The latter in turn can induce type I IFN production and increase the maturation and activation of DCs which can improve TA presentation and lead to a more effective endogenous immune response (Burnette et al., 2011; Crouse et al., 2015). This endogenous immune response can contribute to an abscopal effect which has been reported following radiotherapy and may act synergistically with the CAR T cell therapy (Demaria et al., 2004). Combination with radiotherapy has been found to improve efficacy of CAR T cell therapy in models of GBM as well as some other solid tumors (DeSelm et al., 2018; Jin et al., 2018; Weiss et al., 2018b). Jin et al. (2018) reported that CD70 expression is upregulated on GBM cells following irradiation and that this enhanced CD70-specific CAR T cell mediated tumor cell elimination. Similarly Weiss et al. (2018b) reported increased immune cell infiltration and activity of CAR T cells when combining radiotherapy and NKG2D CAR T cells in murine GBM models.

Chemotherapy provides similar mechanistic changes to the TME as radiotherapy which may enhance CAR T cell efficacy including TA upregulation (Zhang et al., 2007) and immunosuppressive cell elimination (Lutsiak et al., 2005). Furthermore conditioning chemotherapy enhances the persistence and expansion of adoptively transferred T cells, improving the efficacy of CAR T cells (Muranski et al., 2006; Xu J. et al., 2018). Therefore, optimization of the most suitable way to combine chemotherapy and CAR T cells is being explored in several of the current GBM clinical trials.

## Combination With Immune Checkpoint Blockade

Perhaps the most promising combinatorial strategy to be investigated is the synergistic application of CAR T cells with immune checkpoint blockade (ICB) therapy. These drugs block the inhibitory signaling pathways utilized by tumor cells to dampen immune effector cell activity (Korman et al., 2006; Sharma and Allison, 2015). Their use as a monotherapy has shown significant clinical benefit in a proportion of patients with malignant melanoma, lung cancer and renal cell cancer (Topalian et al., 2019), however, results from early trials in GBM patients have not shown significant survival benefit (Romani et al., 2018; Desai et al., 2019). The use of ICB as a monotherapy is limited by the need of a cognate T cell response and presentation of neoantigens by HLA class I antigens on cancer cells. The addition of CAR T cells specific for the tumor can overcome these requirements. Checkpoint inhibitors targeting PD-1/PD-L1 and CTLA4 pathways have been shown to increase the activity of CAR T cells in preclinical models of GBM (Shen et al., 2020). Currently the clinical trial NCT04003649 is investigating whether IL13Ra2 CAR T cells work better alone or in combination with nivolumab (anti-PD-1) and ipilimumab (anti-CTLA4) to treat recurrent and refractory GBM. Similarly, clinical trial NCT03726515 is exploring the combination of EGFRvIII CAR T cells with pembrolizumab, an anti-PD-1 mAb.

## Combination With Oncolytic Viruses

Oncolytic viruses can stimulate immunogenic cell death of cancer cells, production of a type I IFN response in the TME and consequently induction of systemic innate and tumor-specific adaptive immune responses that can promote T-cell trafficking and effector function (Kaufman et al., 2015). Type I IFNs induce clonal expansion, differentiation, development of the cytolytic function, and production of IFN-gamma by CD8 T cells (Curtsinger et al., 2005). In addition, local IFNβ has been shown to disrupt tumor microvasculature (Spaapen et al., 2014), inhibit Treg activation and proliferation (Srivastava et al., 2014), and promote the activity of DCs in TME (Diamond et al., 2011). Therefore, this modulation of the TME is expected to enhance CAR T cell activity (Ajina and Maher, 2017), Huang et al., generated an IL-7-loaded oncolytic adenovirus (oAD-IL7) and used it in combination with B7-H3-specific CAR T cells for the treatment of mice orthotopically grafted with GBM cells (Huang et al., 2021). They demonstrated that the combination of oAD-IL7 and CAR T cells resulted in enhanced T cell proliferation and reduced T cell apoptosis *in vitro*, and prolonged survival, and reduced tumor burden *in vivo*.

## Combination With Small Molecule Inhibitors

There are many pharmacological agents already in clinical use for various diseases which are known to modulate the TME

or have effects on GBM cells. As cancer therapeutics, small-molecule inhibitors are designed to block signaling pathways to inhibit tumor growth, survival, angiogenesis, and metastasis. Tyrosine kinase inhibitors (TKIs) have previously been assessed as monotherapies in clinical trials with GBM patients, however, with limited clinical effect (Kim and Ko, 2020). Combination of TKIs with CAR T cells have shown synergistic effects in the treatment of other types of solid cancer (Wu et al., 2019; Huizhong et al., 2020) and therefore are a viable candidate for assessment in GBM. LB-100 is a small molecule inhibitor of protein phosphatase 2A (PP2A), a molecule involved in cell-cell adhesion. Combination with LB-100 can enhance CAIX-specific CAR T cell treatment efficacy both *in vitro* and in *in vivo* models of GBM (Cui et al., 2020).

## CONCLUSION

CAR T cells are a highly promising therapy for GBM with many potential target TAs identified. Selection of a suitable TA, especially one which is expressed on and will lead to the elimination of GBM CICs, besides that of differentiated GBM cells, but that will allow preservation of normal brain tissue, represents one of the current major challenges in the field. The ability of TA-specific CAR T cells to recognize and eliminate differentiated GBM cells and GBM CICs in preclinical models has been well established. These encouraging results led to the implementation of several clinical trials in patients with advanced GBM (**Tables 2**, **3**).

However, there is still a long way ahead for CAR T cell therapy before it becomes a standard of care for the treatment of patients with GBM. Most clinical trials have proven that CAR T cells as a monotherapy are not particularly effective in solid tumors due to numerous immune escape mechanisms utilized by cancer cells (Lim and June, 2017). So far this appears to also be true for GBM, which additionally presents its own unique challenges to overcome. Among them, optimization of CAR T cell delivery into the brain is an important obstacle to overcome.

Many of the described immune escape mechanisms are not restricted to CAR T cell-based therapy but appear to have a negative impact on many types of immunotherapy. These require the development of combinational strategies to improve the efficacy of immunotherapies and in addition to stimulate the endogenous immune system. As we have described there are many potentially targetable GBM TAs and many CAR-based therapeutic strategies, but it is difficult to decide which strategies are likely to be the most effective. Therefore, the field would benefit from studies which compare the efficacy and associated side effects of each described strategy to determine which one(s) is (are) most likely to translate into significant clinical benefit. Generation of more relevant *in vitro* and *in vivo* models of GBM will help to accelerate and optimize development of CAR T cell treatments (Jacob et al., 2020; Maggs and Ferrone, 2020). For example, patient derived GBM organoids provide a far greater replication of an *in situ* tumor and have been used to model EGFRvIII-specific CAR T cell effects (Jacob et al., 2020). Therefore, whilst there is great promise in the use of CAR T cells to treat GBM, further investigation is needed to optimize the efficacy of this novel therapeutic strategy.

## AUTHOR CONTRIBUTIONS

LM designed the review, collected the data, analyzed and interpreted the data, and wrote and finalized the manuscript. GC and AD collected the data, analysed and interpreted the data, and wrote the manuscript. ASM collected the data, analysed and interpreted the data, and prepared **Figure 1**. SF designed the review and wrote and finalized the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by NIH grants R01DE028172, R03CA239193, R03CA231766, R03CA216114, and R03CA223886 and by DOD grant W81XWH-16-1-0500.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fphar.2021.719313

# Notoginsenoside R1 Reverses Abnormal Autophagy in Hippocampal Neurons of Mice With Sleep Deprivation Through Melatonin Receptor 1A

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**Specialty section:**
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

**Received:** 02 June 2021
**Accepted:** 09 August 2021
**Published:** 16 September 2021

**Citation:**
Cao Y, Li Q, Zhou A, Ke Z, Chen S, Li M, Gong Z, Wang Z and Wu X (2021) Notoginsenoside R1 Reverses Abnormal Autophagy in Hippocampal Neurons of Mice With Sleep Deprivation Through Melatonin Receptor 1A. Front. Pharmacol. 12:719313. doi: 10.3389/fphar.2021.719313

Sleep deprivation (SD) may cause serious neural injury in the central nervous system, leading to impairment of learning and memory. Melatonin receptor 1A (MTNR1A) plays an important role in the sleep regulation upon activation by melatonin. The present study aimed to investigate if notoginsenoside R1 (NGR1), an active compound isolated from *Panax notoginseng*, could alleviate neural injury, thus improve impaired learning and memory of SD mice, as well as to explore its underlying action mechanism through modulating MTNR1A. Our results showed that NGR1 administration improved the impaired learning and memory of SD mice. NGR1 prevented the morphological damage and the accumulation of autophagosomes in the hippocampus of SD mice. At the molecular level, NGR1 reversed the expressions of proteins involved in autophagy and apoptosis, such as beclin-1, LC3B, p62, Bcl-2, Bax, and cleaved-caspase 3. Furthermore, the effect of NGR1 was found to be closely related with the MTNR1A-mediated PI3K/Akt/mTOR signaling pathway. On HT-22 cells induced by autophagy inducer rapamycin, NGR1 markedly attenuated excessive autophagy and apoptosis, and the alleviative effect was abolished by the MTNR1A inhibitor. Taken together, NGR1 was shown to alleviate the impaired learning and memory of SD mice, and its function might be exerted through reduction of excessive autophagy and apoptosis of hippocampal neurons by regulating the MTNR1A-mediated PI3K/Akt/mTOR signaling pathway.

**Keywords: sleep deprivation, autophagy, melatonin receptor 1A, learning and memory, notoginsenoside R1**

## INTRODUCTION

Sleep is an essential and basic physiological activity for optimal body performance and health (Chen et al., 2020). Lack of sleep or sleep deprivation (SD) can lead to significant impairment of cardiovascular, immune, and endocrine health, as well as the central nervous system (Xie et al., 2013). Hippocampus plays a critical role in the modulation of memory, navigation, and cognition (Girardeau et al., 2017), and SD has been revealed to impair cognition, accompanied with ultrastructure damage and pyramidal neuron loss in the hippocampus (Xie et al., 2021).

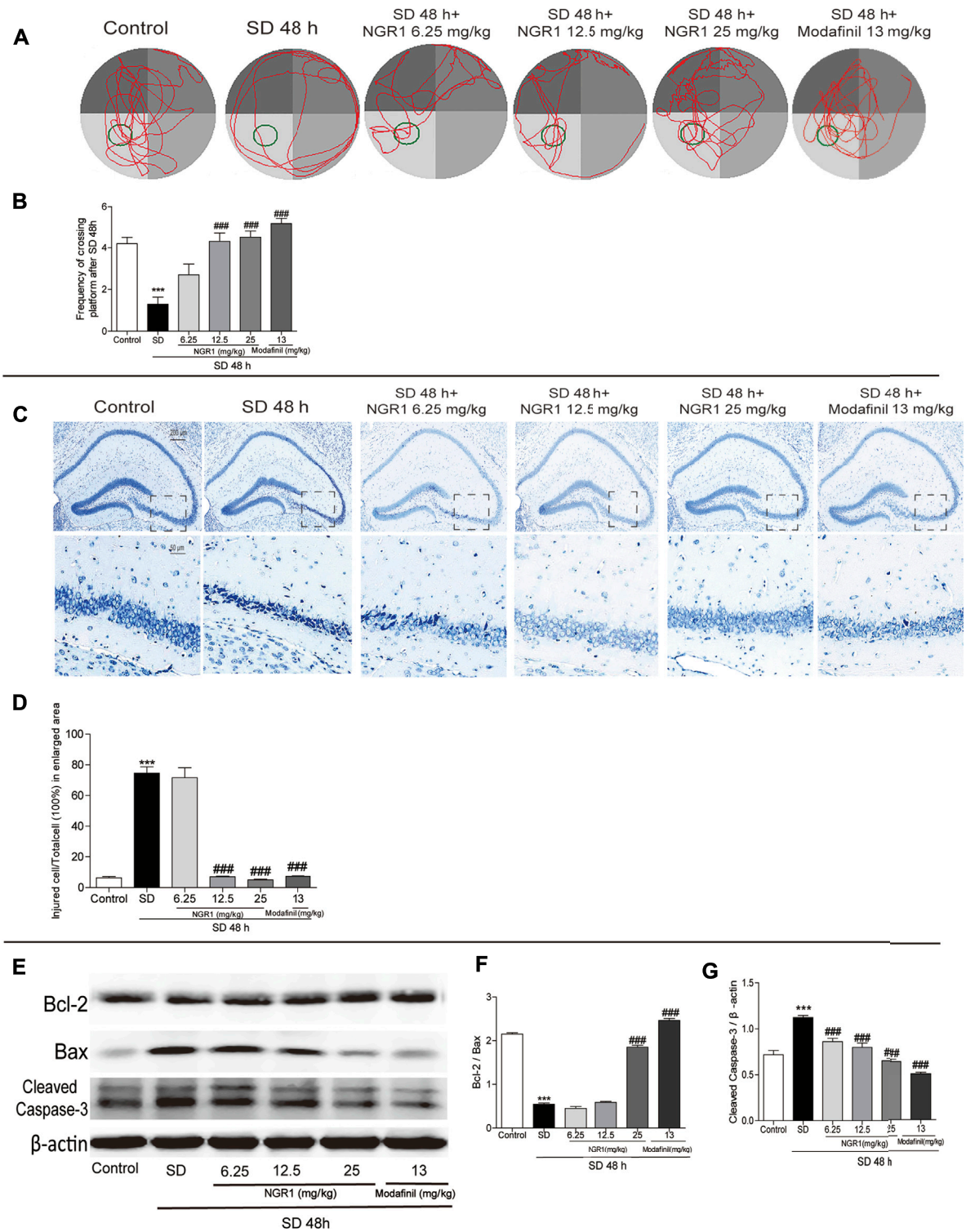


**FIGURE 1 |** NGR1 counteracted the impairment of learning and memory of SD mice. **(A)** Movement tracks of mice in the Morris water maze. **(B)** Comparison of the frequency of mice passing through the target quadrant. **(C)** Nissl staining of hippocampal regions. The enlarged images displayed hippocampal CA3 regions. Scale bar: 200 and 50 μm **(D)** Comparison of the percentage of injured cells in CA3 regions. n = 3/group. **(E)** Hippocampal expressions of proteins involved in apoptosis. **(F–G)** Gray intensity comparison of Bcl-2/Bax and cleaved caspase-3. n = 5/group. ***$p < 0.001$ vs. control group. ###$p < 0.001$ vs. SD group. (NGR1: notoginsenoside R1; SD: sleep deprivation).

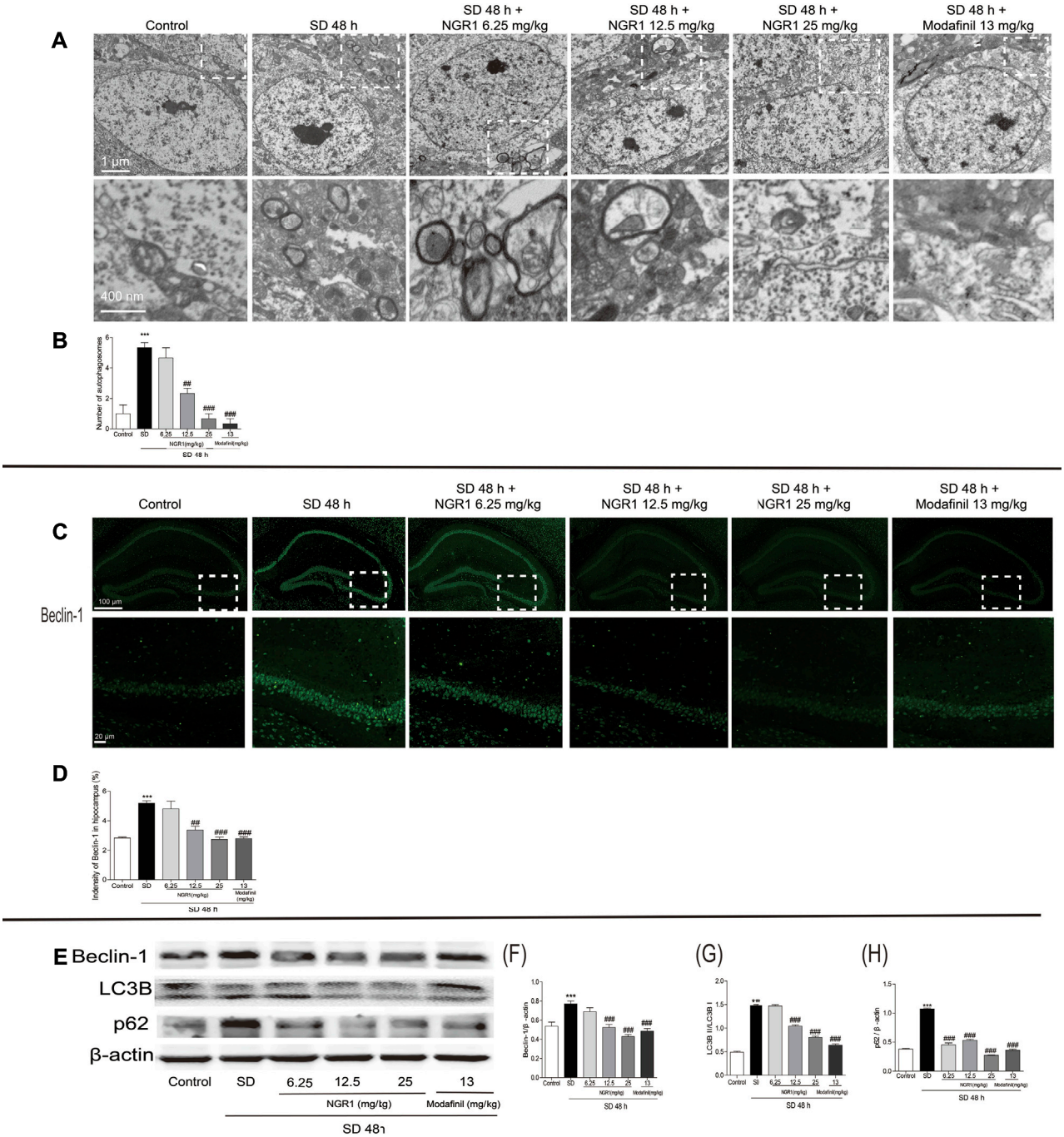


**FIGURE 2 |** NGR1 suppressed the excessive autophagy in the hippocampus of SD mice. **(A)** Autophagosomes in hippocampi examined by transmission electron microscopy. The autophagosomes were denoted by arrows. Scale bar: 1 μm and 400 nm. **(B)** Comparison of the number of autophagosomes. n = 3/group. **(C)** Immunostaining of beclin-1 in the hippocampus. Scale bar: 100 and 20 μm. **(D)** Comparison of beclin-1 immunofluorescence intensity. n = 3/group. **(E)** Hippocampal expressions of proteins involved in autophagy. **(F–H)** Gray intensity comparison of beclin-1, LC3BII/I, and p62. n = 5/group. (NGR1: notoginsenoside R1; SD: sleep deprivation; p62, and ubiquitin-binding protein p62 or sequestosome-1).

Autophagy is a highly conserved cellular catabolism process that acts within cells to achieve substance equilibrium by recycling or degrading proteins and destroying organelles in lysosomal compartments (Lv et al., 2020). It is necessary for maintaining normal neuronal function and counteracting the abnormal changes of neurodegeneration, but dysfunctional autophagy destroys the homeostasis of neuronal cells and makes individuals prone to neurodegenerative or neuropsychiatric disorders (Hylin et al., 2018). When the autophagy process is regulated properly, it promotes cell

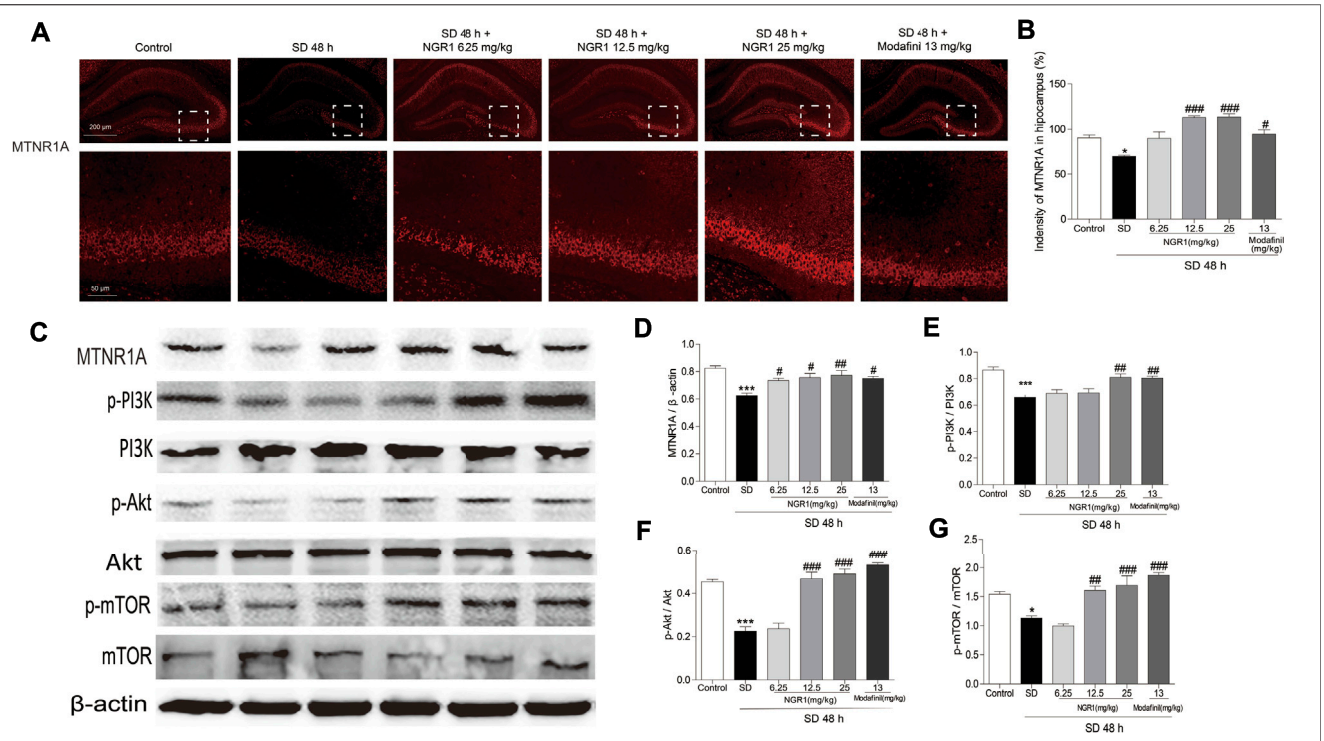


**FIGURE 3 |** NGR1 resumed the decreased MTNR1A in the hippocampus induced by SD. **(A)** Immunostaining of MTNR1A in the hippocampus. Scale bar: 200 and 50 μm. **(B)** Comparison of immunofluorescence intensity of MTNR1A in the hippocampus. n = 3/group. **(C)** Protein expression of MTNR1A in hippocampi. n = 5/group. **(D–G)** Protein expressions of the phosphorylated PI3K, Akt, and mTOR in hippocampi. n = 5/group. *$p < 0.05$; ***$p < 0.001$ vs. control group. ##$p < 0.01$, ###$p < 0.001$ vs. SD group. (NGR1: notoginsenoside R1; MTNR1A: melatonin receptors 1A; SD: sleep deprivation; mTOR, mammalian target of rapamycin; p-Akt, phosphorylated PKB; p-PI3K, phosphorylated PI3K).

survival, while dysregulated excessive autophagy leads to autophagic cell death (Yang et al., 2012). Our previous study found that abnormal autophagy activity in hippocampal neurons caused by SD is related to the impaired cognition; and when the excessive autophagy was suppressed through the PI3K/Akt/mTOR signaling pathway, the neuronal apoptosis in the hippocampus was relieved, implicating an important role of neuronal autophagy in SD-induced neurodegeneration (Cao et al., 2019; Cao et al., 2020).

Melatonin, known as the "dark hormone" in mammals, is mainly secreted by the pineal gland, exhibiting characteristic patterns of daily and seasonal secretion and playing an important role in sleep regulation (Tosches et al., 2014; Hu et al., 2016). It has potential therapeutic effect on neurodegenerative diseases (Pandi-Perumal et al., 2013). Recent studies have reported that melatonin can regulate autophagy through the PI3K/Akt/mTOR pathway (Pandi-Perumal et al., 2013). Melatonin reduction was found to be induced by SD (Pandi-Perumal et al., 2013), while compensatory melatonin can relieve the nerve injury under SD (Lan et al., 2001). Melatonin receptor 1A (MTNR1A) is one of the receptors for melatonin to exerts it neuroprotective function in sleep disorders (Lan et al., 2001).

Notoginsenoside R1 (NGR1) is a saponin extracted from P*anax notoginseng*, which possesses numerous beneficial properties, including antioxidant (He et al., 2012), anti-inflammatory (Son et al., 2009), cardioprotective (Ge et al., 2016), and neuroprotective (Meng et al., 2014) effects. It can increase neuronal excitability and ameliorate synaptic and memory dysfunction induced by amyloid elevation (Yan et al., 2014), and NGR1 exhibits neuroprotection by inhibiting neuronal apoptosis and promoting cell survival *via* PI3K-Akt-mTOR/JNK signaling pathways in neonatal cerebral hypoxic-ischemic brain injury (Tu et al., 2018). However, whether NGR1 can improve neuronal injury induced by SD has not been elucidated. Here, we explored the neuroprotective effects of NGR1 on mice induced by SD and tried to uncover its underlying molecular mechanism from the aspect of alleviating excessive autophagy and apoptosis through the MTNR1A-mediated signaling pathway.

## MATERIALS AND METHODS

### Reagents

Notoginsenoside R1 (purity > 98%), rapamycin, and 3-methyladenine (3-MA) were bought from Dalian Meilun Biotechnology Co. Ltd. (Dalian, Liaoning, China). Selective MTR1A inhibitor was provided by Master of Small Molecules (MCE, Cat No: S26131). Antibodies against beclin-1 (A7353), LC3B (A19665), phosphoinositide 3-kinase (phospho-PI3K, AP0854), phospho-Akt (AP0098), PI3K p85 (A11402), Akt (A18120), Bax (A19684), cleaved caspase-3 (A19654), p62 (A11250), mTOR (A2445), MTNR1A (A13030), and β-actin

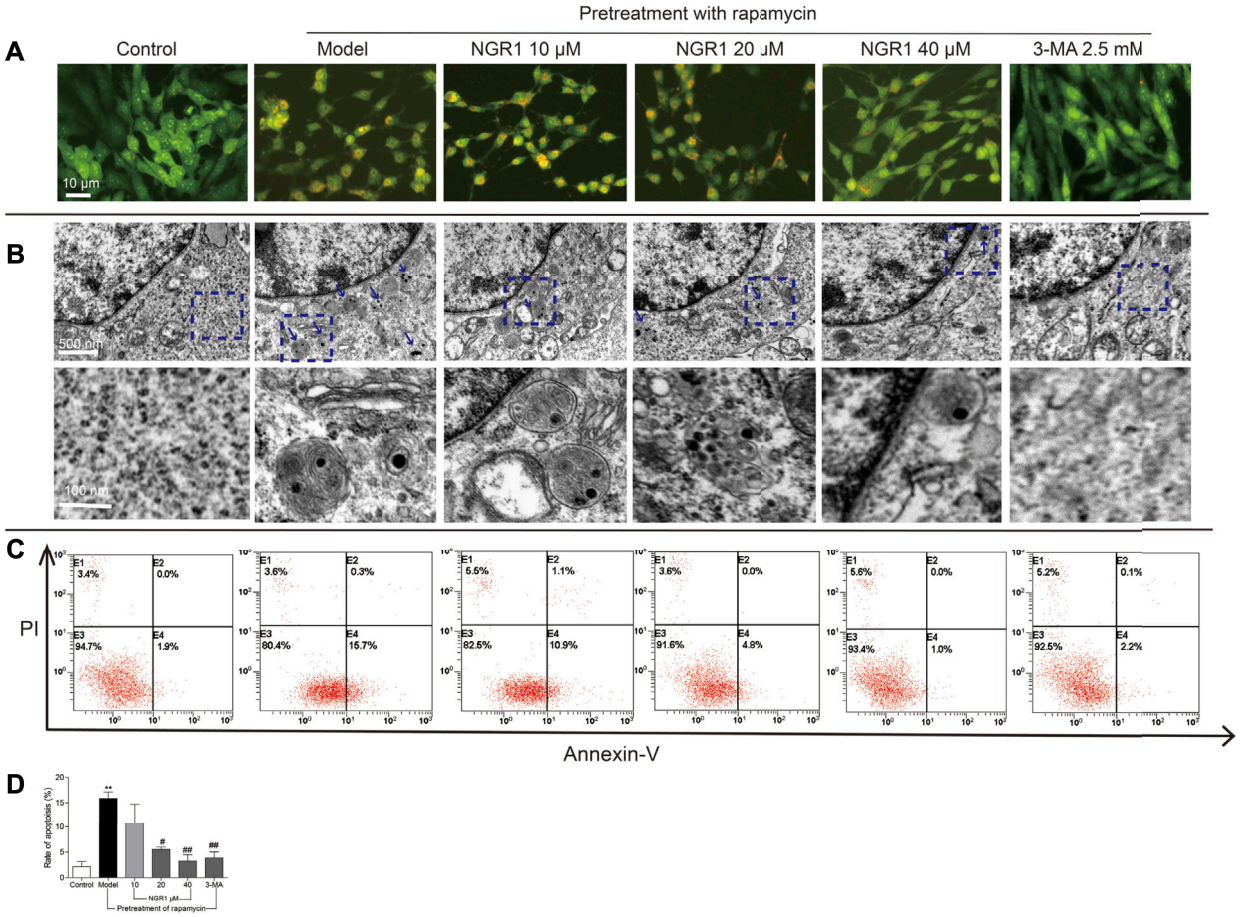


**FIGURE 4 |** NGR1 reduced autophagy and apoptosis of HT-22 cells induced by rapamycin. **(A)** Acidic vesicular organelles stained by acridine orange. Scale bar: 10 μm **(B)** Autophagosomes in cells examined by transmission electron microscopy. The autophagosomes were denoted by arrows. Scale bar: 500 and 100 nm. **(C)** and **(D)** Apoptosis examined by flow cytometry after annexin V/PI staining. n = 3/group. $^{**}p < 0.01$ vs. control group. $\#p < 0.05$, $\#\#p < 0.01$ vs. model group. (NGR1: notoginsenoside R1; 3-MA, 3-methyladenine).

(AC026) were purchased from ABclonal Biotechnology Co. Ltd. (Wuhan, Hubei, China). Bcl-2 antibody (abs131701) was bought from Apicent Biological Technology Co. Ltd. (Shanghai, China). p-mTOR antibody (5536S) was obtained from Cell Signaling Technology (Danvers, MA, United States).

## Animals and Treatment

Sixty male C57BL/6 mice (aged 6 weeks, weighing 18–22 g) were equally and randomly divided into six groups: control group, SD group, SD + modafinil (13 mg/kg) group, and SD + NGR1 groups (6.25 mg/kg, 12.5 mg/kg, and 25 mg/kg). Mice in each group, except the control group, were given respective drugs by gavage for 9 days after adaptive feeding for a week. Except for the control group, the mice in each group were sleep-deprived for 48 h on the eighth and ninth days according to a modified multiple platform method, as reported previously (Tu et al., 2018). All the animals were offered by Animal Research Center of Anhui University of Traditional Chinese Medicine and complied with a protocol approved by the Experimental Animal Ethics of Anhui University of Traditional Chinese Medicine with an approval number (AHUCM-mouse-2020041).

## Morris Water Maze Test

The learning and memory ability of mice was examined by the Morris water maze test. The procedure included adaptive training, visible training, hidden platform training, and space exploring, as reported previously (Suchecki and Tufik, 2000). Before space exploration, the mice, except those in the control group, were sleep-deprived for 48 h using a modified multiple platform method. After the behavioral test, the mice were euthanized with overdose of 1.5% pentobarbital sodium.

## Histopathological Analysis and Immunohistochemistry

Brain tissues of mice were dissected and fixed in 4% of paraformaldehyde solution for 24 h. For immunohistochemistry, the tissues were washed with PBS and soaked in 10 and 30% sucrose solution, respectively, for 24 h. Afterward, the tissues were embedded, frozen, and cut into 20-μm sections. After the infiltration and blocking for 30 min, the sections were incubated with a primary antibody against LC3B overnight at 4°C, followed by the incubation with a

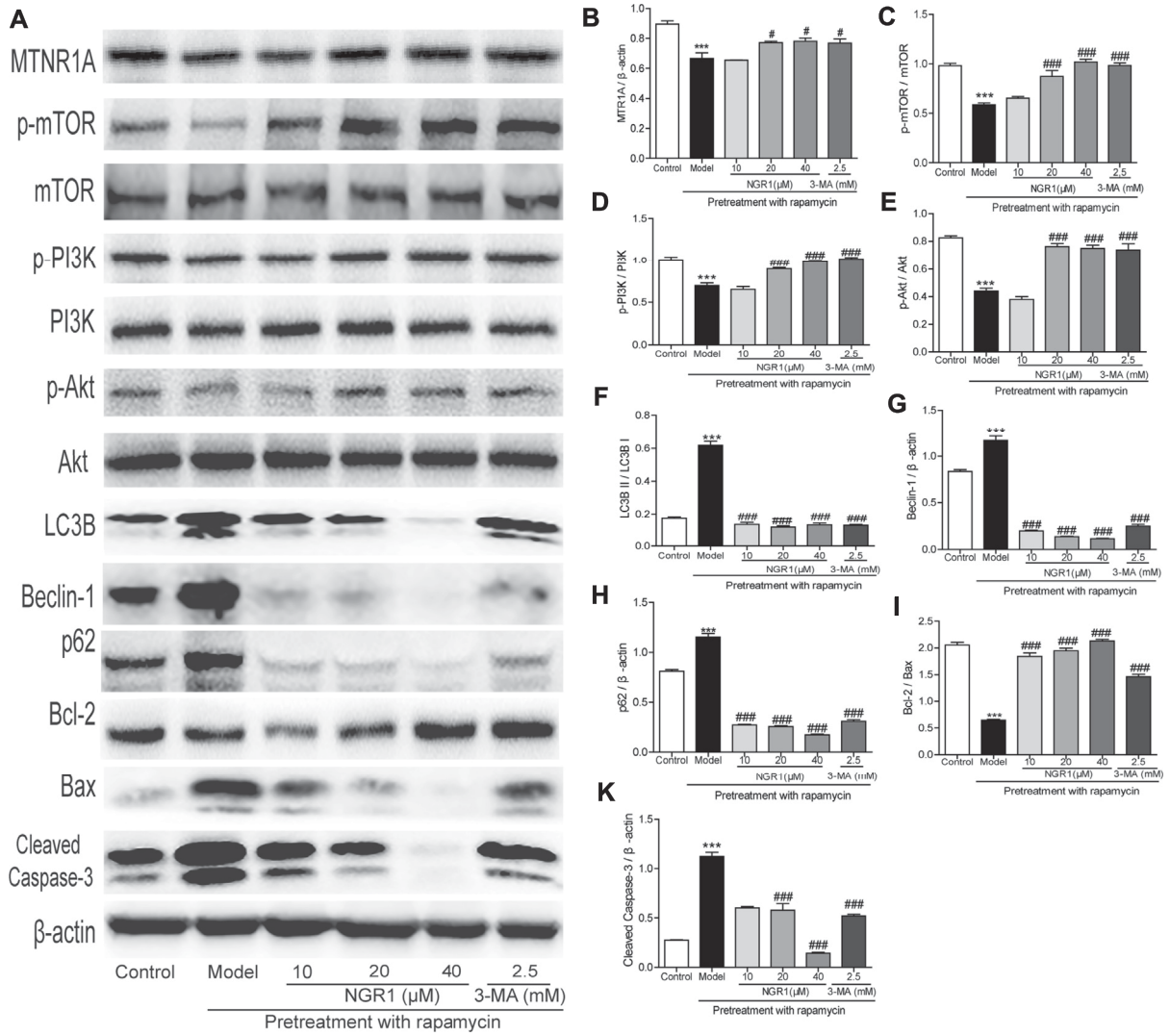


**FIGURE 5 |** NGR1 regulated the expressions of proteins involved in autophagy and apoptosis in HT-22 cells induced by rapamycin. **(A)** Protein bands. **(B)–(K)** Gray intensity analysis of the protein bands. n = 5/group. ***$p < 0.001$ vs. control group. #$p < 0.05$, ###$p < 0.001$ vs. model group. (NGR1: notoginsenoside R1; MTNR1A: melatonin receptors 1A; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; p62, ubiquitin-binding protein p62 or sequestosome-1; p-Akt, phosphorylated PKB; p-PI3K, phosphorylated PI3K).

secondary antibody conjugated with Alexa Fluor 488. Fluorescent images were taken using the Olympus VS120 virtual slide scanner.

## Transmission Electron Microscopy

Following perfusion with phosphate-buffered saline (PBS) and fixation with 2% glutaraldehyde, the hippocampal tissues were cut into ultrathin sections. After dehydration, the sections were stained with uranyl acetate and lead citrate. The ultrastructure images were taken under an HT-7700 transmission electron microscope (Hitachi, Tokyo, Japan).

## Cell Culture and Treatment

Mouse HT-22 cell line was obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM basic medium, supplied with 10% fetal bovine serum and 1% penicillin/streptomycin. Prior to the drug treatment, the cells were seeded at a density of $5 \times 10^5$ cells/ml in cell culture flasks or 24-well plates and cultured overnight. After pretreated with rapamycin (50 μg/ml) for 4 h, the cells were incubated with a complete medium: 3-MA (2.5 mM) or NGR1 at concentrations of 10, 20, and 40 μM for 48 h. Then the cells were lysed in lysis buffer with protease and phosphatase inhibitors for further Western blotting analysis.

## Acridine Orange Staining

HT-22 cells were cultured in 24-well plates and induced with rapamycin and treated with 3-MA or NGR1, as mentioned before. The medium was discarded, and the cells were rinsed with PBS. Afterward, the cells were incubated with acridine orange staining solution for 0.5 h at room temperature. The

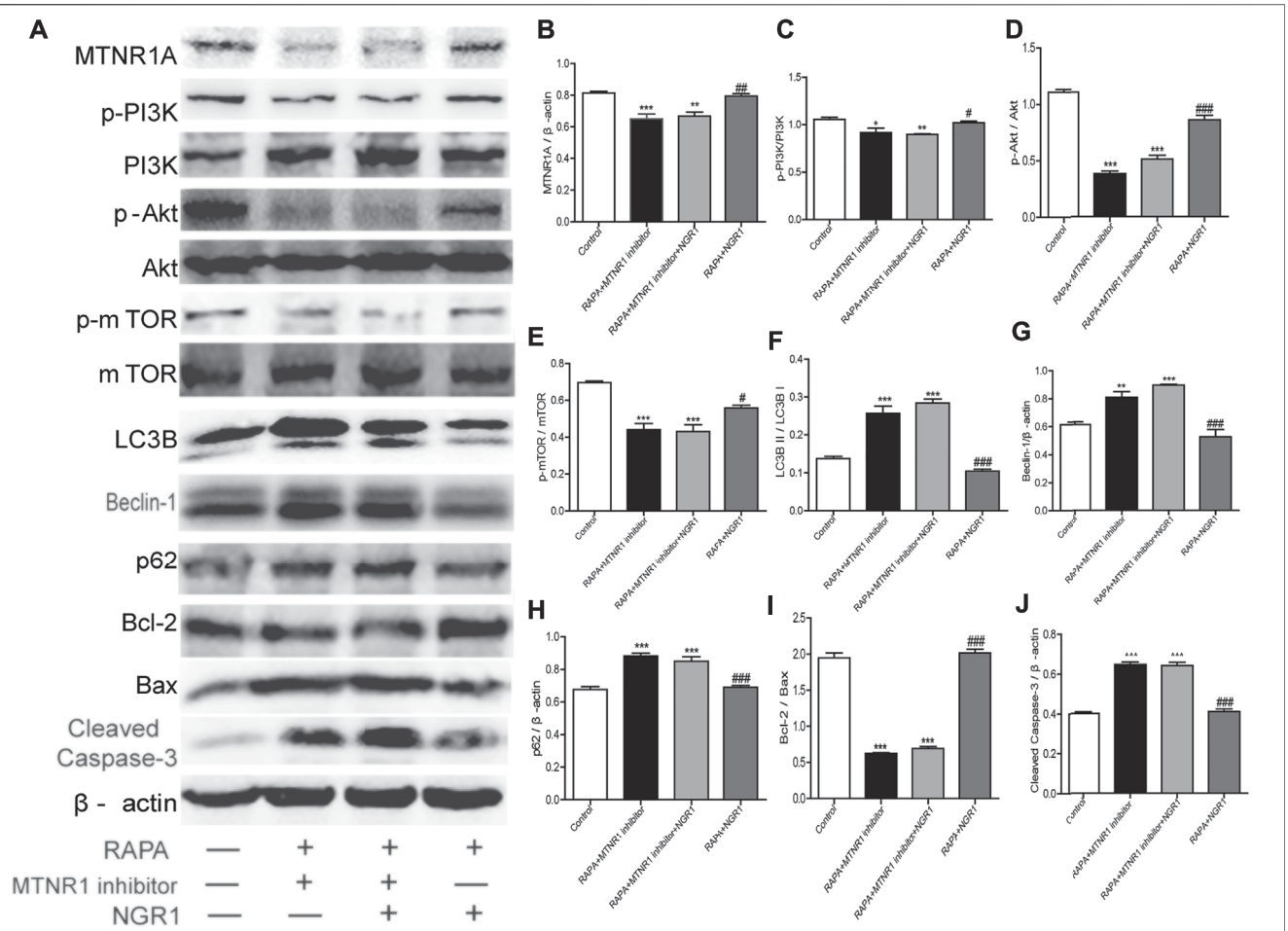


**FIGURE 6 |** MTNR1A inhibitor abolished the alleviative effects of NGR1 on the expressions of proteins involved in autophagy and apoptosis in HT-22 cells induced by rapamycin. After being pretreated with rapamycin (50 μg/ml) for 4 h, the cells were exposed to NGR1 (20 μM) or NGR1 (20 μM) and the MTNR1 inhibitor (10 μM) for 48 h. **(A)** Protein bands. **(B)**–**(J)** Gray intensity analysis of the protein bands. n = ?/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. RAPA + MTNR1 inhibitor + NGR1 group. (NGR1: notoginsenoside R1; MTNR1: melatonin receptors 1; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; p62, ubiquitin-binding protein p62 or sequestosome-1; p-Akt, phosphorylated PKB; p-PI3K, phosphorylated PI3K).

fluorescent images were taken under a fluorescence microscope. Generally, the normal cells were stained green uniformly, while the autophagic cells showed an orange cytoplasm.

## Flow Cytometry

HT-22 cells were induced with rapamycin and treated with 3-MA or NGR1, as described before. Then, the cells were collected with trypsin without EDTA. After washing twice with PBS, the cells were incubated with propidium iodide (PI, 2.5 μg/ml) and annexin V (2 μg/ml) for 10 min. Flow cytometry was carried out on a flow cytometer (Guava easy Cyte HT, Millipore, Germany).

## Western Blotting Analysis

Twenty microgram proteins from each sample were separated on 12% gel and transferred onto PVDF membranes. After blocking with nonfat milk solution (5%) for 1 h, the membranes were incubated with respective primary antibodies overnight at 4°C. Thereafter, they were washed with PBST and incubated with secondary antibodies for 1 h at room temperature. The protein bands were observed by an ECL Prime kit and quantified with ImageJ 1.46r software.

# STATISTICAL ANALYSIS

All the data were presented as mean ± standard error of the mean. One-way analysis of variance (ANOVA) with Dunnett post hoc analysis was performed to analyze the differences among the groups using GraphPad Prism 5.0. A $p$ value < 0.05 was regarded as statistically significant.

# RESULTS

## NGR1 Counteracted the Impairment of Learning and Memory of SD Mice

As shown in **Figures 1A,B**, mice sleep-deprived for 48 h visited the target quadrant (hidden platform) less than the control ones

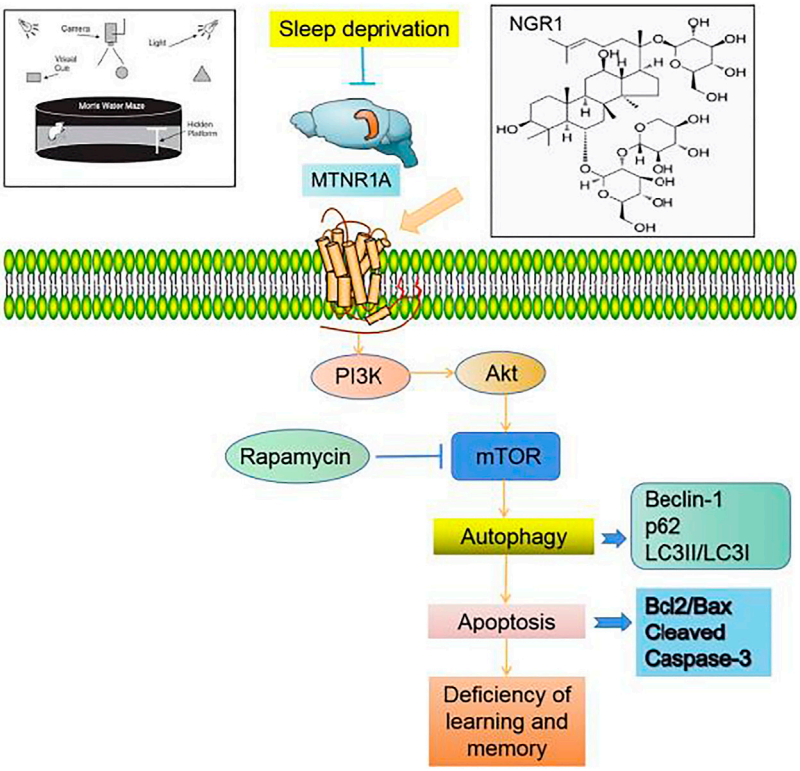


**FIGURE 7 |** Notoginsenoside R1 reverses abnormal autophagy in hippocampal neuron of mice with sleep deprivation through melatonin receptor 1A.

($p < 0.001$). By contrast, after NGR1 treatment, especially at 12.5 mg/kg and 25 mg/kg, the SD mice displayed significantly increased frequency to explore the target quadrant compared with the model group mice ($p < 0.001$). Mice in the modafinil-treated group also showed significantly improved learning and memory capacity ($p < 0.001$). Accordingly, the morphology of the pyramidal cells in CA3 region of the hippocampus of SD group mice changed dramatically. As shown in **Figure 1C**, the cells were shrunken to an irregular shape and stained in deep blue color. NGR1 and modafinil pretreatment significantly prevented the morphological changes of the pyramidal cells induced by SD (**Figure 1D**, $p < 0.001$). Moreover, SD caused the decrease in Bcl-2 but increase in Bax and cleaved caspase-3 in the hippocampus of mice (**Figures 1E–G**). NGR1, particularly at 25 mg/kg, and modafinil counteracted the effect of SD on the ratio of Bcl2/Bax and the expression of cleaved caspase-3 ($p < 0.001$). These results demonstrated that NGR1 pretreatment could alleviate the impairment of learning and memory of SD mice by reducing the neuronal injury.

## NGR1 Decreased the Excessive Neuronal Autophagy in the Hippocampus of SD Mice

As shown in **Figure 2A**, in hippocampal CA3 region of SD mice, the number of autophagosomes increased significantly ($p < 0.001$) compared with the control group. When pretreated with NGR1 (12.5 mg/kg and 25 mg/kg) and modafinil, the numbers were reduced markedly ($p < 0.01$ or $p < 0.001$). In agreement with the changes of autophagosomes, after NGR1 pretreatment, the immunofluorescence intensity of beclin-1 decreased, especially at 12.5 25 mg/kg and 25 mg/kg groups (**Figures 2C,D**, $p < 0.01$ or $p < 0.001$). Furthermore, the protein expressions of beclin-1 and p62 as well as the ratio of LC3BII to LC3BI were all increased in the hippocampus of SD mice, which could be reversed by NGR1 and modafinil pretreatment (**Figures 2E–H**). These results demonstrated that NGR1 could reduce autophagy of hippocampal neurons in SD mice.

## NGR1 Increased the Expression of MTNR1A and Enhanced Its Downstream Signaling in the Hippocampus of SD Mice

As shown in **Figures 3A,B**, the immunofluorescence intensity of MTNR1A was reduced significantly in the hippocampus of SD mice ($p < 0.05$). However, after NGR1 pretreatment (12.5 mg/kg and 25 mg/kg), the immunofluorescence intensity of MTNR1A was increased ($p < 0.001$). Consistently, as revealed in **Figure 3C**, the protein expression of MTNR1A was mitigated in the hippocampus of SD mice. Meanwhile, the phosphorylation of the downstream signaling molecules, such as PI3K, AKT, and mTOR, was also reduced significantly, compared with the control mice ($p < 0.05$ or $p < 0.001$). After NGR1 pretreatment, the changes in the expression of MTNR1A and its downstream signaling pathway molecules induced by SD were abrogated. These results indicated that NGR1 might exert its function through the MTNR1A-mediated signaling pathway.

## NGR1 Reduced the Excessive Autophagy and Apoptosis Induced by Rapamycin in HT-22 Cells

As shown in **Figure 4A**, rapamycin induced the accumulation of acidic vesicular organelles (AVOs) in orange color in HT-22 cells. NGR1, when used at higher concentrations (20 and 40 μM), significantly reduced the intensity of AVOs within the cells. 3-MA, an inhibitor of autophagy, showed similar effect as NGR1. Furthermore, there were more autophagosomes in rapamycin-induced model group cells. However, both NGR1 and 3-MA treatment could reduce the number of autophagosomes. In addition, as shown in **Figures 4C,D**, rapamycin increased the ratio of apoptotic cells ($p < 0.01$), which could be suppressed by the treatment of NGR1 (20 and 40 μM) and 3-MA ($p < 0.05$ or $p < 0.01$). These results demonstrated that NGR1 could prevent excessive autophagy and apoptosis induced by rapamycin in HT-22 cells.

## NGR1 Reversed the Excessive Autophagy and Apoptosis Induced by Rapamycin in HT-22 Cells Through Regulating MTNR1A

As shown in **Figure 5**, rapamycin increased the protein expression of beclin-1 and p62 as well as the ratio of LC3BII to LC3BI in HT-22 cells ($p < 0.001$). Meanwhile, it also decreased the ratio of Bcl-2/Bax but elevated the expression of cleaved caspase-3 ($p < 0.001$). Both NGR1 and 3-MA treatment could reverse the changes of the proteins involved in autophagy and apoptosis ($p < 0.001$). Moreover, NGR1 increased the expression of MTNR1A ($p < 0.05$) and enhanced the phosphorylation of PI3K, AKT, and mTOR in HT-22 cells induced by rapamycin ($p < 0.001$).

To confirm the role of MTNR1A in the protective effect of NGR1, the MTNR1A inhibitor was used. As shown in **Figure 6**, when the inhibitor was added, the alleviative effect of NGR1 on the excessive autophagy and apoptosis induced by rapamycin was abolished in HT-22 cells.

# DISCUSSION AND CONCLUSION

SD can cause deficiency of learning and memory as it induces excessive autophagy and apoptosis in hippocampal neurons according to our previous studies (Suchecki and Tufik, 2000). In the present study, NGR1 was shown to attenuate the impaired learning and memory of SD mice. Moreover, it prevented the morphological changes of the pyramidal layer neurons and reduced excessive autophagy and apoptosis, evidenced by decreased autophagosomes and recovered abnormally expressed proteins, such as beclin-1, p62, LC3B, Bcl-2, Bax, and cleaved caspase-3, that involved in the processes of autophagy and apoptosis. These results clearly demonstrated that NGR1 could alleviate SD-induced hippocampal neuronal injury and thus improve the impaired learning and memory in mice.

Phosphoinositide 3-kinase (PI3K), an intracellular phosphatidylinositol kinase, consists of a catalytic subunit (p110) and a regulatory subunit (p85) (Hennessy et al., 2005), activates protein kinase B (Akt) and mammalian target of rapamycin (mTOR), and actively participates in the regulation of autophagy (Cantley, 2002) (Hennessy et al., 2005). Akt is a serine/threonine kinase and is a primary downstream target in the transduction pathway of PI3K signaling. It is a key signal molecule that promotes cell survival, inhibits apoptosis (Ouyang et al., 1999), and maintains normal functions (Castaneda et al., 2010). mTOR is a serine/threonine protein kinase that controls Atg genes and regulates autophagy negatively (Pattingre et al., 2008). Melatonin is reported to regulate autophagy through the PI3K/Akt/mTOR pathway (Roohbakhsh et al., 2018). Melatonin receptors, MTNR1A and MTNR1B, belong to GPCR family–coupled G proteins. Reducing the level of cAMP, the second messenger in cells, is the most commonly triggered signaling pathway by melatonin (Cecon et al., 2018). The neuroprotective effect of melatonin has been shown to be mediated by the melatonin receptor MTNR1A (Wang et al., 2011). A decrease in the MTNR1A level in the individuals has been revealed to increase the risk to aggravate the pathological process of Alzheimer's disease (Sulkava et al., 2018).

In the present study, SD was shown to suppress the expression of MTNR1A in hippocampal tissue and inhibit the activation of the PI3K/Akt/mTOR pathway. After administration of NGR1, the suppression was alleviated. On HT-22 cells pretreated with rapamycin, NGR1 was demonstrated to reverse the reduced MTNR1A expression and inhibited the PI3K/Akt/mTOR pathway. To confirm the important role of MTNR1A, the inhibitor of MTNR1A was employed, which, at least partly, abolished the effect of NGR1. These results suggested that NGR1 exerted its neuroprotective role through acting on MTNR1A.

In conclusion (**Figure 7**), NGR1 could attenuate the impaired learning and memory of SD mice, which might be exerted by inhibiting the excessive autophagy and apoptosis through MTNR1A-mediated PI3K/Akt/mTOR pathway in hippocampal neurons.

# DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

# ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Anhui University of Chinese Medicine.

# AUTHOR CONTRIBUTIONS

XW, ZW, QL, ZK, ZG, and YC designed the study. YC, ML, SC, and AZ performed the experiment and completed the statistical analysis.

## FUNDING

This work was financially supported by the National Natural Science Foundation of China (81530096), Major International (Regional) Joint Research Project of NSFC (81920108033). Shanghai E-Research Institute of Bioactive Constituent in TCM plan, the Opening Project of Shanghai Key Laboratory of Compound Chinese Medicines (17DZ2273300), and State Key Laboratory of Functions and Applications of Medicinal Plants, Guizhou Medical University (FAMP2020K), Guizhou Science and Technology Platform Talents (QKHRCPT (2019)5106). Youth Project of Anhui Natural Science Foundation (2108085QH372).

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CORRECTION
published: 19 January 2022
doi: 10.3389/fphar.2021.832126

# Corrigendum: Notoginsenoside R1 Reverses Abnormal Autophagy in Hippocampal Neurons of Mice With Sleep Deprivation Through Melatonin Receptor 1A

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**Keywords: notoginsenoside R1, sleep deprivation, autophagy, melatonin receptor 1A, learning and memory**

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***Specialty section:***
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology*

***Received:*** *09 December 2021*
***Accepted:*** *30 December 2021*
***Published:*** *19 January 2022*

***Citation:***
*Cao Y, Li Q, Zhou A, Ke Z, Chen S, Li M, Gong Z, Wang Z and Wu X (2022) Corrigendum: Notoginsenoside R1 Reverses Abnormal Autophagy in Hippocampal Neurons of Mice With Sleep Deprivation Through Melatonin Receptor 1A. Front. Pharmacol. 12:832126. doi: 10.3389/fphar.2021.832126*

## A Corrigendum on

**Notoginsenoside R1 Reverses Abnormal Autophagy in Hippocampal Neurons of Mice With Sleep Deprivation Through Melatonin Receptor 1A**

*by Cao, Y., Li, Q., Zhou, A., Ke, Z., Chen, S., Li, M., Gong, Z., Wang, Z., and Wu X. (2021). Front. Pharmacol. 12:719313. doi:10.3389/fphar.2021.719313*

In the original article, there was an error in the **Funding** statement. The correct number for the funder "Youth Project of Anhui Natural Science Foundation" is 2108085QH372, not 2108085QH3720. There was also a mistake in **Figure 7**, the chemical form of *NGR1* is incorrect. The correct **Figure 7** appears below.

The authors apologize for these errors and state that this do not change the scientific conclusions of the article in any way. The original article has been updated.

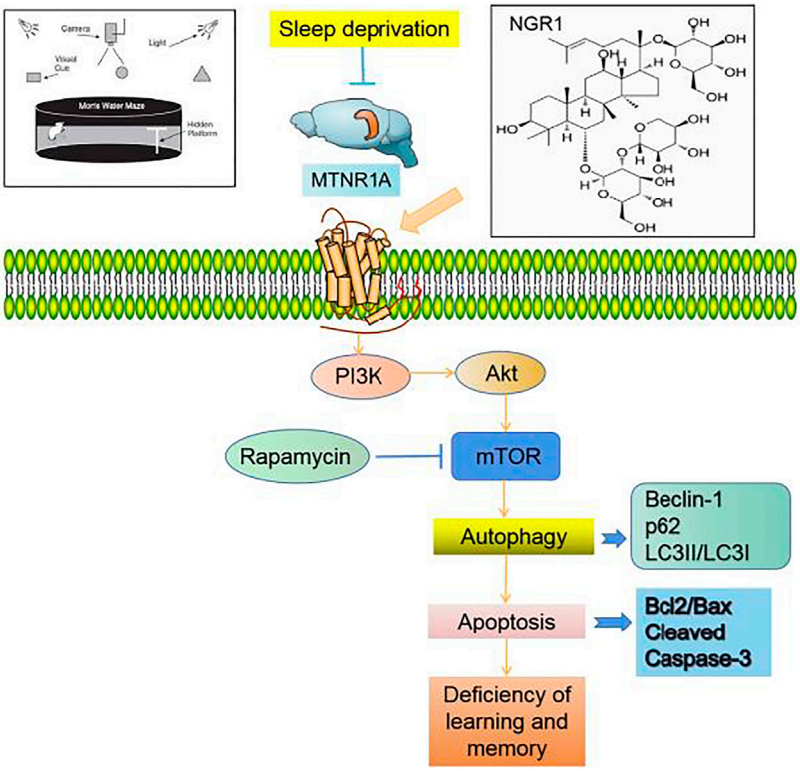


**FIGURE 7 |** Notoginsenoside R1 reverses abnormal autophagy in hippocampal neuron of mice with sleep deprivation through melatonin receptor 1A.

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