

Reviews in pharmacology of anti-cancer drugs 2023

Edited by

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Reviews in pharmacology of anti-cancer drugs: 2023

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Editorial: Reviews in pharmacology of anti-cancer drugs: 2023

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KEYWORDS

cancer, drug delivery systems, antitumor compounds, anticancer therapies,
drug targeting

Editorial on the Research Topic

Reviews in pharmacology of anti-cancer drugs: 2023

Oncological research represents a cornerstone in the global effort to reduce the burden of cancers that are characterized by marked biological complexity and heterogeneity (Ng and Shaffer, 2023; Imodoye et al., 2024). A multifaceted strategy is required to address the aforementioned criticisms, drawing on approaches of molecular and cell biology, genomics, immunology, pharmacology, drug delivery and public health (Liu et al., 2024; Ammar et al., 2025). Each of them provides distinct and complementary insights, allowing a more holistic understanding of tumor development, progression, and response to therapies. In addition, the integration of various methodological approaches—ranging from *in vitro* and *in vivo* studies to clinical trials and epidemiological investigations—enhances the robustness and translational potential of research findings. The interdisciplinary paradigm has proven essential for fostering innovation and for the development of innovative diagnostic and therapeutic therapies in oncology (Mäurer et al., 2023; Grillone et al., 2024).

The contributions included in this Research Topic help shed light on some of these critical aspects, offering valuable perspectives on the various experimental and clinical approaches employed in cancer treatment.

Jiang et al. described the efficacy and safety of various treatments for patients with unresectable colorectal liver metastases (CRLM), with a specific focus on the comparison between first-line and maintenance therapies. Their network meta-analysis included 56 randomized controlled trials (RCTs) encompassing a total of 21,323 patients. Only phase II or III RCTs comparing two or more treatment strategies were included. The analysis revealed that, for first-line therapy, hepatic local treatment or targeted therapy combined with chemotherapy were among the most effective options. In particular, resection or ablation and the use of a single chemotherapeutic agent were associated with the best overall survival, while drug combination, such as atezolizumab + bevacizumab + fluorouracil-based chemotherapy, resulted in the longest progression-free survival. Monotherapy and local treatment were associated with fewer severe side effects, whereas combination therapies were associated with increased toxicity. The described findings provide valuable evidence on the efficacy and safety of therapeutic strategies employed for the treatment of unresectable CRLM.

The treatment of hepatocellular carcinoma (HCC) represents a major global health challenge ranking as the sixth most common cancer worldwide. While surgery is still considered the first-line approach for the management of the disease, pharmacological therapies play an important role, especially in advanced stages of the disease. [Chen et al.](#) reviewed the current landscape of globally approved pharmacological treatments for HCC and highlighted the emerging trends from recent clinical trials, providing a perspective on future therapeutic innovations. In detail, treatment regimens based on single agents—including molecular targeted therapies and immune checkpoint inhibitors used alone or in combination - play a pivotal role in the management of HCC. However, the identification of optimal pharmacological treatments remains an unmet clinical need, and the development of more effective therapeutic agents is required.

Leptomeningeal disease (LMD) is a rare but severe tumor-related condition affecting the central nervous system, characterized by the dissemination of malignant cells within the subarachnoid space and cerebrospinal fluid. It is most commonly associated with lung and breast cancers and represents a complex and multifaceted disease treated with surgery, radiotherapy, systemic and intrathecal drug treatments. [Wang et al.](#) described the state of the art of intrathecal administration of chemotherapeutic, targeted and immunotherapeutic agents for the treatment of LMD, providing safety considerations and recommendations for minimizing adverse effects.

The use of arginine-glycine-aspartic acid (RGD) peptides for tumor targeting represents an innovative and highly selective strategy in cancer therapy. RGD-functionalized nanocarriers enhance drug accumulation and cellular uptake as a consequence of the natural affinity of the RGD motif for integrin receptors, which are frequently overexpressed on tumor cells and their associated endothelial tissue. This targeting can decrease off-target effects on healthy organs. [Wang et al.](#) reviewed recent advances deriving from the conjugation of RGD to various nanosystems, such as liposomes, polymeric or inorganic nanoparticles. Nevertheless, potential immunogenicity, peptide degradation, variability of integrin expression among tumors, the need for scalable procedures, and the possibility to exploit this molecule in personalized therapies remain challenges to be addressed.

In the context of expanding peptide-based therapeutic strategies in oncology and following the promising advances observed with RGD-functionalized systems for targeted drug delivery, antimicrobial peptides (AMPs) emerged as a novel and promising class of anticancer agents. [Zare-Zardini et al.](#) reported the peculiar properties of AMPs proposed for cancer treatment. In detail, it was shown that their physico-chemical features, such as net charge, length, hydrophobicity and Boman index, influence their ability to destabilize the cancer cell membranes, interfere with intracellular pathways and modulate the tumor microenvironment, suggesting a potential role in drug delivery.

An alternative peptide-independent strategy for targeted drug delivery and cancer therapy is represented by porphyrin-based nanoscale metal-organic frameworks (por-nMOFs) due to their ability in generating cytotoxic reactive oxygen species (ROS) for application in photodynamic therapy (PDT).

Unfortunately, por-nMOFs are characterized by a rapid clearance and insufficient tumor targeting. [Zou et al.](#) focused on the biomimetic approaches proposed to overcome these limitations, such as the coating por-nMOFs with cell membrane derived from various cells. This strategy allows to modulate the pharmacokinetic profile of por-nMOFs, helps evade the reticuloendothelial system and increases tumor accumulation, demonstrating the potential of these innovative formulations for the development of advanced PDT.

ROS-mediated cytotoxicity was identified as the key mechanism exploited by *Atractylodes lancea* DC, a traditional Asian medicinal herb, as discussed by [Ahn et al.](#) In detail, the plant extract demonstrated selective cytotoxicity against prostate cancer cells compared to healthy cells, inducing apoptosis through mitochondrial dysfunction characterized by decreased membrane potential and increased intracellular calcium levels.

[Fang et al.](#) investigated the antitumor effects of Schisandrin B, another natural compound obtained by the dried fruits of *Schisandra chinensis*. The study described various molecular targets of Schisandrin B depending on the type of treated tumor, and included a chemoinformatic evaluation of additional intracellular pathways involved in its cytotoxicity. These findings highlight the potential of the active compound as a component of innovative antitumor formulations.

The involvement of CXC chemokine receptor 3 (CXCR3), a G protein-coupled chemokine receptor, in the development of tumor-related diseases was described by [Hou et al.](#) Specifically, these authors discussed the design of small molecule antagonists and their *in vitro* and *in vivo* efficacy with the aim of highlighting the potential impact of this approach in the treatment of various human carcinomas.

In summary, the articles of this Research Topic present a range of therapeutic strategies employed or under investigation for the management of cancer. Although studies on several novel molecules or drug delivery systems herein described are still at an early stage of development, the Guest Editors believe that these approaches may hold promise as future pharmacological formulations.

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A network meta-analysis of efficacy and safety for first-line and maintenance therapies in patients with unresectable colorectal liver metastases

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Background: Evidence comparing the efficacy of different treatments for patients with unresectable colorectal liver metastases (CRLM) receiving first-line or maintenance therapy is sparse. We aimed to assess the efficacy and safety of these treatments, with a distinct focus on evaluating first-line and maintenance treatments separately.

Methods: We conducted Bayesian network meta-analyses, sourcing English-language randomized controlled trials (RCTs) published through July 2023 from databases including PubMed, Embase, the Cochrane Library, [ClinicalTrials.gov](#), and key conference proceedings. Phase II or III trials that assessed two or more therapeutic regimens were included. Primary outcome was overall survival (OS). Secondary outcomes included progression-free survival (PFS), objective response rate (ORR), adverse events graded as 3 or above (SAE), and R0 liver resection rate. Hazards Ratios (HRs) and 95% confidence intervals (CI) were used as effect size for OS and PFS, Odds Ratios (ORs) and 95% CI were used for ORR, SAEs and R0 resection rate. Subgroup and sensitive analyses were conducted to analysis the model uncertainty (PROSPERO: CRD42023420498).

Results: 56 RCTs were included (50 for first-line treatment, six for maintenance therapies), with a total of 21,323 patients. Regarding first-line, for OS, the top three mechanisms were: local treatment + single-drug chemotherapy (SingleCT), Targeted therapy (TAR)+SingleCT, and TAR + multi-drug chemotherapy (MultiCT). Resection or ablation (R/A)+SingleCT, S1, and Cetuximab + intensified fluorouracil-based combination chemotherapy (ICTFU) were identified as the best treatments. For PFS, the top three mechanisms were: Immune therapy + TAR + MultiCT, multi-targeted therapy (MultiTAR), TAR + SingleCT. The top three treatments were: Atezolizumab + Bevacizumab + fluorouracil-based combination chemotherapy (CTFU), TAS-102+bevacizumab, Bevacizumab + ICTFU. Cetuximab + CTFU was the best choice for RAS/RAF wild-type patients. Regarding maintenance treatment, Bevacizumab + SingleCT and Adavosertib were the best options for OS and PFS, respectively. For safety, MultiCT was the safest, followed by local treatment + MultiCT, TAR + MultiCT caused the most SAEs. Bevacizumab plus chemotherapy was found to be the safest among all targeted combination therapies.

Conclusion: In first-line, local treatment or targeted therapy plus chemotherapy are the best mechanisms. R/A + SingleCT or CTFU performed the best for OS, Atezolizumab + Bevacizumab + ICTFU was the best option regarding PFS. For RAS/RAF wild-type patients, Cetuximab + CTFU was the optimal option. Monotherapy may be preferred choice for maintenance treatment. Combination therapy resulted in more SAEs when compared to standard chemotherapy.

KEYWORDS

metastatic colorectal cancer, unresectable liver metastases, network meta-analysis, firstline treatment, maintenance treatment

1 Background

Colorectal cancer (CRC) is a common malignant digestive tract tumor, and in recent years, its incidence and mortality rates have shown an increasing trend year by year. The global incidence of CRC has been escalating, experiencing yearly growth rates of 3.2% (Zhou et al., 2022). Worldwide, it is the second leading cause of cancer-related mortality and ranks as the third most common cancer (Wang et al., 2023). Approximately 15%–25% of patients are found to have colorectal liver metastases (CRLM) at their initial diagnosis, while 70%–80% of patients with CRLM are initially deemed unresectable. For resectable patients, 50%–60% may experience recurrence after surgery, potentially progressing to unresectable disease (Xu et al., 2019).

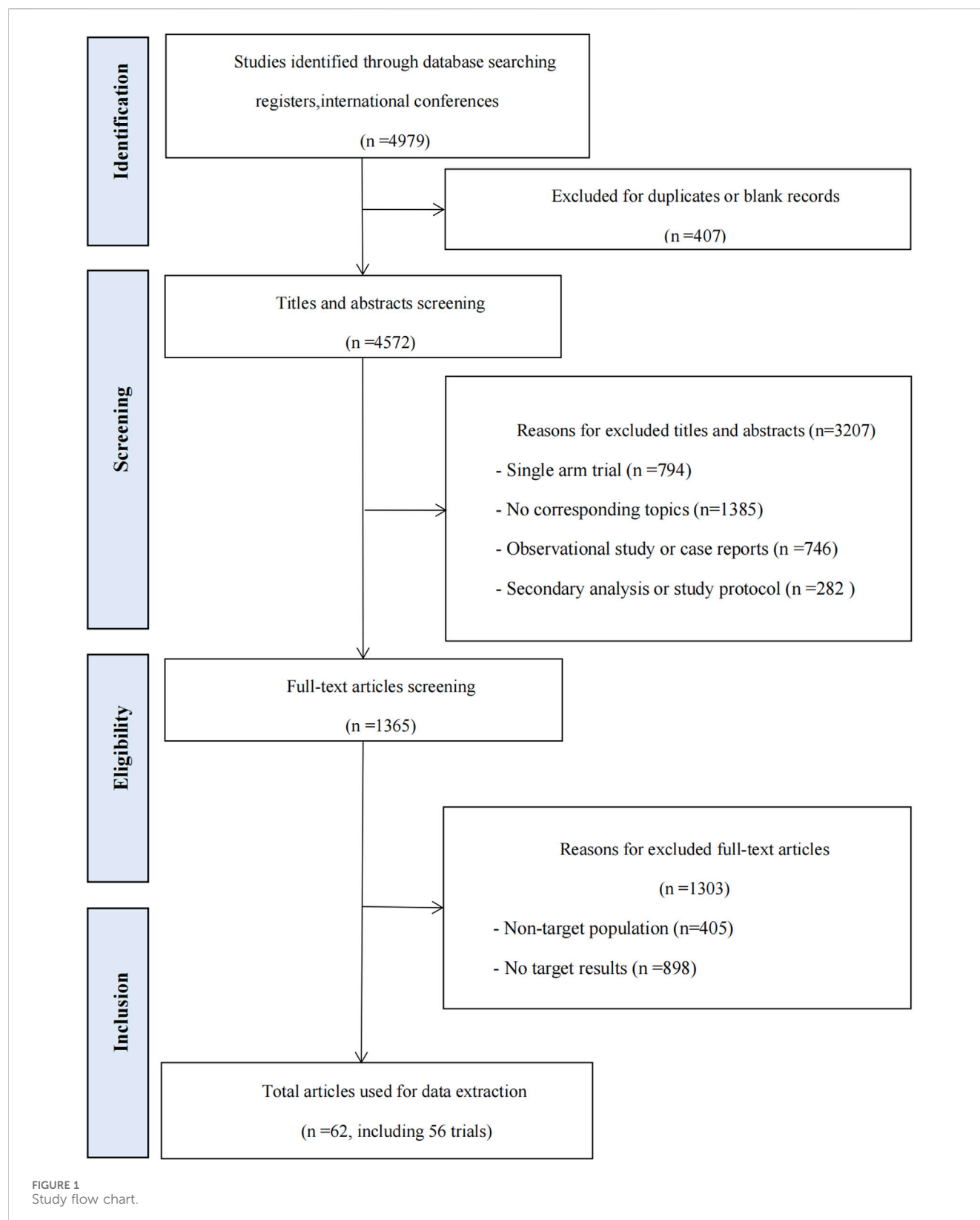
When suspected liver metastasis is found in clinical examinations, it is recommended to perform liver-enhanced magnetic resonance imaging (MRI) scans. For initial unresectable CRC, it is recommended to test the patient's gene statuses. For patients with unresectable CRLM, conversion therapy can be considered after multidisciplinary team discussion, with preoperative chemotherapy or chemotherapy combined with targeted drugs recommended. Whether the primary lesion of CRLM, without bleeding or obstruction symptoms, should be removed is still under debate (NCCN Guidelines; NCCN Guidelines; Scherman et al., 2021). A multicenter prospective study showed that there was no statistically significant difference in overall survival (OS) between patients with primary synchronous CRLM who underwent resection of the primary lesion tumor followed by systemic chemotherapy and those who only received chemotherapy (Park et al., 2020). For most LM that cannot be surgically removed, radiofrequency or microwave ablation can be used to control local lesions. Transarterial chemoembolization (TACE) is an effective minimally invasive treatment that is widely used for unresectable CRLM. However, TACE-induced hypoxia microenvironment and increased neovascularization may potentially promote early progression (Fiorentini et al., 2018). Systemic therapy is a preferable treatment choice for unresectable CRLM due to its ability to improve both quality-of-life and survival. Furthermore, effective systemic therapy—which includes chemotherapy, targeted therapy, and other systemic treatments—has the potential to convert unresectable lesions into resectable ones (Tomasello et al., 2017). FOLFOX, CAPEOX, FOLFIRI, and 5-fluorouracil/leucovorin or capecitabine are the recommended initial chemotherapy treatments for eligible patients who require intensified therapy. Effective cancer therapy enables about

12.5% of patients with unresectable CRLM to undergo liver resection and consequently improves their survival rates, however, it is essential to carefully consider the potential adverse effects (AEs) associated with this regimen (Adam et al., 2004). Patients who are able to tolerate aggressive therapy may experience improved outcomes by combining chemotherapy with targeted therapy. The combination of chemotherapeutic drugs is commonly used along with drugs that target epithelial growth factor receptor (EGFR) and VEGF (Li et al., 2014). Cetuximab and panitumumab are frequently used as EGFR inhibitors, while bevacizumab plays a vital role in anti-angiogenesis by targeting VEGF (Xie et al., 2020). Nevertheless, the response to anti-angiogenic therapy differs among patients, whereby some individuals do not experience any benefits, while others may develop tolerance or encounter more severe consequences (Abdalla et al., 2018; Lugano et al., 2020). Blocking immune checkpoints directly to prevent immune escape is the most established approach in immunotherapy, which has shown outstanding efficacy in treating various types of cancer (Hoos et al., 2010). The patients' response to immune checkpoint blockade (ICB) varies depending on whether they have DNA microsatellite instability (MSI) or mismatch repair (MMR) status, unlike patients with other types of cancer (Johdi and Sukor, 2020).

At present, there are numerous treatment choices accessible for unresectable CRLM patients who have not received treatment or receive maintenance therapies, caused by various mechanisms. However, there is insufficient information regarding the comparative outcomes of these options. As a result, we undertook this study to comprehensively evaluate the influence of all current treatment regimens on the survival outcomes of patients with unresectable CRLM receiving first-line or maintenance therapies. Our objective was to determine the relative efficacy and safety of these regimens and provide healthcare clinicians, patients, and relevant guidelines with valuable references for clinical medication and disease management.

2 Methods

Our study was conducted following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) extension statement (Hutton et al., 2015). See [Supplementary File S1](#). This systematic review protocol was registered on PROSPERO (CRD42023420498).



2.1 Data sources and search strategy

The search strategy is provided in [Supplementary File S2](#). In 31 July 2023, we conducted a comprehensive search on PubMed,

EMBASE, Cochrane Library, and [ClinicalTrials.gov](https://clinicaltrials.gov) to find relative RCTs and published studies. There were no restrictions on the publication date, and language was limited to English. Moreover, abstracts from the European Society for Medical Oncology,

American Society of Clinical Oncology since 2021 were also included in the search.

2.2 Selection criteria

Two researchers (YJ and TS) independently screened all articles identified through the database search by title and abstract. Articles that met the inclusion criteria were then subjected to full-text screening. Discrepancies were sorted out through discussions involving other researchers (YJ, TS, MZ, YX, and XZ). The eligibility criteria based on the PICOS framework were as follows:

- (1) Population: Adult patients with confirmed CRLM, diagnosed either histologically or cytologically. Patients also need to meet the requirement of receiving first-line treatment or maintenance treatment after prior system treatment stabilization. No limitations were imposed regarding individual-level characteristics. Due to the fact that some RCTs only reported results for mCRC patients with 2 or more organ metastases, we assumed that such patients had liver metastasis, considering liver metastasis is present in more than 90% of these patients (Riihimäki et al., 2016; Reboux et al., 2022).
- (2) Interventions and comparisons: We evaluated any systematic interventions, including pharmaceutical, surgical, radiological, and combination therapies.
- (3) Outcomes: The trials included in the analysis reported on clinical outcome measures such as OS, progression-free survival (PFS), objective response rate (ORR), AEs graded as 3 or above (SAE) according to the National Cancer Institute Common Terminology Criteria for Adverse Events, and R0 liver resection.
- (4) Study design: Phase II or III studies that compared multiple distinct treatments were primarily considered.

To avoid redundancy, we focused on trials that provided the most recent and significant insights. Moreover, we dismissed trials that explored treatments unrelated to any comparisons. Additionally, trials that specifically investigated varying dosages but implemented the same administrations were also eliminated.

2.3 Data extraction and quality assessment

Two independent researchers (YJ and TS) were responsible for extracting the required data. The extracted information encompassed the characteristics of eligible trials (publication year, registration information, etc.), characteristics of populations (age, sample size, countries, etc.), and characteristics of the program (interventions, outcomes of endpoints, etc.). The clinical outcomes extracted included OS, PFS, ORR, SAEs, and R0 liver resection. For studies that only published Kaplan-Meier curves without providing hazard ratios (HRs) or a 95% confidence interval (CI), Liu et al.'s tool was used to extract OS or PFS rates, and number-at-risk from Kaplan-Meier curves. Individual patient data (IPD) were then reconstructed, and HR and their 95% CI were calculated based on reconstructed IPD (Liu et al., 2021).

Cochrane Collaboration's risk of bias (ROB) tool was used to evaluate the quality of the studies included (Higgins et al., 2011). The eligible studies were categorized into three groups: high, low, or unclear risk (Lin and Chu, 2018). To evaluate the publication bias, the Egger regression test was utilized, with p -values <0.05 being interpreted as evidence of bias.

2.4 Statistical analyses

The primary outcomes analysed are OS and PFS, while the secondary outcomes included ORR and SAE, as well as R0 liver resection. Network plots were created to compare and visually represent the different treatment options. Pooled hazard ratios (HRs) with 95% CI were computed for OS and PFS. Pooled odds ratios (ORs) with 95% CI were calculated for ORR, SAE, and R0 resection rate. The analysis of synthesized HRs or ORs utilized the Bayesian approach, taking into account that the majority of direct evidence stemmed from a single trial. Therefore, the fixed effects consistency model was chosen (Zhao et al., 2019). The Bayesian network meta-analysis (NMA) using the R statistical packages Gemtc was carried out by employing four sets of Markov chains. Each set consisted of 50,000 samples with 10,000 burn-in samples. Non-informative prior distributions were utilized: specifically, a uniform prior distribution (Uniform (0, 1)) was used for parameter theta, and a normal prior distribution (Normal (0, 106)) was used for parameter mu (Sutton et al., 2008). In addition, we calculated the probability ranking for each available treatment and represented it using the surface area under the cumulative ranking (SUCRA). A higher SUCRA value indicated a greater rank.

The I^2 statistic was used to evaluate heterogeneity among studies, with a value greater than 50% indicating a moderate level of heterogeneity (Zhao et al., 2019). The edge-splitting method was used to assess the inconsistency of models, taking into account direct and indirect evidence (Zhao et al., 2019). To ensure the robustness of this study, several comparisons were conducted using pairwise meta-analysis. To confirm the convergence of Markov chains, trace plots and Gelman-Rubin diagnostic statistics were utilized (Brooks and Gelman, 1998).

To assess the influence of the number of metastatic organs, subgroup and sensitivity analyses were conducted to evaluate the dependability of the findings. We classified the population receiving first-line treatment into two categories: those with liver-limited metastasis and those with multiple metastasis sites, and then conducted subgroup analysis separately. Due to limited evidence for patients receiving maintenance treatment, we chose not to classify this group. In the sensitivity analyses, we studied the potential impact of mutation target levels on the efficacy of intervention strategies.

3 Results

3.1 Characteristics of the included studies

A total of 4979 records were identified from database searches. 3614 records were excluded during the title and abstract screening,

and 1365 records were screened in full text. Sixty-two articles, comprising 56 RCTs, were included in the review. The flow chart can be seen in [Figure 1](#). Details about the included studies are presented in [Supplementary File S3](#).

This research study included a total of 21,323 patients diagnosed with metastasis CRC (mCRC). Of these, 50 RCTs are applicable for the comparative analysis of first-line treatment, and six RCTs were specifically designed for maintenance treatment. To form a complete indirect comparison, we classified chemotherapy into single-drug chemotherapy (SingleCT), fluorouracil-based combination chemotherapy (CTFU), capecitabine-based combination (CTCA), and intensified CTFU (ICTFU) which contained four drugs. We uniformly referred to the best supportive treatment, observation, or placebo as BSC without distinction. Briefly, 12 mechanisms have been included, including: BSC, immune checkpoint inhibitor (ICI)+multi-drug chemotherapy (MultiCT), ICI + targeted therapy (TAR)+MultiCT, local treatment (defined as hepatic artery infusion [HAI], Selective internal radiation therapy [SIRT], transcatheter arterial chemoembolization [TACE], resection or ablation [R/A]), local treatment + MultiCT, local treatment + SingleCT, MultiCT, multi-targeted therapy (MultiTAR), RNA therapy + TAR + MultiCT, SingleCT, TAR + MultiCT, TAR + SingleCT. Besides, 29 treatments were involved, comprising of Adavosertib, Atezolizumab + Bevacizumab + ICTFU, Bevacizumab, Bevacizumab + CTCA, Bevacizumab + CTFU, Bevacizumab + Erlotinib, Bevacizumab + ICTFU, Bevacizumab + SingleCT, Cediranib + CTCA, Cetuximab + CTFU, Cetuximab + ICTFU, CTCA, CTFU, CTFU + SIRT, GOLFIG (defined as combination of gemcitabine, oxaliplatin, levofolinate, 5-fluorouracil, granulocyte-macrophage colony-stimulating factor, and interleukin-2), ICTFU, R/A + CTFU, R/A + SingleCT, panitumumab + CTFU, panitumumab + ICTFU, pelareorep + bevacizumab + CTFU, S1 (combined chemotherapy of tegafur, gimeracil, and oteracil), S1+bevacizumab + singleCT, S1+singleCT, SingleCT, sorafenib + CTFU, TAS-102 (trifluridine/tipiracil) + bevacizumab, tivozanib + CTFU, TSU-68 (Orantinib) + S1 + singleCT. We did not classify capecitabine and fluorouracil as the same drug category. Despite similar mechanisms, their differences in clinical application and side effect management warranted separate analyses ([Twelves et al., 2005](#); [Cassidy et al., 2008](#)). This yielded more accurate results and better supported clinical decisions.

3.2 Risk of bias

The assessment of ROB is presented in [Supplementary File S4](#). Overall, ROB in all RCTs was generally low. However, multiple RCTs were open-label in our study ([Cassidy et al., 2008](#); [Ducreux et al., 2011](#); [Hoff et al., 2012](#); [Hong et al., 2012](#); [Ruers et al., 2012](#); [Yamada et al., 2013](#); [Correale et al., 2014](#); [Heinemann et al., 2014](#); [Lee et al., 2014](#); [Loupakis et al., 2014](#); [Gruenberger et al., 2015](#); [Simkens et al., 2015](#); [Tournigand et al., 2015](#); [Benson et al., 2016](#); [Luo et al., 2016](#); [Stintzing et al., 2016](#); [Aparicio et al., 2018](#); [Jonker et al., 2018](#); [Qin et al., 2018](#); [Yamada et al., 2018](#); [Aranda et al., 2020](#); [Cremolini et al., 2020](#); [Van Cutsem et al., 2020](#); [Avallone et al., 2021](#); [Kanemitsu et al., 2021](#); [Antoniotti et al., 2022](#); [Modest et al., 2022](#); [Rossini et al., 2022](#); [Ychou et al., 2022](#); [Bond et al., 2023](#);

[Watanabe et al., 2023](#)), this raised concerns about the blinding of participants and personnel, assessment of outcomes, and concealment of allocation. Furthermore, several RCTs were found to have potential bias because of insufficient availability of outcome data. The network's results from the Egger test showed no publication bias, and the funnel plots can be found in [Supplementary File S5](#).

3.3 Efficacy outcomes

3.3.1 Primary analysis of overall survival for first-line treatments

For the analysis of OS and PFS, Fire-4.5 (a phase III trial, also known as AIO KRK-0116) was not considered due to its focus on BRAF mutations (which only account for 5% of all mCRC patients), resulting in excessive heterogeneity ([Stintzing et al., 2016](#)).

For OS, network plot is presented in [Figure 2A](#) and [Figure 2B](#). In the 9 intervention mechanisms, the top five rankings were, in order: local treatment + SingleCT (SUCRA, 0.938), TAR + SingleCT (SUCRA, 0.886), TAR + MultiCT (SUCRA, 0.732), Local treatment + MultiCT (SUCRA, 0.537), MultiCT (SUCRA, 0.411). Compared to MultiCT, the mechanisms with significant advantages were: local treatment + SingleCT (HR 0.45%, 95% CI 0.3–0.67), TAR + SingleCT (HR 0.49%, 95% CI 0.24–0.99), TAR + MultiCT (HR 0.74%, 95% CI 0.65–0.84). More details are shown in [Table 1](#) and [Supplementary File S6](#) ([Supplementary Figure S1](#)). In all 19 intervention schemes, the top five rankings were: R/A + SingleCT (SUCRA, 0.894), S1 (SUCRA, 0.836), Cetuximab + ICTFU (SUCRA, 0.797), Bevacizumab + SingleCT (SUCRA, 0.794), Cetuximab + CTFU (SUCRA, 0.765). Compared to CTFU, the ones with significant advantages, ranked from high to low, were as follows: R/A + SingleCT (HR 0.45%, 95% CI 0.3–0.67), Cetuximab + ICTFU (HR 0.53%, 95% CI 0.34–0.83), Cetuximab + CTFU (HR 0.56%, 95% CI 0.42–0.76), Bevacizumab + ICTFU (HR 0.58%, 95% CI 0.45–0.76), R/A + CTFU (HR 0.65%, 95% CI 0.49–0.87), Panitumumab + CTFU (HR 0.68%, 95% CI 0.58–0.81), and Bevacizumab + CTFU (HR 0.75%, 95% CI 0.64–0.88). Moreover, CTFU had a significant advantage compared to HAI (HR 1.61, 95% CI 1.12–2.31) and BSC (HR 2.55, 95% CI 1.17–5.54). Detailed results are provided in [Figure 3A](#) and [Supplementary File S6](#) ([Supplementary Table S3](#)).

3.3.2 Primary analysis of progression-free survival for first-line treatments

For PFS, network plots are shown in [Figures 2C,D](#). The top five ranked among the 11 intervention mechanisms were: ICI + TAR + MultiCT (SUCRA, 0.834), MultiTAR (SUCRA, 0.784), TAR + SingleCT (SUCRA, 0.675), Local treatment + SingleCT (SUCRA, 0.669), and ICI + MultiCT (SUCRA, 0.625). Compared to MultiCT, the mechanisms with significant advantages were: ICI + TAR + MultiCT (HR 0.51%, 95% CI 0.27–0.98), MultiTAR (HR 0.6%, 95% CI 0.49–0.75), TAR + SingleCT (HR 0.65 95% CI 0.43–1), TAR + MultiCT (HR 0.71%, 95% CI 0.64–79) and local treatment + MultiCT (HR 0.84 95% CI 0.75–0.95). Among all 26 intervention plans, the top five rankings were respectively Atezolizumab + Bevacizumab + ICTFU (SUCRA, 0.919), TAS-102+bevacizumab (SUCRA, 0.853), Bevacizumab + ICTFU (SUCRA, 0.838),

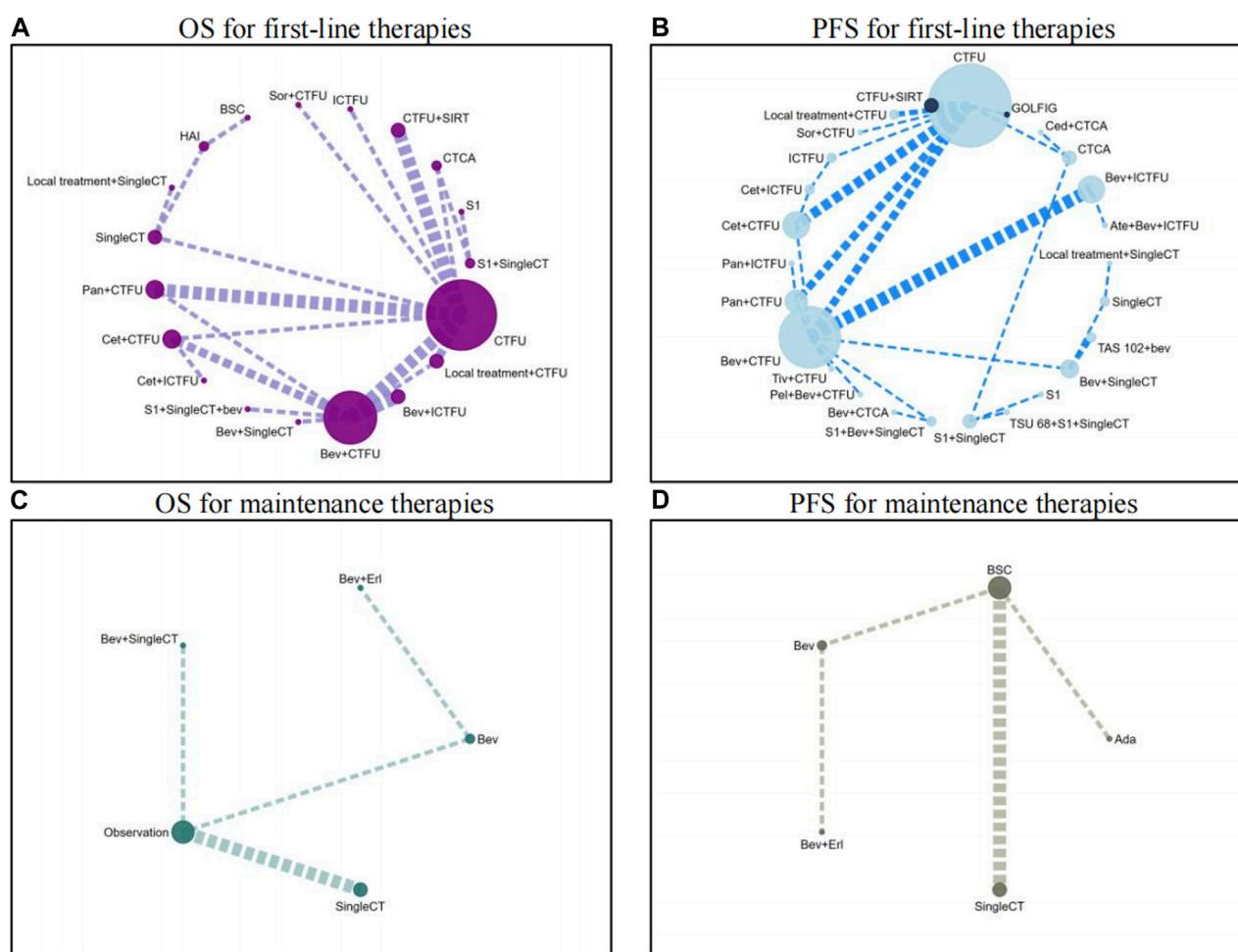


FIGURE 2

Network plots for first-line treatment and maintenance treatment in patients with unresectable colorectal liver metastases. Abbreviation: Ada, Adavosertib; ATE, Atezolizumab; BEV, Bevacizumab; BSC, best supportive care; CED, Cediranib; CET, Cetuximab; CTCA, capecitabine-based combination chemotherapy; CTFU, fluorouracil-based combination chemotherapy; ERL, Erlotinib; HAI, hepatic artery infusion; ICTFU, intensified fluorouracil-based combination chemotherapy; PAN, panitumumab; SingleCT, single-drug chemotherapy; SIRT, Selective internal radiation therapy; SOR, sorafenib; TIV, tivozanib. (A) OS for first-line therapies; (B) PFS for first-line therapies; (C) OS for maintenance therapies; (D) PFS for maintenance therapies.

Bevacizumab + SingleCT (SUCRA, 0.746), R/A + SingleCT (SUCRA, 0.742). Compared to CTFU, the scheme with significant advantages were: Atezolizumab + Bevacizumab + ICTFU (HR 0.35%, 95% CI 0.17–0.68), TAS-102+bevacizumab (HR 0.42%, 95% CI 0.19–0.89), Bevacizumab + ICTFU (HR 0.48, 95% CI 0.38–0.61), Cetuximab + CTFU (HR 0.57, 95% CI 0.47–0.69), S1+Bevacizumab + SingleCT (HR 0.57%, 95% CI 0.43–0.76), Cetuximab + ICTFU (HR 0.59%, 95% CI 0.43–0.79), Tivozanib + CTFU (HR 0.58%, 95% CI 0.38–0.9), R/A + CTFU (HR 0.6%, 95% CI 0.45–0.8), Bevacizumab + CTFU (HR 0.62%, 95% CI 0.54–0.73), and Panitumumab + CTFU (HR 0.71%, 95% CI 0.61–0.82). Other schemes, such as CTCA, showed no significant differences compared to CTFU. More details are shown in Table 1 and Figure 3B and Supplementary File S6 (Supplementary Table S3).

3.3.3 Primary analysis of maintenance treatments

Network plots are provided in Figures 2C,D. Seven intervention strategies were included for comparison. For OS, compared to BSC, the significant improvements were:

Bevacizumab + SingleCT (HR 0.71%, 95% CI 0.56–0.89) and SingleCT (HR 0.95%, 95% CI 0.91–0.98). Bevacizumab or Bevacizumab + Erlotinib did not show significant superiority when compared to BSC; In terms of PFS, Adavosertib (HR 0.35%, 95% CI 0.17–0.72) and SingleCT (HR 0.73%, 95% CI 0.65–0.81) had a significant advantage over BSC; Similarly, Bevacizumab or Bevacizumab + Erlotinib did not show significant differences compared to BSC. For more details, see Table 2. Due to the lack of safety data for CRLM patients, a quantitative comparison could not be made. However, the safety results of the overall patients indicated that monotherapy was a safer choice for maintenance treatment. See more in Supplementary File S9.

3.3.4 Primary analysis of safety, ORR, and R0 resection rate for first-line treatments

Network plots are presented in Supplementary File S6, Supplementary Figure S2. In terms of safety, for first-line treatments, MultiCT was the safest choice (SUCRA, 0.998), followed by local treatment + MultiCT (SUCRA, 0.795), TAR + MultiCT was the

TABLE 1 Comparative results of overall survival and progression-free survival of first-line treatment mechanism in patients with unresectable colorectal liver metastases.

Progression – free Survival	Overall Survival											
	BSC	NA	NA	0.63 (0.32, 1.24)	0.37 (0.17, 0.8)	0.18 (0.08, 0.4)	0.39 (0.18, 0.85)	NA	NA	0.42 (0.2, 0.88)	0.29 (0.13, 0.63)	0.19 (0.07, 0.55)
	NA	ICI + MCT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	NA	1.29 (0.46, 3.6)	ICI + TAR + MCT	NA	NA	NA	NA	NA	NA	NA	NA	NA
	NA	NA	NA	LT	0.58 (0.4, 0.86)	0.28 (0.18, 0.44)	0.62 (0.43, 0.9)	NA	NA	0.67 (0.49, 0.9)	0.46 (0.31, 0.68)	0.3 (0.14, 0.68)
	NA	0.79 (0.35, 1.75)	0.61 (0.32, 1.18)	NA	LT + MCT	0.48 (0.31, 0.73)	1.07 (0.95, 1.2)	NA	NA	1.14 (0.9, 1.46)	0.79 (0.67, 0.93)	0.52 (0.25, 1.08)
	NA	1.03 (0.36, 2.96)	0.8 (0.31, 2.05)	NA	1.31 (0.65, 2.65)	LT + SCT	2.22 (1.48, 3.34)	NA	NA	2.38 (1.68, 3.37)	1.64 (1.08, 2.52)	1.09 (0.48, 2.48)
	NA	0.66 (0.3, 1.46)	0.51 (0.27, 0.98)	NA	0.84 (0.75, 0.95)	0.64 (0.32, 1.29)	MCT	NA	NA	1.07 (0.86, 1.32)	0.74 (0.65, 0.84)	0.49 (0.24, 1)
	NA	1.09 (0.48, 2.49)	0.85 (0.44, 1.65)	NA	1.39 (1.09, 1.77)	1.06 (0.54, 2.09)	1.65 (1.34, 2.04)	MTAR	NA	NA	NA	NA
	NA	0.54 (0.23, 1.29)	0.42 (0.2, 0.86)	NA	0.69 (0.48, 0.99)	0.53 (0.25, 1.13)	0.82 (0.58, 1.15)	0.49 (0.34, 0.72)	RNA + TAR + MCT	NA	NA	NA
	NA	0.55 (0.2, 1.48)	0.42 (0.18, 1.02)	NA	0.69 (0.37, 1.29)	0.53 (0.38, 0.75)	0.83 (0.45, 1.52)	0.5 (0.28, 0.9)	1.01 (0.51, 2)	SCT	0.69 (0.54, 0.88)	0.46 (0.22, 0.96)
	NA	0.93 (0.42, 2.07)	0.72 (0.38, 1.37)	NA	1.18 (1.01, 1.38)	0.9 (0.45, 1.8)	1.41 (1.27, 1.55)	0.85 (0.71, 1.02)	1.72 (1.24, 2.39)	1.7 (0.93, 3.1)	TAR + MCT	0.66 (0.33, 1.33)
	NA	1.01 (0.41, 2.48)	0.78 (0.36, 1.69)	NA	1.28 (0.83, 2)	0.98 (0.57, 1.71)	1.53 (1, 2.34)	0.92 (0.62, 1.38)	1.87 (1.1, 3.18)	1.85 (1.2, 2.86)	1.09 (0.72, 1.65)	TAR + SCT

Abbreviation: BSC, best supportive care; ICI, immune checkpoint inhibitor; LT, local treatment; MCT, multi-drug chemotherapy; MTAR, multi-targeted therapy; SCT, single-drug chemotherapy; TAR, targeted therapy.
Note: This table presents the comparative results for overall survival (right section) and progression-free survival (left section) among various first-line treatment mechanisms in patients with unresectable colorectal liver metastases. Each cell contains a comparison between two treatments, expressed as a ratio (95% confidence interval). Ratios in the lower-left cells (below the diagonal) represent the outcome of the treatment in the row compared to the treatment in the column; Ratios in the upper-right cells (above the diagonal) represent the outcome of the treatment in the column compared to the treatment in the row. NA, indicates that data is not available for a particular comparison. Bold values denote statistically significant results ($p < 0.05$). The interpretation method described above applies consistently to Tables 2–4.

worst (SUCRA, 0.007). MultiCT showed significant advantages in terms of safety compared to other targeted combination therapies and local combination therapies. CTFU is the safest choice, and the combined mechanism therapy increased the incidence of SAEs compared to chemotherapy. Specifically, the treatment plans with significant differences compared to CTFU were: Bevacizumab + ICTFU (OR 4.5 95% CI 2.29–8.92), Panitumumab + CTFU (OR 4.07 95% CI 2.3–7.29), Cetuximab + CTFU (OR 3.27 95% CI 1.35–7.88), Bevacizumab + CTFU (OR 1.99 95% CI 1.23–3.24), and CTFU + SIRT (OR 1.43 95% CI 1.1–1.86). For ORR, compared to CTFU, combined mechanism therapies have significant advantages, ranked from high to low were: Panitumumab plus CTFU (OR 6.98, 95% CI 3.3–15.01), Cetuximab + ICTFU (OR 6.5%, 95% CI 2.97–12.5), Panitumumab + ICTFU (OR 5.26%, 95%

CI 1.72–16.16), Bevacizumab + ICTFU (OR 4.71%, 95% CI 2.49–8.92), Cetuximab + CTFU (OR 3.99%, 95% CI 2.3–7.02), Bevacizumab + CTFU (OR 1.93%, 95% CI 1.21–3.08), and CTFU + SIRT (OR 1.55%, 95% CI 1.21–2). There was no significant difference between CTFU, CTCA, and ICTFU. In terms of the R0 resection rate, the significant advantages in order compared to CTFU were: Bevacizumab + ICTFU (OR 15.81%, 95% CI 5.87–45.53), Bevacizumab + CTFU (OR 5.12%, 95% CI 2.53–11.13), and Cetuximab + CTFU (OR 4.09%, 95% CI 1.92–9.16). Cetuximab + ICTFU was better than BSC (OR 2.69, 95% CI 0.69–11.61), but the difference was not statistically significant. CTFU, ICTFU, and CTCA had almost no difference. More details are provided in Tables 3, 4 and Supplementary File S6, Supplementary Figure S3.

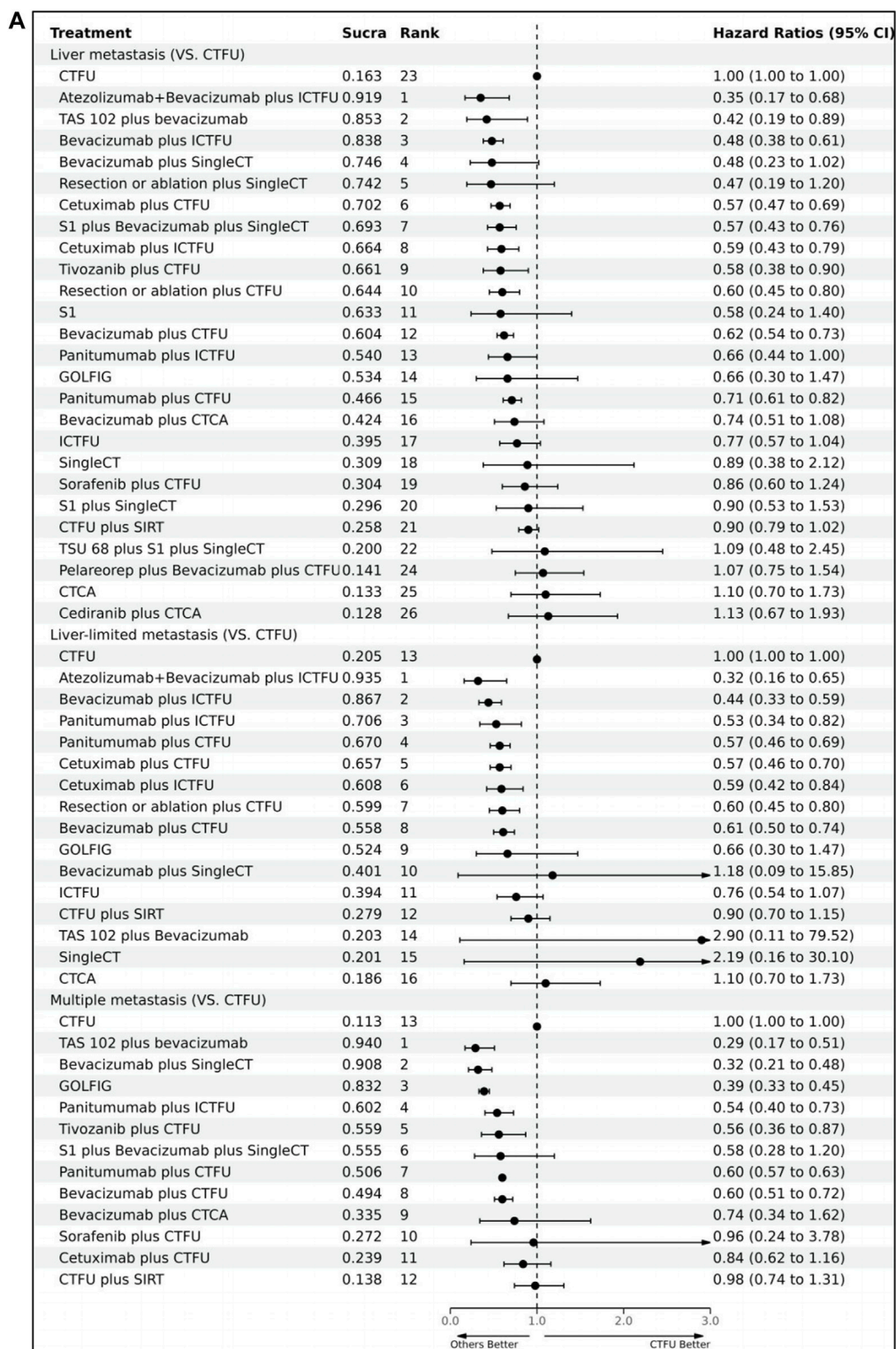


FIGURE 3
(Continued).

3.3.5 Subgroup analysis

For patients with liver-limited metastatic, the top five ranked regimens in terms of PFS were Atezolizumab + Bevacizumab + ICTFU (SUCRA 0.935), Bevacizumab + ICTFU (SUCRA 0.867), Panitumumab + ICTFU (SUCRA 0.706), Panitumumab + CTFU

(SUCRA 0.67), and Cetuximab + CTFU (SUCRA 0.657). Compared to CTFU, the schemes that possessed significant advantages were: Atezolizumab + Bevacizumab + ICTFU (HR 0.32%, 95% CI 0.16–0.65), Bevacizumab + ICTFU (HR 0.44%, 95% CI 0.33–0.59), Panitumumab + ICTFU (HR 0.53%, 95% CI

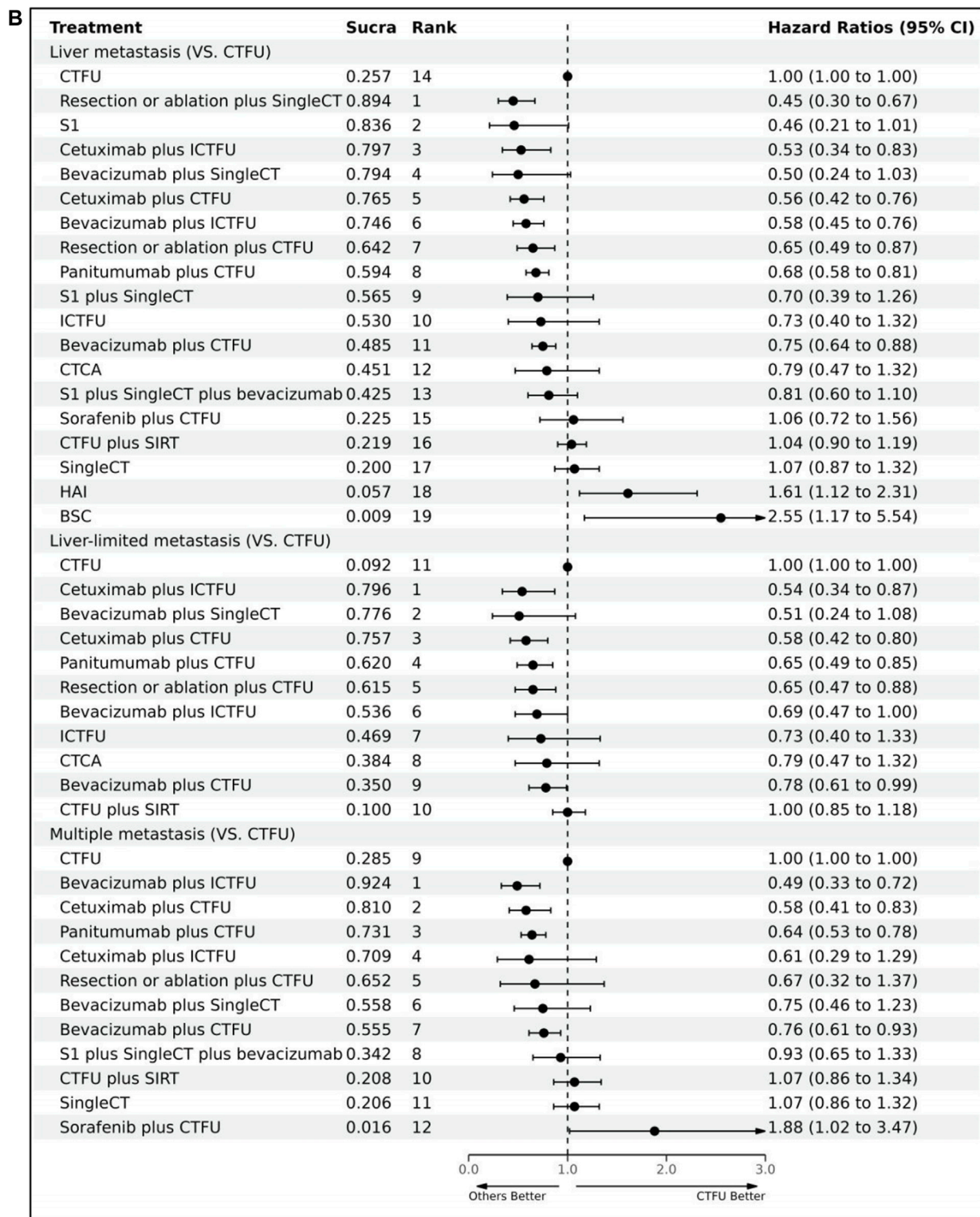


FIGURE 3
(Continued). Forest plots illustrating the comparison results of primary and secondary endpoints Abbreviation: CTFU, fluorouracil-based combination chemotherapy; CTCA, capecitabine-based combination chemotherapy; ICTFU, intensified fluorouracil-based combination chemotherapy; SIRT, Selective internal radiation therapy; SingleCT, single-drug chemotherapy. (A) OS for first-line treatments; (B) PFS for first-line treatments.

0.34–0.82), Panitumumab + CTFU (HR 0.57%, 95% CI 0.46–0.69), Cetuximab + CTFU (HR 0.57%, 95% CI 0.46–0.7), Cetuximab + ICTFU (HR 0.59%, 95% CI 0.42–0.84), R/A + CTFU (HR 0.6%, 95%

CI 0.45–0.8), and Bevacizumab + CTFU (HR 0.61%, 95% CI 0.5–0.74). In terms of OS, the top five ranked options were: Cetuximab + ICTFU (SUCRA 0.796), Bevacizumab + SingleCT (SUCRA 0.776),

TABLE 2 Comparative results of overall survival and progression-free survival of maintenance treatment in patients with unresectable colorectal liver metastases.

Progression – free Survival	Overall Survival					
	ADA	NA	NA	NA	NA	NA
	0.41 (0.19, 0.87)	BEV	1.05 (0.81, 1.36)	0.71 (0.13, 3.88)	1 (0.19, 5.4)	0.95 (0.18, 5.1)
	0.46 (0.2, 1.04)	1.13 (0.84, 1.52)	BEV + ERL	0.68 (0.12, 3.79)	0.95 (0.17, 5.25)	0.9 (0.16, 4.97)
	NA	NA	NA	BEV + SCT	1.41 (1.12, 1.77)	1.33 (1.06, 1.68)
	0.35 (0.17, 0.72)	0.86 (0.68, 1.08)	0.76 (0.52, 1.1)	NA	BSC	0.95 (0.91, 0.98)
	0.48 (0.23, 1)	1.18 (0.91, 1.53)	1.04 (0.7, 1.54)	NA	1.37 (1.23, 1.54)	SCT

Abbreviation: ADA, adavosertib; BEV, bevacizumab; BSC, best supportive care; ERL, erlotinib; SCT, single-drug chemotherapy.

TABLE 3 Comparative results of safety of first-line treatment in patients with unresectable colorectal liver metastases.

BEV + CTFU					
0.44 (0.27, 0.71)	BEV + ICTFU				
0.61 (0.29, 1.27)	1.38 (0.57, 3.33)	CET + CTFU			
1.99 (1.23, 3.24)	4.5 (2.29, 8.92)	3.27 (1.35, 7.88)	CTFU		
1.39 (0.81, 2.42)	3.15 (1.53, 6.56)	2.28 (0.91, 5.72)	0.7 (0.54, 0.91)	CTFU + SIRT	
0.49 (0.3, 0.79)	1.11 (0.56, 2.18)	0.8 (0.33, 1.94)	0.25 (0.14, 0.44)	0.35 (0.19, 0.66)	PAN + CTFU

Abbreviation: BEV, bevacizumab; CET, cetuximab; CTFU, fluorouracil-based combination chemotherapy; ICTFU, intensified fluorouracil-based combination chemotherapy; PAN, panitumumab; SIRT, selective internal radiation therapy.

Cetuximab + CTFU (SUCRA 0.757), Panitumumab + CTFU (SUCRA 0.62), and R/A + CTFU (SUCRA 0.615). Compared to CTFU, the plans with significant advantages were: Cetuximab + ICTFU (HR 0.54, 95% CI 0.34–0.87), Cetuximab + CTFU (HR 0.58%, 95% CI 0.42–0.8), Panitumumab + CTFU (HR 0.65%, 95% CI 0.45–0.85), R/A + CTFU (HR 0.65%, 95% CI 0.47–0.88), Bevacizumab + ICTFU (HR 0.69%, 95% CI 0.47–1.00), and Bevacizumab + CTFU (HR 0.78%, 95% CI 0.61–0.99).

For patients with multiple-site metastases, the top five treatments in terms of PFS were: TAS-102+bevacizumab (SUCRA 0.94), Bevacizumab + SingleCT (SUCRA 0.908), GOLFIG (SUCRA 0.832), Panitumumab + ICTFU (SUCRA 0.602), and Tivozanib + CTFU (SUCRA 0.559). Compared to CTFU, the solutions with significant advantages were as follows: TAS-102+bevacizumab (HR 0.29%, 95% CI 0.17–0.51), Bevacizumab + SingleCT (HR 0.32%, 95% CI 0.21–0.48), GOLFIG (HR 0.39%, 95% CI 0.33–0.45), Panitumumab + ICTFU (HR 0.54%, 95% CI 0.4–0.73), Tivozanib + CTFU (HR 0.56%, 95% CI 0.36–0.87), Panitumumab + CTFU (HR 0.6%, 95% CI 0.57–0.63), Bevacizumab + CTFU (HR 0.6%, 95% CI 0.51–0.72). In terms of OS, the top five ranked solutions were: Bevacizumab + ICTFU (SUCRA 0.924), Cetuximab + CTFU (SUCRA 0.81), Panitumumab + CTFU (SUCRA 0.731), Cetuximab + ICTFU (SUCRA 0.709), and R/A + CTFU (SUCRA 0.652). Compared to CTFU, the treatments with significant advantages were as follows: Bevacizumab + ICTFU (HR 0.49%, 95% CI 0.33–0.72), Cetuximab + CTFU (HR 0.58%, 95% CI 0.41–0.83), Panitumumab + CTFU (HR 0.64%, 95% CI 0.53–0.78), and Bevacizumab + CTFU (HR 0.76%, 95% CI 0.61–0.93).

Detailed results are presented in [Figure 3](#) and [Supplementary File S7](#), [Supplementary Tables S4](#), [S5](#).

3.3.6 Heterogeneity and inconsistency assessment

Most of the comparisons showed minimal or low heterogeneity, as observed in the results of the heterogeneity test summarized in [Supplementary File S10](#), [11](#). Nevertheless, comparisons of demonstrated moderate to high heterogeneity were as follows:

- A. Bevacizumab + CTFU VS Bevacizumab + ICTFU (54.2%), Panitumumab + CTFU (78.3%), or CTFU (65.8%) for PFS in liver metastasis;
- B. Bevacizumab + CTFU (54.3%) or Panitumumab + CTFU (56.9%) VS CTFU and Monotherapy VS NAT (66% for the long-term and 68% for the short-term) for PFS in multiple organ metastasis;
- C. Bevacizumab + CTFU VS Bevacizumab + ICTFU (65%), Panitumumab + CTFU (60.5%), or CTFU (70.4%) for liver-limited PFS;
- D. SingleCT VS BSC for Maintenance Treatment (OS, 98.4%; PFS, 98.6%).
- E. In the network of mechanism comparison, MultiCT VS Local treatment + MultiCT (50.4%) and Target + MultiCT VS MultiCT (54.1%).

After conducting pairwise meta-analyses, good consistency was observed between direct and indirect evidence. During the analysis of node splitting, we found no significant discrepancies between direct and indirect estimates, as all *p* values in the inconsistency test

TABLE 4 Comparative results of R0 liver resection rate and objective response rate of first-line treatment in patients with unresectable colorectal liver metastases.

ORR	R0									
	BEV + CTFU	3.06 (1.55, 6.38)	0.8 (0.41, 1.54)	0.52 (0.11, 2.66)	0.2 (0.06, 0.67)	0.2 (0.09, 0.39)	NA	0.23 (0.07, 0.81)	NA	NA
	0.41 (0.27, 0.63)	BEV + ICTFU	0.26 (0.1, 0.68)	0.17 (0.03, 0.99)	0.06 (0.01, 0.26)	0.06 (0.02, 0.17)	NA	0.08 (0.02, 0.32)	NA	NA
	0.48 (0.26, 0.89)	1.18 (0.55, 2.49)	CET + CTFU	0.66 (0.14, 3.45)	0.25 (0.07, 0.87)	0.24 (0.11, 0.52)	NA	0.29 (0.08, 1.05)	NA	NA
	0.32 (0.15, 0.68)	0.78 (0.32, 1.87)	0.66 (0.39, 1.1)	CET + ICTFU	0.37 (0.06, 2.03)	0.37 (0.09, 1.44)	NA	0.45 (0.16, 1.16)	NA	NA
	1.85 (0.7, 4.93)	4.54 (1.56, 13.13)	3.84 (1.39, 10.69)	5.84 (1.91, 17.91)	CTCA	0.98 (0.36, 2.7)	NA	1.18 (0.3, 4.97)	NA	NA
	1.93 (1.21, 3.08)	4.71 (2.49, 8.92)	3.99 (2.3, 7.02)	6.05 (2.97, 12.5)	1.04 (0.44, 2.44)	CTFU	NA	1.2 (0.47, 3.37)	NA	NA
	1.24 (0.73, 2.12)	3.03 (1.53, 6.03)	2.56 (1.4, 4.77)	3.89 (1.84, 8.4)	0.67 (0.27, 1.63)	0.64 (0.5, 0.83)	CTFU + SIRT	NA	NA	NA
	1.76 (0.75, 4.28)	4.31 (1.66, 11.57)	3.65 (1.58, 8.7)	5.53 (2.31, 13.86)	0.95 (0.3, 3.02)	0.92 (0.44, 1.98)	1.42 (0.65, 3.21)	ICTFU	NA	NA
	0.28 (0.15, 0.5)	0.67 (0.32, 1.4)	0.57 (0.24, 1.33)	0.87 (0.33, 2.29)	0.15 (0.05, 0.46)	0.14 (0.07, 0.3)	0.22 (0.1, 0.49)	0.16 (0.05, 0.44)	PAN + CTFU	NA
	0.37 (0.13, 1.02)	0.9 (0.3, 2.72)	0.76 (0.23, 2.5)	1.15 (0.32, 4.19)	0.2 (0.05, 0.82)	0.19 (0.06, 0.58)	0.3 (0.09, 0.93)	0.21 (0.05, 0.8)	1.33 (0.58, 3.09)	PAN + ICTFU

Abbreviation: BEV, bevacizumab; CET, cetuximab; CTFU, fluorouracil-based combination chemotherapy; CTCA, capecitabine-based combination chemotherapy; ICTFU, intensified fluorouracil-based combination chemotherapy; PAN, panitumumab; SIRT, Selective internal radiation therapy.

exceeded 0.05. The trace plots indicated a favorable convergence of iterations (Supplementary File S12).

3.3.7 Sensitivity analysis

Firstly, only RCTs that did not differentiate patients based on the level of target gene mutations were included, and the results were generally consistent with the baseline analysis: in terms of PFS, with CTFU as the reference, Atezolizumab + Bevacizumab + ICTFU (HR 0.34%, 95% 0.16–0.7) still ranked first, followed by Bevacizumab + ICTFU (HR 0.47%, 95% 0.33–0.66), and TAS-102+bevacizumab (HR 0.43%, 95% 0.19–0.94). Other treatments showed significant advantages in comparison to CTFU were S1+Bevacizumab + SingleCT (HR 0.59%, 95% 0.41–0.84), R/A + CTFU (HR 0.6%, 95% 0.45–0.8), Tivozanib + CTFU (HR 0.6%, 95% 0.37–0.97), Cetuximab + CTFU (HR 0.61, 95% 0.46–0.81), Cetuximab + ICTFU (HR 0.64%, 95% 0.43–0.95), and Bevacizumab + CTFU (HR 0.64%, 95% 0.49–0.84). OS results were consistent with the results of the base-case analysis, and treatments with significant advantages compared to CTFU were: R/A + SingleCT (HR 0.6%, 95% 0.37–0.97), Bevacizumab + SingleCT (HR 0.44%, 95% 0.21–0.94), Bevacizumab + ICTFU (HR 0.52%, 95% 0.37–0.73), R/A + CTFU (HR 0.54%, 95% 0.48–0.85), and Bevacizumab + CTFU (HR 0.67%, 95% 0.51–0.88).

Secondly, focus on patients with wild-type RAS/RAF. In terms of PFS, compared to CTFU, it was ranked from high to low as follows: Cetuximab + CTFU (HR 0.53%, 95% 0.39–0.72), Bevacizumab + ICTFU (HR 0.71%, 95% 0.45–1.14), Panitumumab + ICTFU (HR 0.74%, 95% 0.49–1.13), Panitumumab + CTFU (HR 0.79%, 95% 0.69–0.95), and Bevacizumab + CTFU (HR 0.79%, 95% 0.61–1.03).

For OS, compared to CTFU, the best choices were still Cetuximab + CTFU (HR 0.61%, 95% 0.43–0.85), followed by Panitumumab + CTFU (HR 0.74%, 95% 0.61–0.91) and Bevacizumab + CTFU (HR 0.88%, 95% 0.68–1.14). More details are available in Supplementary File S8, Supplementary Tables S6, S7.

4 Discussion

4.1 Main findings

This study is the first to systematically evaluate the efficacy and safety of different treatment options for patients with CRLM. The key findings are summarized as follows:

1. For CRLM patients, the optimal treatment options were local treatment + chemotherapy and TAR + chemotherapy. In terms of overall survival (OS), the best choices were R/A+ SingleCT or CTFU, Cetuximab + ICTFU or CTFU, Bevacizumab + ICTFU or CTFU, and Panitumumab + CTFU. For progression-free survival (PFS), the top options were Immune + TAR + chemotherapy, MultiTAR, local treatment + chemotherapy, and TAR + chemotherapy. For patients with liver-limited metastasis, Cetuximab, Bevacizumab, and Panitumumab + chemotherapy were the best choices for both OS and PFS. For those with multiple metastatic sites, Bevacizumab + ICTFU, Cetuximab or Panitumumab + CTFU were the best for OS, while TAS-102 + Bevacizumab, Bevacizumab + SingleCT, and GOLFIG were optimal for PFS.

2. For maintenance treatment, Bevacizumab + SingleCT was the best choice for OS. For PFS, Adavosertib and SingleCT showed significant advantages compared to BSC.
3. For first-line treatments, combination therapy caused more SAEs compared to CTFU. Bevacizumab + chemotherapy was the safest among targeted combination therapies. For ORR, Panitumumab or Cetuximab + CTFU or ICTFU, and Bevacizumab + ICTFU showed significant advantages over CTFU. Bevacizumab + ICTFU had the best R0 resection rate, followed by Bevacizumab or Cetuximab + CTFU.
4. For RAS/RAF wild-type patients, Cetuximab + CTFU was the best choice for both PFS and OS.

In the base-case analysis, heterogeneity was observed in some networks, such as the PFS of the overall population, likely due to lack of limitation on target mutation levels. Sensitivity analysis confirmed that controlling for target expression levels reduced heterogeneity across all networks.

TAR + chemotherapy can produce a higher remission rate and improve resectability. EGFR or VEGF inhibitors combined with chemotherapy are the best choices for patients with unresectable CRLM. EGFR inhibitors, such as Cetuximab and Panitumumab, are associated with higher tumor response and expedited symptom relief. Anti-EGFR therapy may induce tumor-specific adaptive immune responses and immunogenic cell apoptosis. VEGF inhibitors, such as Bevacizumab and Aflibercept, normalize tumor vasculature, increasing tumor blood supply (Xie et al., 2020). Combining chemotherapy with targeted therapy can enhance patient survival, but it also increases SAEs, making it challenging to find a solution that is both effective and safe. Surprisingly, adding Cetuximab to ICTFU did not worsen safety compared to CTFU, and Bevacizumab + ICTFU is also acceptable in terms of safety. Thus, Bevacizumab + ICTFU and Cetuximab + ICTFU are optimal for multiple metastasis sites and liver-limited metastasis, respectively. For the WT population, Cetuximab + CTFU is the best choice. New therapies like ICI + TAR and MultiTAR showed great PFS performance but had poor safety profiles, and more OS data are needed to confirm their efficacy. More RCTs are needed to identify the applicable population for these new mechanisms in precision treatment for CRC.

Despite using fewer drugs, monotherapy has been relatively effective in maintaining treatment efficacy for both PFS and OS. Bevacizumab combined with SingleCT is the most effective for OS, but its safety has been poor (Simkens et al., 2015). On the other hand, Adavosertib has demonstrated the best PFS, but more data is needed to establish its safety profile. Overall, monotherapy may still be the ideal choice at present.

Right- and left-sided colorectal tumors exhibit distinct epidemiological, clinicopathological characteristics, gene expression profiles, genetic alterations, and prognoses. Thus, efficacy of EGFR inhibitors is significantly influenced by the primary tumor site. Moretto et al. (2016) demonstrated that anti-EGFR therapies are less effective in right-sided tumors compared to left-sided ones. Takayuki's meta-analysis (Yoshino et al., 2024) further confirmed the superior efficacy of EGFR inhibitors in patients with left-sided primary tumors. Due to data limitations, our study could not differentiate the primary tumor site in patients

with liver metastases. Future research should address this aspect more thoroughly.

Recent trials have explored the use of anti-EGFR monoclonal antibodies in maintenance therapy. Filippo et al.'s phase II trial indicated that using panitumumab alone was less effective for PFS compared to a combination of panitumumab with fluorouracil-leucovorin (Pietrantonio et al., 2019). Similarly, the ERMES study concluded that cetuximab alone was not as effective for maintenance following FOLFIRI/cetuximab induction and thus is not recommended (Pinto et al., 2024). Due to the focus on liver metastasis in our study and limited available data, we could not evaluate additional maintenance therapies, including anti-EGFR monoclonal antibodies.

The advantage of this research is clear. It is the first study to systematically compare the efficacy and safety of all first-line and maintenance treatment regimens for CRLM patients, providing significant reference value for clinical practice and guidelines. Unlike previous studies, this study meticulously analyzed the type and mechanism of chemotherapy to minimize heterogeneity. The low heterogeneity of the study population enhances the reliability of the conclusions. Sensitivity analysis and in-depth examination of heterogeneity sources confirmed the robustness of the basic analysis. Multiple subgroup analyses, including distinctions between liver-limited and multiple-site metastases and analyses of wild-type patients, support precision treatment for CRC. Our innovative conclusions offer a new direction for future clinical research and provide substantial evidence for clinical decision-making.

Due to the availability of data, this study has some limitations. First, we cannot analyze the RAS/KRAS or RAF mutation population. Second, limited by a lack of individual data, the majority of studies only reported HR. Therefore, we utilized the time-invariant HR methods for indirect comparison, as opposed to using other risk variable models. Third, in order to form more comparisons, we consider patients with multiple-site metastases to have liver metastasis, even though this proportion exceeds 90%, it also introduces some uncertainty. Fourth, the relative efficacy between a certain number of schemes is obtained through indirect comparison, and more direct evidence from RCTs is needed to validate our findings. Fifth, for the results related to immunotherapy, a more cautious interpretation is required, as the evidence is based on a small subset of patients. Additionally, more clinical evidence is needed to validate the conclusions of this study.

5 Conclusion

For unresectable CRLM patients without prior systemic therapy, local treatment or targeted therapy plus chemotherapy are optimal. R/A combined with SingleCT or CTFU performed best for OS, while Atezolizumab + Bevacizumab + ICTFU was the best for PFS. For maintenance treatment, Bevacizumab + SingleCT was optimal for OS, and Adavosertib for PFS. Cetuximab + CTFU was the best choice for RAS/RAF wild-type patients. Combination therapy resulted in more SAEs compared to standard chemotherapy, with Bevacizumab + chemotherapy being the safest among targeted combinations. Our findings offer additional supporting evidence for current guideline recommendations.

Data availability statement

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

Author contributions

YJ: Writing–original draft, Writing–review and editing. TS: Writing–original draft, Writing–review and editing. MZ: Software, Writing–review and editing. YX: Supervision, Writing–review and editing. XZ: Funding acquisition, Supervision, Writing–review and editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2024.1374136/full#supplementary-material>

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Glossary

CRLM	colorectal liver metastases
RCTs	randomized controlled trials
OS	overall survival
PFS	progression-free survival
ORR	objective response rate
SAE	adverse effects graded as 3 or above/severe adverse effects
HRs	Hazards Ratios
CI	confidence intervals
ORs	odds ratios
SingleCT	single-drug chemotherapy
TAR	Targeted therapy
MultiCT	multi-drug chemotherapy
R/A	Resection or ablation
ICTFU	intensified fluorouracil-based combination chemotherapy
MultiTAR	multi-targeted therapy
CTFU	fluorouracil-based combination chemotherapy
CTCA	capecitabine-based combination
CRC	colorectal cancer
MRI	magnetic resonance imaging
TACE	transarterial chemoembolization
AEs	adverse effects
EGFR	epithelial growth factor receptor
ICB	immune checkpoint blockade
MSI	microsatellite instability
MMR	mismatch repair
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
IPD	individual patient data
ROB	risk of bias
NMA	network meta-analysis
SUCRA	surface area under the cumulative ranking
mCRC	metastasis CRC
ICI	immune checkpoint inhibitor
HAI	hepatic artery infusion
SIRT	selective internal radiation therapy
TSU-68	Orantinib
TAS-102	trifluridine/tipiracil
S1	combined chemotherapy of tegafur, gimeracil, and oteracil



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Diversified applications of hepatocellular carcinoma medications: molecular-targeted, immunotherapeutic, and combined approaches

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Hepatocellular carcinoma (HCC) is one of the primary forms of liver cancer and is currently the sixth most prevalent malignancy worldwide. In addition to surgical interventions, effective drug treatment is essential for treating HCC. With an increasing number of therapeutic drugs for liver cancer undergoing clinical studies, the therapeutic strategies for advanced HCC are more diverse than ever, leading to improved prospects for HCC patients. Molecular targeted drugs and immunotherapies have become crucial treatment options for HCC. Treatment programs include single-agent molecular-targeted drugs, immunotherapies, combinations of immunotherapies with molecular-targeted drugs, and dual immune checkpoint inhibitors. However, further exploration is necessary to determine the optimal pharmacological treatment regimens, and the development of new effective drugs is urgently needed. This review provides an overview of the current globally approved drugs for liver cancer, as well as the latest advances in ongoing clinical research and drug therapies. Additionally, the review offers an outlook and discussion on the prospects for the development of drug therapy approaches for HCC.

KEYWORDS

hepatocellular carcinoma, molecular-targeted drugs, sorafenib, immunotherapeutic agents, combination therapies

Highlights

- Drug treatment remains an essential approach to treating HCC besides surgical interventions, and the therapeutic strategies for advanced HCC are more diversified than before.
- Molecular targeted drugs and immunotherapies used alone or in combination with each other have been crucial therapeutic options for the treatment of HCC.
- The optimal pharmacological treatment regimens for HCC necessitate further exploration, and the new effective drugs need to be developed urgently.

Introduction

Liver cancer remains a major global health challenge, and its incidence is increasing worldwide (Vogel et al., 2022). It is projected that the annual incidence of new liver cancer cases will rise by over half in the next 10–20 years (Rumgay et al., 2022). Hepatocellular carcinoma (HCC) is the most common form of liver cancer, accounting for the majority of cases (Llovet et al., 2021). Viral hepatitis and cirrhosis are significant risk factors for HCC, with the Hepatitis B virus (HBV) being a more prominent risk factor than Hepatitis C virus (HCV) (Rumgay et al., 2022). HBV can integrate into the host genome, causing direct carcinogenic effects. Even in the absence of cirrhosis, HBV can induce hepatocarcinogenesis through mechanisms such as the activation of oncogenes and the inhibition of tumor suppressor genes (Jiang et al., 2021). HCV promotes HCC through chronic inflammation, oxidative stress, and fibrosis (Guntipalli et al., 2021). The burden of HCC caused by viral hepatitis is substantial. Non-alcoholic fatty liver disease (NAFLD) is also rapidly becoming the primary cause of HCC (Yip et al., 2022). Indeed, HCC is the fourth leading cause of cancer-related death across the world. The highest incidence and mortality of HCC are observed in East Asia and Africa, and these rates are also increasing in different parts of Europe and the USA (McGlynn et al., 2015). According to the Surveillance Epidemiology End Results (SEER), HCC is projected to become the third leading cause of cancer-related death by 2030 (Rahib et al., 2014). This review focuses specifically on the recent advancements in drug therapies for hepatocellular carcinoma (HCC) and their impact on treatment outcomes.

Accurate staging is essential for determining the most appropriate treatment for HCC due to its diverse causes and risk factors. The Barcelona Liver Cancer Clinic (BCLC) staging algorithm, proposed in 1999, is the most widely used system. It classifies patients into five stages (BCLC-0, A, B, C, or D) (Llovet et al., 1999; European Association for the Study of the Liver/European Association for the Study of the Liver, 2018). Curative treatment options such as surgical resection, ablation, and liver transplantation are recommended for patients with early-stage liver cancer (single liver tumors or up to 3 nodules $\leq$ 3 cm), which have been shown to produce the best results with a 5-year survival rate of about 70%–80% (European Association for the Study of the Liver/European Association for the Study of the Liver, 2018; Vogel et al., 2018; Marrero et al., 2018; Omata et al., 2017). While surgical resection and liver transplantation are curative options for early-stage HCC, many patients present with advanced disease where systemic therapies play a critical role.

Multiple systemic therapies have been approved in phase III clinical. These include first-line treatments like sorafenib, lenvatinib, atezolizumab plus bevacizumab, and tremelimumab plus durvalumab, as well as second-line treatments like regorafenib, cabozantinib, and ramucirumab (Llovet et al., 2021; Sonbol et al., 2020). On this basis, the U.S. Food and Drug Administration (FDA) has granted accelerated approval of nivolumab, pembrolizumab, and nivolumab plus ipilimumab as second-line treatments. Following systematic evaluation, the atezolizumab and bevacizumab combination is now considered the standard of care for first-line treatment of advanced HCC patients (Sonbol et al., 2020). Moreover, combination therapies involving anti-angiogenesis agents with immune checkpoint inhibitors (ICIs), dual ICIs, and targeted

agents in conjunction with surgery or other loco-regional therapies have shown promise and provided the basis for clinical trials (El-Khoueiry et al., 2022).

Despite the availability of several systemic therapies, challenges such as drug resistance and limited efficacy in certain patient populations underscore the urgent need for novel therapeutic approaches. New therapeutic strategies may focus on enhancing treatment efficacy and overcoming drug resistance to expand treatment options, ultimately improving the survival of patients with HCC. This review summarizes the drugs approved for first-line and second-line treatment of HCC in recent years, including their approval dates, targets and mechanisms of action, limitations, and adverse reactions. The purpose is to provide information to support doctors and patients in choosing the best treatment drugs by summarizing the basic situation of existing drugs for liver cancer treatment and to provide directions for researchers to develop drugs with more significant effects and safer use based on current drug foundations.

Mechanisms of molecular and immunologically targeted therapies

Approved systemic therapies for liver cancer currently focus on targeting molecular receptors or the immune system. The mechanisms underlying the development of liver cancer are complicated and diverse. Preventing hepatitis and fatty liver can somewhat reduce the risk of HCC. However, for patients already diagnosed with cancer, molecular and immune therapies are effective measures.

Molecular targeted therapies, which aim to inhibit molecular pathways critical for tumor growth and maintenance, are the primary treatment approach for advanced HCC.

Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are crucial factors that play significant roles in the neovascularization, invasiveness, and metastatic potential of HCC. The VEGF receptors (VEGFR) are found on endothelial cells and initiate a cascade essential for angiogenesis, making them crucial drivers of tumor vascularization (Mabeta and Steenkamp, 2022). PDGF contributes to angiogenesis by recruiting pericytes and smooth muscle cells to nascent vascular sprouts. The aberrant expression of VEGFR and PDGF receptor (PDGFR) during hepatocarcinogenesis leads to increased angiogenesis formation and activation of the downstream RAS/MAPK pathway, promoting cellular proliferation (Zou et al., 2022; Babina and Turner, 2017). Therefore, drugs that inhibit VEGF and PDGF, such as sorafenib and lenvatinib, are effective therapeutic options for liver cancer (Wilhelm et al., 2008; Zhao et al., 2020).

The fibroblast growth factor (FGF) family comprises at least five fibroblast growth factor receptors (FGFR1–FGFR5) and over 20 homologous ligands. FGFR4 is the predominant receptor found in the human liver, and its endogenous ligand FGF19 is overexpressed to promote HCC survival and enhance its resistance to apoptosis (Yang et al., 2023). FGF8, FGF17, and FGF18 are involved in autocrine and paracrine signaling in hepatocellular carcinoma, augmenting tumor cell survival, angiogenesis, and neovascularization (Xie et al., 2020). These mechanisms underpin the use of FGFR-targeted inhibitors like lenvatinib and regorafenib in treating liver cancer (Zhao et al., 2020; Wilhelm et al., 2011).

MET is a tyrosine kinase receptor for hepatocyte growth factor (HGF). The activation of the HGF-MET pathway is closely associated with the development and progression of HCC. This pathway promotes cell proliferation by regulating cell morphology and motility (Guo et al., 2020). Additionally, elevated expression of HGF-MET correlates with poor survival outcomes in HCC patients (Wang et al., 2020). For HCC with high MET expression, cabozantinib is a therapeutic option. However, selective inhibitors targeting MET specifically are more likely to effectively suppress MET activity while minimizing off-target toxicity, making them the focus of current clinical research (Yang et al., 2023).

The RAS/RAF/MEK/ERK (MAPK) signaling cascade is an important pathway that regulates tumor cell proliferation and differentiation and is closely associated with the metastasis and progression of HCC (Moon and Ro, 2021). The aberrant RAS/MAPK pathway activation is strongly linked to various growth factors, such as epidermal growth factor (EGF), VEGF, PDGF, and HGF (Degirmenci et al., 2020). Consequently, blocking the MAPK signaling pathway represents a potentially effective therapeutic strategy for HCC. Sorafenib and regorafenib are effective inhibitors of the MAPK pathway by targeting RAF, providing a valuable approach in the treatment of liver cancer.

Other receptor tyrosine kinases, including KIT, RET, and AXL, also play crucial roles in developing and progressing HCC (Huang et al., 2020). Aberrant expression of KIT in some HCC patients is associated with specific HCC subtypes, impacting cancer invasiveness and prognosis. Additionally, KIT can influence HCC invasion and metastasis by regulating the expression of genes related to epithelial-mesenchymal transition (EMT) (Pathania et al., 2021). RET, an oncogene-encoded receptor tyrosine kinase, is known for its role in the development and maintenance of the nervous system. However, increasing evidence indicates that RET is also involved in tumor growth and metastasis across various cancer types (Takahashi, 2022). AXL, a TAM receptor tyrosine kinase family member, was initially identified for its role in embryonic development and immune regulation. Nonetheless, its abnormal expression is associated with tumor invasiveness, resistance, and poor prognosis in several cancers, including HCC (Zhu et al., 2019). Cabozantinib, which can target and inhibit KIT, RET, and AXL receptor tyrosine kinases, has been approved as a second-line treatment for HCC that is resistant to sorafenib.

Tumor cells can evade the immune system by hijacking the programmed death-1 (PD-1)/programmed death ligand 1 (PD-L1) pathway or other mechanisms. This aids in tumor development and progression (Yi et al., 2022). To enhance tolerance to harmless foreign molecules, the liver typically maintains an anti-inflammatory environment by expressing and secreting inhibitory molecules (Heymann and Tacke, 2016). Similarly, HCC exhibits an inflammatory yet suppressive immune environment, which is conducive to stronger and more durable responses to immune checkpoint inhibitors. Moreover, the liver contains a substantial number of immune cells, making HCC patients suitable candidates for immunotherapy (Sangro et al., 2021). Several immunotherapy drugs have been approved for first- and second-line treatment of HCC. These drugs primarily target established checkpoints such as PD-1 ligands, PD-L1/2 receptors, and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) receptors (Ringelhan et al., 2018).

Developing immune-based treatments for HCC has also accelerated the emergence of combination therapies involving

immune-related approaches. For instance, dual immune checkpoint inhibitors have proven to be an effective combination therapy. This targets the PD-1/PD-L1 pathway and CTLA-4 receptors, significantly suppressing immune evasion in HCC (Song X. et al., 2023; Yau et al., 2020). Additionally, VEGF inhibitors can create an inflammatory microenvironment that enhances the efficacy of immune checkpoint inhibitors. The use of this combined therapy has demonstrated strong therapeutic effects. It is the mechanism behind the current standard therapy for HCC, atezolizumab plus bevacizumab (Rimini et al., 2022).

Furthermore, we have depicted the targets of drugs currently approved for first-line or second-line treatment of HCC in Figure 1.

Molecular-targeted monotherapies

Molecular targeted therapy has been a focal point in HCC research. Since the multikinase inhibitor sorafenib was approved for HCC treatment, an increasing number of molecular drugs have shown excellent therapeutic effects in clinical trials for HCC. These include anti-angiogenic drugs such as bevacizumab, tyrosine kinase inhibitors like cabozantinib, and epidermal growth factors inhibitors such as lenvatinib and regorafenib. The application of molecular targeted drugs in liver cancer treatment offers patients more precise, personalized, and effective therapeutic options, with the potential to improve both the quality of life and survival period. However, it is crucial to closely monitor patients' treatment responses and drug side effects to ensure the safety and efficacy of the treatment. This section focuses on molecular targeted drugs as a standalone therapy, as shown in Table 1.

Sorafenib

Sorafenib, the first approved treatment for HCC, owes its efficacy to its inhibition of multiple kinase targets, which promotes cell apoptosis, reduces angiogenesis, and inhibits tumor cell proliferation. Therefore, sorafenib has been the mainstay of treatment for a decade (Tang et al., 2020). The approval of subsequent drugs has primarily been based on comparisons with the efficacy of sorafenib, further underscoring its pivotal role in treating HCC. Sorafenib targets and inhibits several key kinases and receptors involved in cancer cell growth and survival, including RAF kinase, (Liu et al., 2006), VEGFR1-2, PDGFR, KIT, and RET proto-oncogene receptor tyrosine kinase (Wilhelm et al., 2008). It can be used to treat various cancers, including liver, (Llovet et al., 2021), kidney, (Escudier, 2007), and thyroid cancers (Brose et al., 2014). In advanced HCC, sorafenib is currently the effective first-line treatment drug (Tang et al., 2020). It is worth noting that sorafenib is the first targeted therapy drug that has shown efficacy in patients with advanced HCC.

In one key SHARP study, the median overall survival (OS) for HCC patients in the sorafenib group was 10.7 months, while the patients who received a placebo were 7.9 months (Llovet et al., 2008). A parallel Phase 3 study also showed similar results (Ren et al., 2021; Cheng et al., 2009). Although several clinical trials have demonstrated the efficacy of sorafenib in improving OS and progression-free survival in patients with advanced HCC, the

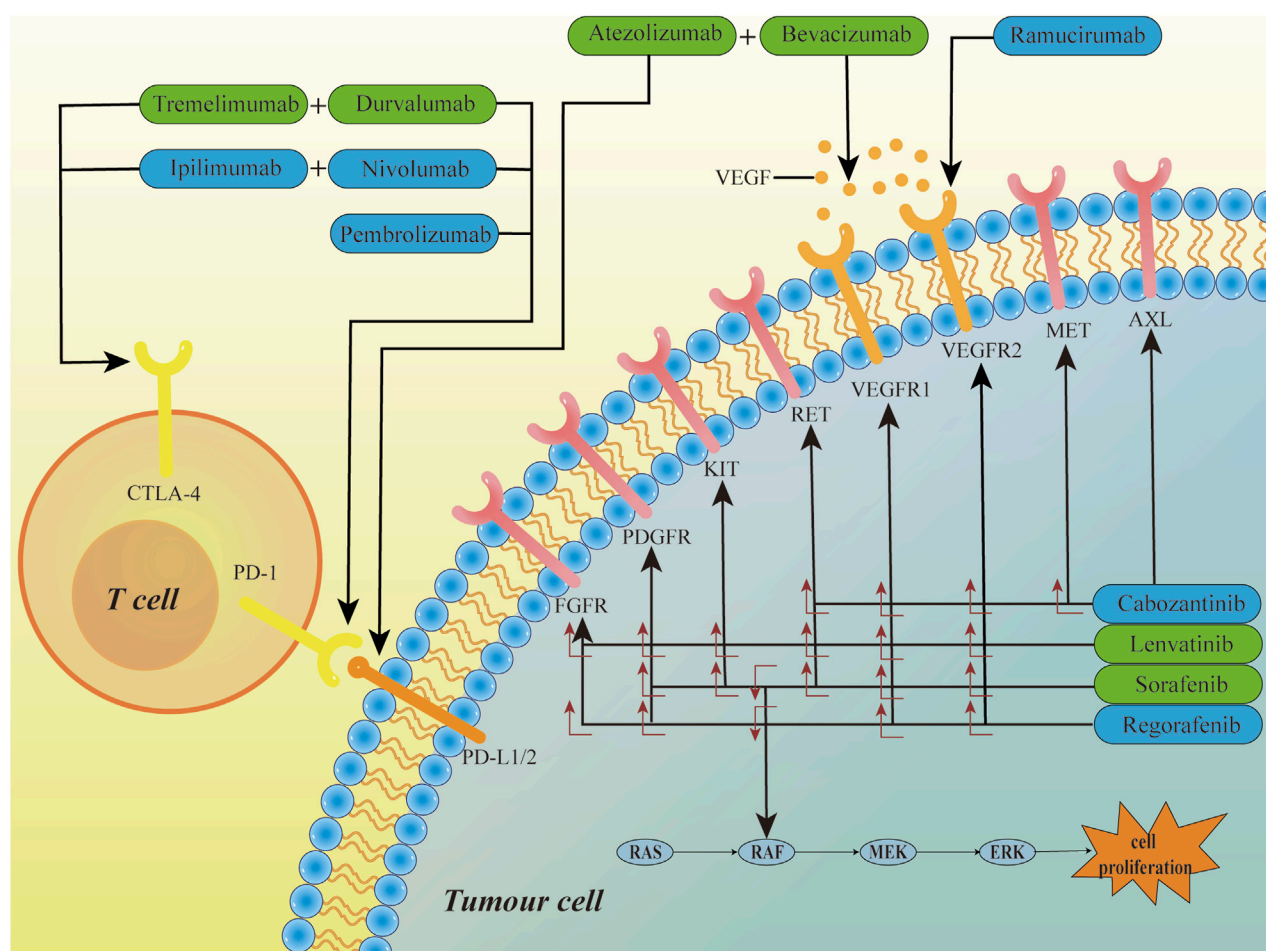


FIGURE 1

The mechanism of HCC targeted therapy drugs. Sorafenib, lenvatinib, cabozantinib, and regorafenib are small-molecule multi-kinase inhibitors that target multiple kinase receptors, inhibiting tumor growth by blocking biological processes such as angiogenesis. Bevacizumab and Ramucirumab are kinase inhibitors, targeting the VEGF ligand and VEGFR2, respectively. Atezolizumab, Durvalumab, Nivolumab, and Pembrolizumab are immune checkpoint inhibitors that exert their effects by inhibiting the PD-1/PD-L1/2 signaling pathway. Tremelimumab and Ipilimumab exert their effects by inhibiting the CTLA-4 receptors. Green boxes represent first-line treatment drugs, blue boxes represent second-line treatment drugs, and the symbol “+” indicates combination therapy.

reasons for the extension of survival are likely multifactorial, including differences in inclusion criteria and the use of effective sequential therapies (Huang et al., 2020; Cheng et al., 2009). Currently, the clinical benefit of sorafenib is still limited, with only about 30% of patients benefiting from it, and this population usually develops resistance within 6 months (Cheng et al., 2020; Xia et al., 2020; Méndez-Blanco et al., 2018). Sorafenib may cause adverse reactions in some patients, and the frequency and severity of these adverse reactions vary among individuals, mainly including gastrointestinal (diarrhea and weight loss) and hand-foot skin reactions (Rimassa et al., 2019; Li Y. et al., 2015). In severe cases, sorafenib can cause hypertension and abdominal pain, leading to treatment discontinuation (Colagrande et al., 2015; Fu et al., 2018).

However, sorafenib remains an important treatment option for advanced HCC patients, and ongoing research is focused on developing more effective and personalized treatment strategies for this disease. The complex molecular pathogenesis of HCC has stimulated research on combinations of sorafenib with other molecularly targeted drugs. Sorafenib has been combined with

antiangiogenic agents, (Morse et al., 2019), MEK/ERK pathway inhibitors, (Huynh et al., 2019), mTOR pathway inhibitors, (Wang C. et al., 2019), histone deacetylase inhibitors, (Chang et al., 2020; Freese et al., 2019) EGF/EGF receptor (EGFR) pathway inhibitors, (Dong et al., 2020), and HGF/c-MET pathway inhibitors (Goyal et al., 2013). Other agents such as interferon, (Tan et al., 2019; Wang M. et al., 2019), selumetinib, (Tai et al., 2016), capecitabine, (Patt et al., 2017), tegafur-uracil, (Azim et al., 2018), gemcitabine and oxaliplatin (GEMOX), (Assenat et al., 2019), and gemcitabine alone have also been evaluated. Still, to date, no treatments involving combinations containing sorafenib have succeeded in phase III trials.

Lenvatinib

Before lenvatinib was approved for the treatment of HCC, sorafenib has consistently been the preferred first-line treatment drug for HCC. Lenvatinib was approved based on its non-inferiority

TABLE 1 Summary of molecular-targeted monotherapies for advanced HCC.

Drugs	Classification	Target and mechanism of action	Clinical stage	Study name	Application	MOS (months)	MPFS (months)	TRAES	Refs
Sorafenib	protein kinase inhibitors	RAF, VEGFR1-2, PDGFR, KIT and RET	FDA-approved (2007)	SHARP	First-Line	10.7	4.1	Gastrointestinal, hand-foot skin reactions, abdominal pain, and hypertension	Llovet et al. (2008)
Lenvatinib		VEGFR1-2, FGFR, PDGFR, RET and KIT	FDA-approved (August 2018)	REFLECT	First-Line	13.6	7.4	Liver failure, heart failure, renal failure, hypertensive crisis, and bleeding	FDA, 2018a; Kudo et al. (2018)
Regorafenib		VEGFR1-2, FGFR, PDGFR, RAF, BRAF and KIT	FDA-approved (April 2017)	RESORCE	Second-line	10.6	3.1	Fatigue, hypertension, diarrhea, hand-foot skin reaction, hepatotoxicity, and bleeding	Bruix et al., 2017; FDA (2017a)
Cabozantinib		VEGFR1-2, RET, MET and AXL	FDA-approved (March 2019)	CELESTIAL	Second-line	10.2	5.2	Diarrhea, hypertension, hand-foot skin reaction, bleeding, hypothyroidism, ONJ and RPLS.	Abou-Alfa et al. (2018), FDA (2019a)
Donafenib ^a		VEGFR, PDGFR and RAF	UMPA-approved (January 2021)	ZGDH3	First-Line	12.1	3.7	Hand-foot skin reactions, diarrhea, hypertension, alopecia, rash, decreased appetite	Qin et al. (2021a)
Ramucirumab	monoclonal antibodies	VEGFR2	FDA-approved (May 2019)	REACH-2	Second-line	8.5	2.8	Fatigue, peripheral edema, hypertension, abdominal pain, decreased appetite, proteinuria, ascites, hyponatremia, hypoalbuminemia, and thrombocytopenia	Chau et al., 2017; FDA (2019b)

HCC, Hepatocellular carcinoma; FDA, Food and Drug Administration; MOS, Median overall survival; MPFS, Median progression-free-survival; TRAES, Treatment-related adverse events; ONJ, Osteonecrosis of the jaw; RPLS, Reversible posterior leukoencephalopathy syndrome.

^a: Approved by the National Medical Products Administration (NMPA) of China.

to sorafenib (Vogel et al., 2021). Lenvatinib is also a multitargeted tyrosine kinase inhibitor (TKI) that targets various receptors, including VEGFR1-2, FGFR, PDGFR, the RET proto-oncogene receptor tyrosine kinase, and KIT proto-oncogene receptor tyrosine kinase (Zhao et al., 2020). By inhibiting these receptors, lenvatinib can help reduce the growth and spread of liver cancer cells. Specifically, it works by blocking the formation of new blood vessels that provide nutrients and oxygen to the tumor, a process known as angiogenesis. Without adequate blood supply, the ability of the tumor to grow and spread is diminished. Lenvatinib can be used to treat various types of cancer, including thyroid (Ferrari et al., 2021; Fogli et al., 2021), kidney, (Roviello et al., 2018), and liver cancer. The FDA has approved lenvatinib for the first-line treatment of advanced HCC (Yamazaki et al., 2019; FDA, 2018a).

In clinical trials, lenvatinib has been shown to be effective in treating advanced HCC. A parallel Phase 3 study found a statistically significant improvement in OS with lenvatinib compared to placebo in patients with unresectable HCC who had not previously received systemic therapy (Kudo et al., 2018). In the phase 3 REFLECT study, which primarily recruited Asian patients, lenvatinib demonstrated noninferiority to sorafenib in first-line treatment, the median OS in the lenvatinib group was 13.6 months compared to 12.3 months in the sorafenib group (Vogel et al., 2021). For the key secondary endpoints, median progression-free survival, and overall response rate, lenvatinib was superior to sorafenib (Arai et al., 2017). Recently, it has been reported that compared to sorafenib, lenvatinib has demonstrated even better clinical outcomes in the treatment of unresectable hepatitis B virus-related HCC (Choi et al., 2022).

Lenvatinib's effectiveness against HCC is encouraging, but adverse events during treatment are common and often lead to dose interruption or treatment discontinuation. Common side effects of lenvatinib include fatigue, nausea, diarrhea, hypertension, and decreased appetite. In addition to these common side effects, lenvatinib may cause more severe side effects in some patients, including bleeding or blood clots, liver problems (such as elevated liver enzymes or liver failure), heart problems (such as heart attacks or heart failure), kidney problems (such as renal failure or proteinuria), and hypertensive crisis (Rimassa et al., 2019; Fogli et al., 2021; Arai et al., 2017; Reed et al., 2020; Kim et al., 2022; Nervo et al., 2021). A lower dose that can guarantee efficacy and improve tolerance is being explored, considering also the potential activity of lenvatinib in controlling primary and secondary brain tumors. (Wang R. et al., 2019).

Overall, lenvatinib is considered an important treatment option for advanced HCC patients who are not candidates for surgery or liver transplantation and is typically used as a first-line therapy. However, more research is still needed to explore whether available biomarkers can predict lenvatinib's efficacy response and resistance to define patient populations better and avoid unnecessary risks (Catalano et al., 2021).

Regorafenib

Regorafenib is a small molecule inhibitor of multiple kinases, including those involved in angiogenesis and tumor growth (Grothey et al., 2020). Specifically, regorafenib inhibits the activity of VEGFR, PDGFR, and FGFR, all of which are involved in angiogenesis, and other kinases involved in cell proliferation and

survival, such as RAF, BRAF and KIT (Wilhelm et al., 2011). Clinical studies have shown that regorafenib is effective in treating HCC patients who have received sorafenib. In a phase III clinical trial, regorafenib was shown to improve OS in HCC patients previously treated with sorafenib significantly. In this study, the median OS of patients receiving regorafenib was 10.6 months compared to 7.8 months for patients receiving the placebo (Bruix et al., 2017). In 2017, regorafenib was approved by the FDA for the treatment of advanced HCC in patients who are intolerant to sorafenib and have progressed on that therapy (FDA, 2017a). The subsequent success of regorafenib in HCC patients who progress on sorafenib treatment heralded a new era of second-line treatment and was quickly followed by ramucirumab, cabozantinib, and the most influential, ICIs. Besides, regorafenib has also been approved for the treatment of metastatic colorectal cancer that has progressed on other therapies, as well as for the treatment of advanced gastrointestinal stromal tumors that are no longer sensitive to imatinib and sunitinib successively (Grothey et al., 2013; Huemer et al., 2020; Demetri et al., 2013). Overall, regorafenib has demonstrated significant efficacy in various types of cancers that are resistant or have progressed after treatment, making it an important second-line therapy for multiple cancers, including HCC (Grothey et al., 2020).

Like all cancer treatments, regorafenib has side effects. Some common side effects of regorafenib in HCC include fatigue, hypertension, diarrhea, and hand-foot skin reaction (also known as palmar-plantar erythrodysesthesia) (McLellan et al., 2015). Additionally, regorafenib can cause more serious side effects in some patients. These include hepatotoxicity (liver damage or failure), bleeding (especially in patients with liver cancer or those previously treated with anticoagulants or antiplatelet agents), and worsening of cardiac issues (especially in patients with a history of heart disease or hypertension). To avoid serious adverse events, a strict treatment plan is necessary before starting regorafenib therapy (Li J. et al., 2015; Maeda et al., 2019).

Cabozantinib

Cabozantinib is a small molecule inhibitor of multiple tyrosine kinases, including VEGFR, hepatocyte growth factor receptor (HGFR/c-MET), AXL, and RET, cabozantinib works by inhibiting the activity of these tyrosine kinases, resulting in decreased cancer cell growth and proliferation, reduced angiogenesis, and increased cancer cell death (Yakes et al., 2011). Clinical trials have shown that cabozantinib is effective in treating advanced HCC, particularly in patients who have previously received sorafenib (Abou-Alfa et al., 2018). In 2019, cabozantinib was approved by the FDA for the treatment of advanced HCC in patients who have previously received sorafenib (FDA, 2019a). Cabozantinib is also used to treat renal cell carcinoma (RCC), (Tannir et al., 2017) and medullary thyroid carcinoma (MTC) (Brose et al., 2021). As a second-line therapy for HCC, cabozantinib exhibits side effects that are highly similar to those of regorafenib, including diarrhea, hypertension, hand-foot skin reaction, and bleeding (Rimassa et al., 2019; Zuo et al., 2015). Although cabozantinib has less hepatotoxicity than regorafenib, it may cause serious adverse reactions such as hypothyroidism

(low thyroid hormone levels), osteonecrosis of the jaw (a rare but serious condition that can cause jaw pain, swelling, and infection), and reversible posterior leukoencephalopathy syndrome (RPLS, a rare and potentially serious neurological disorder that can cause seizures, headaches, and changes in vision) (McGregor et al., 2022). Therefore, cabozantinib is a prescription drug that can only be taken under the guidance and supervision of a healthcare provider.

Donafenib

Donafenib is a deuterated sorafenib derivative and a novel multikinase inhibitor, including VEGFR, PDGFR, and Raf kinases (Keam and Duggan, 2021). The results of the phase III clinical trial, ZGDH3, have demonstrated that donafenib is superior to sorafenib in improving OS and has good safety and tolerability in Chinese patients with advanced HCC. It is expected to be a potential first-line monotherapy for these patients (Qin et al., 2021a). Based on these results, in June 2021, donafenib was approved for the first time in China for the treatment of uHCC in patients who have not received prior systemic therapy (Administration, 2021).

Ramucirumab

Ramucirumab is a monoclonal antibody that targets VEGFR-2. By binding to VEGFR-2, ramucirumab blocks signaling pathways that promote tumor angiogenesis, which helps slow cancer cells' growth and spread (Poole and Vaidya, 2014). Ramucirumab has been approved by the FDA for the treatment of various types of cancer, including gastric or gastroesophageal junction adenocarcinoma, (Fuchs et al., 2019), non-small cell lung cancer, (Arrieta et al., 2017) and colorectal cancer (Debeuckelaere et al., 2019). It is typically used in combination with chemotherapy but may also be used as a single agent in certain cases.

In a phase III clinical trial, patients with advanced HCC treated with ramucirumab had a median OS of 8.5 months and a median progression-free survival (PFS) of 2.8 months, which was better than the group of patients treated with the placebo (Chau et al., 2017). Therefore, ramucirumab was approved by the FDA for the treatment of advanced HCC in 2019, (FDA, 2019b) becoming an effective second-line treatment for HCC that is not a TKI.

Immunotherapy monotherapies

The normal human liver contains numerous immune cells, but immune suppression has been identified in HCC. This makes immunotherapy a potentially ideal treatment approach for HCC patients, particularly those who experience disease progression with traditional first-line drugs, especially those with severe cirrhosis or cardiovascular diseases and who are intolerant to other TKIs.

Immunotherapeutic drugs approved for HCC treatment mainly target PD1-PDL1/2 or CTLA-4. In this section, we will discuss immunotherapeutic drugs as a standalone therapy for HCC, as shown in Table 2. Additionally, immunotherapy may be associated with specific side effects, necessitating close monitoring of patients' responses during treatment.

Nivolumab

Nivolumab, marketed as Opdivo, is a PD-1 antibody, and the FDA granted accelerated approval in September 2017 for second-line treatment of advanced HCC patients who are resistant to sorafenib (FDA, 2017b). This is the first FDA-approved immune checkpoint inhibitor for HCC. The efficacy of immunotherapy for HCC is low, but the duration of effectiveness is relatively long. The function of nivolumab is to block the interaction between PD-1 and its ligands, PD-L1 and PD-L2, which are expressed in cancer cells and other cells in the tumor microenvironment (Abedi et al., 2022). By blocking PD-1, nivolumab helps release the immune system to attack cancer cells and shrink tumors.

In the CheckMate-459 trial, nivolumab was compared with sorafenib as a first-line treatment for advanced HCC. Compared to sorafenib, nivolumab did not improve OS in the overall population (Yau et al., 2022). However, nivolumab showed a significant improvement in OS in patients with high PD-L1 expression, a biomarker associated with better response to ICIs.

Overall, nivolumab is an important treatment option for advanced HCC, especially for patients with high PD-L1 expression levels. Ongoing research focuses on identifying biomarkers and combination therapies to improve the response of HCC and other types of cancer to nivolumab and other ICIs.

Pembrolizumab

Pembrolizumab is also an immune checkpoint inhibitor that targets the PD-1 receptor on immune cells. On 9 November 2018, the US FDA granted accelerated approval of the immunotherapy drug pembrolizumab (Keytruda) for the treatment of HCC (second-line therapy) in patients who are resistant to sorafenib (FDA, 2018b). In terms of clinical data, there is little difference between pembrolizumab and another drug, Opdivo (Zhu et al., 2018).

Recently, in the KEYNOTE-240 trial, the anti-tumor activity and safety of pembrolizumab have been further confirmed. The study showed that compared with placebo, pembrolizumab improved the OS of advanced HCC patients who had previously received sorafenib treatment (Finn et al., 2020a). Similarly, the latest clinical trial has demonstrated that Pembrolizumab can significantly improve OS and PFS in Asian patients (Qin et al., 2023). Overall, pembrolizumab is an important treatment option for advanced HCC, serving as a second-line therapy after sorafenib. Ongoing research is focused on identifying biomarkers and combination therapies to improve the response to pembrolizumab and other ICIs for HCC and other types of cancer.

Combination therapies

With the increasing resistance of HCC to single drugs, the effectiveness of a single agent becomes more limited. The spotlight is turning toward the combined use of drugs. Combining therapies contributes to better-individualized treatment. Depending on the patient's immune status, tumor genotype, phenotype, and other characteristics, a suitable combination of immune checkpoint inhibitors and molecular targeted drugs can be selected to maximize

TABLE 2 Summary of immunotherapy monotherapies for advanced HCC.

Drugs	Target and mechanism of action	Clinical stage	Study name	Application	MOS (months)	MPFS (months)	TRAES	Refs
Nivolumab ^a	Target: PD-1 Mechanism: immune checkpoint inhibitor	FDA-accelerated approval (September 2017)	CheckMate 459	Second-line	16.4	3.7	Fatigue, rash, pruritus, diarrhea, nausea, asthenia, cough, dyspnea, constipation, decreased appetite, arthralgia, upper respiratory tract infection, and pyrexia	Yau et al., 2020; FDA (2017b), Yau et al. (2022)
Pembrolizumab ^a	Target: PD-1 Mechanism: immune checkpoint inhibitor	FDA-accelerated approval (December 2018)	KEYNOTE 240	Second-line	13.9	3.0	Fatigue, nausea, constipation, dyspnea, vomiting, diarrhea, rash, and acute kidney injury	FDA, 2018b; Zhu et al. (2018), Finn et al. (2020a)

HCC, Hepatocellular carcinoma; FDA, Food and Drug Administration; MOS, Median overall survival; MPFS, Median progression-free-survival; TRAES, Treatment-related adverse events; ^a, Drugs granted accelerated approval for second-line treatment of HCC.

TABLE 3 Summary of combination therapies for advanced HCC.

Drugs	Advantages of combination	Clinical stage	Study name	Application	MOS (months)	MPFS (months)	TRAES	Refs
Atezolizumab + bevacizumab	Targeting both angiogenesis and PD-L1 signaling	FDA-approved (May 2020)	IMbrave150	First-Line	19.2	6.8	Hypertension, fatigue, bleeding and proteinuria	Finn et al., 2020b; FDA (2020a)
Tremelimumab + Durvalumab	Targeting PD-1 and CTLA-4 receptors on T cells	FDA-approved (October 2022)	HIMALAYA	First-Line	16.4	3.8	Rash, diarrhea, fatigue, pruritus, musculoskeletal pain, and abdominal pain	FDA (2022b)
Nivolumab ^a + Ipilimumab ^a	Targeting PD-1 and CTLA-4 receptors on T cells	FDA-accelerated approval (March 2020)	CheckMate040	Second-line	12.5–22.8	—	Diarrhea, fatigue, rash, pruritus, nausea, pyrexia, cough, decreased appetite, vomiting, abdominal pain, decreased weight, hypothyroidism, and dizziness	Yau et al., 2020; FDA (2020b)
Cabozantinib + Atezolizumab	Targets multiple kinase receptors and PD-L1 signaling	Phase III (September 2017)	COSMIC-312	First-Line	15.4	6.8	Diarrhoea, palmar-plantar erythrodysesthesia syndrome, decreased appetite, fatigue, hypertension, hypothyroidism	Kelley et al. (2022)
Pembrolizumab + Lenvatinib	Targets multiple kinase receptors and PD-1 receptor	Phase III (December 2018)	LEAP-002	First-Line	21.2	8.2	Hypothyroidism, diarrhoea, palmar-plantar erythrodysesthesia syndrome, decreased appetite, hypertension, fatigue, dysphonia	Llovet et al. (2023)

HCC, Hepatocellular carcinoma; FDA, Food and Drug Administration; MOS, Median overall survival; MPFS, Median progression-free-survival; TRAES, Treatment-related adverse events. ^a: Drugs granted accelerated approval for second-line treatment of HCC.

treatment effectiveness while minimizing toxic side effects. The most groundbreaking combination involves the use of the immune checkpoint inhibitor atezolizumab and the molecularly targeted drug bevacizumab, which has now become the standard therapy for advanced HCC. Additionally, dual immune checkpoint inhibitors and dual molecular targeted drugs demonstrate promising applications in liver cancer treatment. The relevant content of the combination therapy approach is illustrated in Table 3.

Atezolizumab plus bevacizumab

Following the approval of sorafenib and lenvatinib, the approval of atezolizumab plus bevacizumab indicates a new era in HCC treatment, where combination therapy is gradually becoming a more prominent treatment choice, replacing monotherapy. Atezolizumab is a monoclonal antibody that targets the PD-L1 protein on cancer cells and disrupts the interaction between PD-L1 and the PD-1

receptor on immune cells. PD-1 is a protein that helps regulate immune responses and prevents them from attacking normal cells in the body. In many types of cancer, including liver cancer, cancer cells can hijack the PD-1 pathway to evade detection and destruction by the immune system (Zhu et al., 2022). This interaction typically prevents immune cells from attacking cancer cells, but by blocking it, atezolizumab can activate the immune system and help it identify and destroy cancer cells (Markham, 2016; Frampton, 2020). Bevacizumab is a monoclonal antibody that targets the VEGF protein. By blocking VEGF, bevacizumab can reduce the blood supply to the tumor and prevent its growth (Garcia et al., 2020). However, due to an increased risk of bleeding with bevacizumab use, endoscopy is required within 6 months before enrollment, and it is strongly recommended that patients with portal hypertension be screened for varices prior to treatment (Cao et al., 2019; Li and Kroetz, 2018; Hsu et al., 2021).

In comparison to sorafenib, the combination of the VEGF-A antibody bevacizumab and the PD-L1 antibody atezolizumab demonstrated an improvement in OS (Ringelhan et al., 2018; Rimini et al., 2022). The IMbrave150 clinical trial, the first positive phase 3 study using an ICI-based regimen, (Galle et al., 2021; Qin et al., 2021b; Finn et al., 2020b), showed the combination therapy significant survival benefits compared to sorafenib. Atezolizumab in combination with bevacizumab was approved by the United States FDA in May 2020 for the treatment of unresectable or metastatic HCC (FDA, 2020a). The combination of atezolizumab and bevacizumab has been recognized by international guidelines as the new standard of care for first-line treatment of advanced HCC (Cheng et al., 2022).

Tremelimumab plus durvalumab

In recent times, immunotherapy has gained a significant role in the management of cancer. With Atezolizumab plus bevacizumab becoming the new first-line standard therapy for HCC, the combination of various drugs in treating HCC is now being given more attention. As clinical trials progress, dual immune checkpoint inhibitors have shown significant promise in the treatment of HCC. Dual ICIs such as tremelimumab plus durvalumab and nivolumab plus ipilimumab target proteins named PD-1 and CTLA-4 on T cells, respectively, helping to activate them to identify and attack cancer cells (Arru et al., 2021). Tremelimumab and durvalumab were approved by the FDA in the fall of 2022 for the treatment of metastatic non-small cell lung cancer (mNSCLC) in adult patients with sensitizing epidermal growth factor receptor mutations or anaplastic lymphoma kinase genomic tumor aberrations and unresectable HCC (uHCC) in adult patients with the same (FDA, 2022a; FDA, 2022b; Keam, 2023). In addition, a randomized phase III clinical trial named KESTREL showed that tremelimumab and durvalumab in combination displayed durable responses and reduced treatment-related adverse events (TRAEs) in recurrent or metastatic head and neck squamous cell carcinoma (R/M HNSCC), highlighting the promising potential of the combination therapy in multiple cancers (Psyrry et al., 2023).

In a phase III clinical trial named HIMALAYA, tremelimumab plus durvalumab was compared with sorafenib, the standard therapy

for advanced HCC. The trial included advanced HCC patients who had not received systemic therapy before. The results showed that the combination therapy improved OS and had manageable safety compared to sorafenib (Song et al., 2023; Kelley et al., 2021). Based on these positive results, the FDA granted breakthrough therapy designation for the combination therapy for the treatment of advanced HCC in 2019, (FDA, 2022b), following the latest standard first-line therapy atezolizumab plus bevacizumab. However, it is worth noting that tremelimumab plus durvalumab is currently limited to adult patients with uHCC (Song et al., 2023; FDA, 2022b).

Currently, the durvalumab monotherapy has not been approved. Some patients have contraindications to ICIs therapy, including those with severe autoimmune diseases and those who have undergone major organ transplants (Wichelmann et al., 2021). For these patients, monotherapy with sorafenib or lenvatinib is an appropriate first-line therapy.

Nivolumab plus ipilimumab

The mechanism of action of nivolumab and ipilimumab is to enhance the immune system's ability to attack cancer cells. By blocking the PD-1 receptor, nivolumab enables T cells to more effectively recognize and attack cancer cells. On the other hand, CTLA-4 is a protein that inhibits the immune system's response. By blocking CTLA-4 with ipilimumab, the immune system's response to cancer cells is enhanced. The combination of these two drugs has been demonstrated to effectively treat liver cancer, especially HCC.

Multiple clinical trials have studied the combination of nivolumab and ipilimumab for the treatment of HCC. In a recent phase II trial, the combination of nivolumab and ipilimumab was compared to sorafenib, the standard of care for advanced HCC (Yau et al., 2020). The study showed that the combination therapy significantly improved OS compared to sorafenib. It is important to emphasize that not all patients are suitable for this treatment.

In March 2020, the FDA granted accelerated approval for the use of the PD-1 inhibitor nivolumab in combination with the CTLA-4 inhibitor ipilimumab for the treatment of patients with HCC who have previously received sorafenib treatment (FDA, 2020b). Nivolumab plus ipilimumab is the first dual ICI therapy approved by the FDA for patients with HCC, and is the most effective immunotherapy regimen, even for lung cancer, (Hellmann et al., 2019) kidney cancer, (Motzer et al., 2018), and colon cancer, (Overman et al., 2018) with outstanding efficacy.

Cabozantinib plus atezolizumab

Currently, cabozantinib plus atezolizumab is being investigated as an emerging combination therapy for the treatment of advanced HCC (Esteban-Fabré et al., 2022). In a phase III clinical trial named COSMIC-312, previously untreated advanced HCC patients were randomly assigned to receive cabozantinib plus atezolizumab or sorafenib, the standard treatment for advanced HCC (Kelley et al., 2022). The study found that combination therapy was associated with improved OS and progression-free survival compared to

sorafenib. Based on these hopeful results, the FDA has granted breakthrough therapy designation for the use of cabozantinib plus atezolizumab to treat advanced HCC. This designation aims to accelerate the development and review of new therapies for severe or life-threatening diseases. However, further evaluation of the efficacy of cabozantinib plus atezolizumab in the treatment of advanced HCC is needed. In addition, the combination of cabozantinib and atezolizumab has shown promising results in the treatment of advanced RCC for patients who have previously received anti-angiogenic therapy (Pal et al., 2021).

Pembrolizumab plus lenvatinib

To improve the response to pembrolizumab or other ICIs for HCC. Combination therapies have become an essential choice in the treatment of HCC. Recently, in the KEYNOTE-524 trial, pembrolizumab was also studied as a first-line treatment for advanced HCC (Finn et al., 2020c). In this study, pembrolizumab was combined with the targeted therapy drug lenvatinib and compared with lenvatinib alone. The study found that the combination of pembrolizumab and lenvatinib improved progression-free survival and OS compared to lenvatinib alone (Sun et al., 2022).

Pembrolizumab showing promise in combination with lenvatinib as a new first-line treatment approach (Rizzo et al., 2022). To further evaluate the safety and efficacy of the pembrolizumab plus lenvatinib therapy, a phase III clinical trial named LEAP-002 is currently underway, lenvatinib plus pembrolizumab has promising antitumor activity in uHCC (Llovet et al., 2023).

Future perspectives

With the continuous in-depth research on liver cancer, an increasing number of emerging therapeutic methods and drugs are being discovered. Despite multiple first- and second-line regimens being approved for systemic treatment of HCC, the development of resistance and limitations of existing drug therapies for HCC remind us that we cannot stop researching HCC and the urgent need for more precise drug selection, the discovery, and development of drugs with more significant efficacy and safer usage to curb the incidence and progression of HCC in the population. A crucial aspect is the identification and discovery of biomarkers for drug treatment. For instance, mutations in the PI3K-AKT-mTOR pathway serve as genomic biomarkers for sorafenib treatment, while serum biomarkers like angiopoietin-2 (ANG2) and fibroblast growth factor 21 (FGF21) are associated with lenvatinib treatment (Choi et al., 2022; Song et al., 2023). In the realm of immunotherapy, in addition to the established PD-1 and PD-L1, there is potential for CD3 and CD8 to serve as biomarkers (Duffy et al., 2017; Sangro et al., 2020). Biomarkers facilitate personalized treatment, contributing to the specificity and efficacy of drug therapies. Detecting biomarkers enables the monitoring of tumor progression, facilitating adjustments in treatment plans. Furthermore, biomarkers are crucial in new drug development, aiding in assessing drug safety and efficacy.

Immunotherapy, as an emerging and effective cancer solution, holds tremendous potential. Expanding current immunotherapies in drug treatment for HCC is of significant importance. Different from traditional immunotherapeutic drugs, adoptive cell therapy (ACT) enhances the anti-tumor immune response by injecting immune cells, making it more suitable for solid tumors (Roddy et al., 2022). Therefore, ACT is considered feasible in treating liver cancer, and related clinical trials have shown promising results (Shi et al., 2020). Peptide vaccines can induce T-cell responses, enhancing specific anti-tumor immunity and represent a hopeful immunotherapeutic approach for HCC (Mizukoshi et al., 2015).

In addition to general combination therapies, sequential combination treatment is an emerging therapeutic approach that can maintain the synergistic effects of drugs while reducing the combined toxicity. Another similar strategy is alternate treatment, primarily targeting tumor sensitivity to one drug at the cost of developing resistance to another. However, the effectiveness of this approach in HCC is still under debate.

An iterative approach to targeted drugs has provided a wealth of data and encouraging results, particularly in those patients where resistance to sorafenib develops. The understanding and management of HCC have changed significantly due to extensive basic and clinical research over the last decade. However, HCC remains a devastating disease with a widespread and enormous impact on healthcare systems worldwide. In recent years, there have been significant advances in understanding the mechanisms that lead to rapid progress in treating patients with cancer and liver diseases. This collaborative effort involves oncologists, hepatologists, and basic scientists, and it provides hope for continued improvement in patient prognosis.

Author contributions

HC: Conceptualization, Data curation, Investigation, Methodology, Project administration, Software, Visualization, Writing—original draft, Writing—review and editing. HL: Conceptualization, Investigation, Methodology, Supervision, Writing—review and editing. XZ: Conceptualization, Investigation, Methodology, Supervision, Writing—review and editing. SW: Conceptualization, Funding acquisition, Investigation, Supervision, Writing—review and editing. CL: Conceptualization, Investigation, Methodology, Supervision, Writing—review and editing. KA: Conceptualization, Investigation, Supervision, Writing—review and editing. RL: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing—original draft, Writing—review and editing. XT: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing—original draft, Writing—review and editing.

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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.

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Glossary

HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
NAFLD	Non-alcoholic fatty liver disease
SEER	Surveillance Epidemiology End Results
BCLC	Barcelona Liver Cancer Clinic
FDA	Food and Drug Administration
ICIs	Immune checkpoint inhibitors
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
HGF	Hepatocyte growth factor
RET	Rearranged during transfection
PD-1	Programmed death-1
PD-L1	Programmed death ligand 1
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
OS	Overall survival
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GEMOX	Gemcitabine and oxaliplatin
TKI	Tyrosine kinase inhibitor
mNSCLC	Metastatic non-small cell lung cancer
uHCC	Unresectable HCC
TRAEs	Treatment-related adverse events
R/M HNSCC	Recurrent or metastatic head and neck squamous cell carcinoma
HGFR	Hepatocyte growth factor receptor
RCC	Renal cell carcinoma
MTC	Medullary thyroid carcinoma
RPLS	Reversible posterior leukoencephalopathy syndrome
PFS	Progression-free-survival
METIV-HCC	High MET expressing HCC
ANG2	Angiopoietin-2
FGF21	Fibroblast growth factor 21
ACT	Adoptive cell therapy



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RGD-based self-assembling nanodrugs for improved tumor therapy

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RGD-based self-assembling nanodrugs are a promising advancement in targeted cancer therapy, combining the specificity of RGD peptides with the benefits of nanotechnology. These nanodrugs enhance tumor targeting and cellular uptake while reducing off-target effects. RGD peptides facilitate the self-assembly of stable nanostructures, ensuring efficient drug delivery. Despite their potential, challenges such as immunogenicity, stability, tumor heterogeneity, and manufacturing scalability need to be addressed. Future research should focus on improving biocompatibility, advanced targeting strategies, personalized medicine approaches, and innovative manufacturing techniques. Overcoming these challenges will pave the way for the successful clinical translation of RGD-based nanodrugs, offering more effective and safer cancer treatments.

KEYWORDS

RGD peptides, self-assembling nanodrugs, tumor targeting, cancer therapy, integrin receptors

1 Introduction

Cancer remains a major global health challenge, responsible for millions of deaths each year. Traditional chemotherapy, while effective, often suffers from significant limitations including non-specific distribution, severe side effects, and drug resistance (Fang et al., 2023; Zhong et al., 2021; Yan et al., 2024; Boumahdi and de Sauvage, 2020). These issues have prompted the development of advanced drug delivery systems designed to enhance the efficacy and safety of cancer treatments (Agiba et al., 2024; Zhao et al., 2023; Zhang et al., 2023a; Liu N. et al., 2023; Zhang et al., 2023b; Tu et al., 2024). One such promising approach involves the use of nanotechnology to create self-assembled nanodrugs (Tian and Lu, 2022; Lu et al., 2024; Qiao et al., 2022).

Nanodrugs leverage the unique properties of nanoparticles to improve drug delivery. They can encapsulate chemotherapeutic agents, protecting them from degradation, enhancing their solubility, and allowing for controlled release (Chen et al., 2022; van der Meel et al., 2019; Haist et al., 2022). Among various strategies, self-assembled nanodrugs have gained particular attention due to their ability to form well-defined structures through spontaneous organization of their components (Huang et al., 2021; Liu Y. et al., 2023; Li et al., 2024). This process, driven by non-covalent interactions such as hydrophobic effects, electrostatic interactions, and hydrogen bonding, enables the precise engineering of nanoparticle size, shape, and functionality.

A critical aspect of effective cancer therapy is the ability to target tumor cells specifically, thereby minimizing harm to healthy tissues (Leng et al., 2024; Liu et al., 2024). This is where RGD (arginine-glycine-aspartic acid) peptides come into play. RGD peptides are known to bind with high affinity to several integrin receptors, including $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha 5\beta 1$, $\alpha IIb\beta 3$, and $\alpha 8\beta 1$, all of which play crucial roles in tumor biology and beyond. Although $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins have been extensively studied in tumor targeting, emerging research highlights the importance of other RGD-binding integrins such as $\alpha 5\beta 1$ and $\alpha v\beta 6$, which may offer more specific and effective targeting strategies in cancer therapy (Kesharwani et al., 2024; Sanati et al., 2023; Nader et al., 2020; Rana et al., 2024). It is important to note that $\alpha v\beta 3$ integrin is not exclusively expressed by tumor cells. It is also found in various physiological processes, particularly in the vasculature, making tumor-specific targeting using $\alpha v\beta 3$ -directed approaches challenging. This poses a limitation in achieving selective tumor accumulation and may lead to off-target effects (Hosseinihah et al., 2024; Sheikh et al., 2021).

Integrin receptors play a crucial role in tumor angiogenesis, metastasis, and survival, making them attractive targets for cancer therapy (Liu F. et al., 2023; Marelli et al., 2013; Moreno-Layseca et al., 2019). The RGD motif mimics the natural ligands of these receptors, allowing for selective binding and internalization by tumor cells. This targeted approach not only improves the concentration of the drug at the tumor site but also reduces systemic toxicity and adverse side effects (Van Hove et al., 2021; Slack et al., 2022).

Recent advances in nanotechnology and peptide engineering have enabled the development of sophisticated RGD-based self-assembled nanodrugs (Kesharwani et al., 2024). These nanodrugs combine the targeting capabilities of RGD peptides with the versatile drug delivery properties of nanoparticles. They can be engineered to encapsulate a wide range of therapeutic agents, including small-molecule drugs, proteins, and nucleic acids, and deliver them directly to tumor cells.

In summary, RGD-based self-assembled nanodrugs represent a cutting-edge approach in cancer therapy. By harnessing the specificity of RGD peptides and the advantages of self-assembled nanostructures, these nanodrugs offer the potential for more effective and safer cancer treatments. The following sections will explore the mechanisms of RGD-based targeting, the process of self-assembly, the therapeutic benefits, and the recent advancements and future prospects in this exciting field.

2 Distinct advantages of RGD-based self-assembled nanodrugs

RGD-based self-assembled nanodrugs combine the advantages of targeted delivery with the benefits of nanotechnology, resulting in a highly effective therapeutic approach for cancer treatment. The RGD peptides play crucial roles in both tumor targeting and the self-assembly process, leading to unique advantages. Here, we detail the structure and distinct benefits of RGD-based self-assembled nanodrugs.

2.1 Types and variations of RGD

RGD peptides can exist in several forms and configurations, including linear and cyclic versions (Li et al., 2022). The primary

types of RGD peptides include linear RGD peptides and cyclic RGD peptides. Linear RGD peptides are straightforward to synthesize and modify. Alternatively, the cyclic RGD are cyclized through the formation of a disulfide bond or through head-to-tail cyclization, enhancing their stability and binding affinity by reducing the flexibility of the molecule. RGD peptides exist in various forms, including linear, cyclic, and multivalent configurations. Linear RGD peptides, while easy to synthesize, tend to be less stable in physiological environments. Cyclic RGD peptides, such as c(RGDfK), c(RGDyK), c(RGDyC), c(RGDfC), and iRGD, where “f” denotes D-phenylalanine, “y” denotes tyrosine, and “C” denotes cysteine, providing additional stabilization through disulfide bonds, offer improved stability and binding affinity due to their conformational rigidity. In addition to these, multivalent RGD peptides, which present multiple RGD motifs in a single structure, are being explored to enhance binding specificity and avidity for integrin-rich tumor cells, further expanding the toolbox for integrin-targeted therapies (Park et al., 2012; Sani et al., 2021). Notably, studies have shown that cyclic RGD peptides (cRGD) exhibit superior binding efficacy compared to linear RGD peptides due to their enhanced stability and conformational rigidity. Fu et al. (2021) demonstrated that liposomes modified with c(RGDfK) exhibited significantly higher cellular uptake and tumor inhibition in lung cancer models than those with linear RGD. Moreover, Bogdanowich-Knipp et al. (1999) found that cyclic peptides, such as cyclo-(1,6)-Ac-Cys-Arg-Gly-Asp-Phe-Pen-NH₂, displayed greater stability, particularly at neutral pH, contributing to their prolonged biological activity and enhanced integrin binding.

2.2 Tumor targeting ability of RGD peptides

2.2.1 High affinity and specificity

RGD peptides are known for their strong binding affinity to integrin receptors, particularly $\alpha v\beta 3$ and $\alpha v\beta 5$, which are overexpressed on the surface of various tumor cells and their vasculature. This high specificity ensures that RGD-based nanodrugs selectively accumulate in tumor tissues, enhancing the local concentration of the therapeutic agent and reducing systemic exposure. Recent studies suggest that integrins such as $\alpha 5\beta 1$ and $\alpha v\beta 6$, which are involved in critical tumor processes such as invasion, metastasis, and survival, may offer better specificity for tumor targeting. $\alpha 5\beta 1$, in particular, plays a key role in fibronectin-mediated cell adhesion, making it an attractive target in aggressive tumor types. Similarly, $\alpha v\beta 6$ integrin, which is upregulated in many epithelial cancers, has been identified as a potential marker for tumor progression and metastasis, providing a more selective targeting option in certain cancer types (Zhou et al., 2023; Niu and Li, 2017).

2.2.2 Enhanced cellular uptake

The interaction between RGD peptides and integrin receptors facilitates receptor-mediated endocytosis, significantly increasing the internalization of nanodrugs by tumor cells. This enhanced uptake ensures that a larger proportion of the drug reaches its intracellular targets, thereby improving its therapeutic efficacy. In addition to the design aspects of RGD-modified NPs, comparative studies between RGD-modified and non-RGD-modified

nanoparticles have demonstrated significant differences in cellular uptake and tumor targeting. RGD peptides, particularly in their cyclic forms, significantly enhance the interaction with integrin receptors (such as $\alpha\beta3$ and $\alpha\beta5$) that are overexpressed in tumor cells. For example, studies have shown that RGD-modified NPs exhibit increased cellular internalization through receptor-mediated endocytosis, resulting in higher drug accumulation in tumor tissues. In contrast, non-RGD-modified NPs primarily rely on passive targeting, which limits their cellular uptake and reduces their overall therapeutic efficacy (Chen et al., 2012).

2.2.3 Reduced off-target effects

By specifically targeting integrin receptors that are primarily expressed on tumor cells, RGD-based nanodrugs minimize interactions with normal tissues. This selective targeting reduces the adverse side effects commonly associated with conventional chemotherapy, leading to a better safety profile. While RGD-modified NPs exhibit superior targeting and cellular uptake, their biocompatibility and toxicity profiles are equally important for clinical translation. The biocompatibility of RGD-modified NPs has been shown to be generally favorable, with minimal immune response in short-term studies. However, long-term biocompatibility and potential toxicity, especially due to repeated administration, require further investigation. Some studies suggest that the use of cyclic RGD peptides reduces immunogenicity due to their stability, but systemic toxicity remains a concern if off-target interactions occur (Xu et al., 2023; Wang et al., 2022).

2.3 Self-assembly promotion of RGD peptides

2.3.1 Facilitated nanostructure formation

RGD peptides can influence the self-assembly process by promoting the formation of well-defined nanostructures. Their amphiphilic nature, with both hydrophilic and hydrophobic regions, assists in the spontaneous organization of drug molecules into stable nanoparticles through non-covalent interactions such as hydrophobic effects and hydrogen bonding.

2.3.2 Enhanced stability and solubility

The incorporation of RGD peptides into nanodrug formulations can improve the solubility and stability of hydrophobic chemotherapeutic agents. Self-assembled nanodrugs protect these agents from premature degradation and enhance their solubility in aqueous environments, facilitating their delivery to the tumor site.

2.3.3 Controlled drug release

Self-assembled nanodrugs can be engineered to provide controlled and sustained release of the encapsulated therapeutic agents. The presence of RGD peptides in the nanostructure can influence the release kinetics, ensuring a steady and prolonged delivery of the drug, which can enhance its therapeutic efficacy while minimizing peak-related toxicity.

2.3.4 Multifunctional nanocarriers

RGD-based self-assembled nanodrugs offer the potential for multifunctionality. They can be designed to carry multiple therapeutic agents, imaging agents, or targeting moieties within a

single nanocarrier. This multifunctionality enables simultaneous cancer treatment and diagnosis (theranostics), providing a comprehensive approach to cancer management.

In summary, the distinct advantages of RGD-based self-assembled nanodrugs lie in their ability to combine targeted delivery with the efficient encapsulation and controlled release of therapeutic agents. The RGD peptides not only enhance tumor specificity and cellular uptake but also promote the self-assembly of stable and functional nanostructures. These unique benefits make RGD-based self-assembled nanodrugs a promising approach for improving the outcomes of cancer therapy.

3 Recent advances and applications

Recent studies have demonstrated the efficacy of RGD-based self-assembled nanodrugs in various cancer models, showing significant improvements in targeting, penetration, and therapeutic outcomes. These nanodrugs leverage the high affinity of RGD peptides for integrin receptors, facilitating precise delivery to tumor cells and reducing off-target effects. Advances in nanotechnology have enabled the development of multifunctional nanodrugs that combine chemotherapy, phototherapy, and imaging capabilities. *In vitro* and *in vivo* experiments have confirmed their potential in enhancing drug accumulation at tumor sites, improving cellular uptake, and achieving sustained drug release, ultimately leading to superior antitumor activity and reduced systemic toxicity.

3.1 Linear RGD-based self-assembling nanodrugs

Ma et al. (2023) developed and synthesized the RGD-targeted self-assembling nanodrug involved the synthesis of an amphiphilic lipopeptide named P17 (Figure 1A), which integrated the RGD peptide and KLA peptide. The RGD peptide was chosen for its ability to target the $\alpha\beta3$ integrin receptor, overexpressed in tumor cells, enhancing tumor targeting and permeability. The KLA peptide was selected for its mitochondrial apoptosis-inducing properties (Figure 1B). P17 self-assembled into stable spherical aggregates in aqueous solution, capable of encapsulating anticancer drugs such as doxorubicin (Dox). The α -helical projection of P17 indicated amphiphilic properties, with one side hydrophilic and the other hydrophobic (Figure 1C). The 3D structure confirmed the α -helical formation, crucial for its membrane interaction (Figure 1D). TEM images showed regular monodisperse spheres with an average diameter of about 50 nm, supporting its suitability as a drug carrier (Figure 1E). Molecular docking patterns revealed that the RGD motif in P17 effectively interacted with the integrin $\alpha\beta3$ receptor through multiple hydrogen bonds, facilitating targeted delivery and penetration into tumor cells (Figure 1F). This interaction enhanced the therapeutic efficacy of the encapsulated Dox, resulting in effective tumor growth inhibition and reduced toxic side effects. The study had several strengths in developing the KLA-RGD integrated lipopeptide for targeted cancer therapy, but also exhibited some limitations. The major drawbacks included the relatively low encapsulation efficiency of the lipopeptide for the anticancer drug Dox and the limited *in vivo*

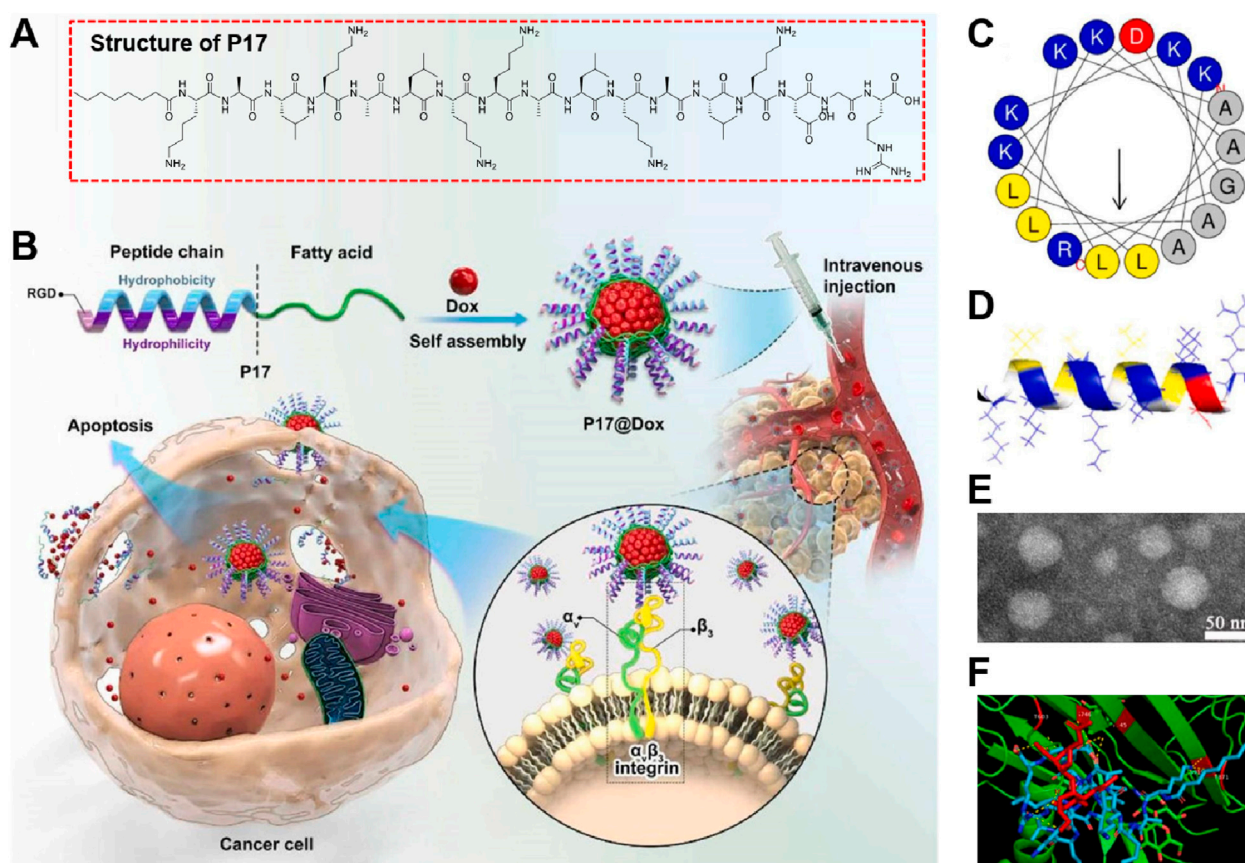


FIGURE 1

(A) Structure of P17 lipopeptide, showing the integration of RGD and KLA peptides modified with *n*-octanoic acid for improved stability and membrane binding capacity. (B) Schematic representation of P17 lipopeptide self-assembly and its encapsulation of doxorubicin (Dox) to form P17@Dox nanomedicine. The nanomedicine targets $\alpha v \beta 3$ integrin on cancer cells, facilitating drug delivery and inducing apoptosis. (C) α -Helical projection of the P17 lipopeptide, indicating its amphiphilic nature with distinct hydrophilic and hydrophobic sides. (D) 3D structure of P17, highlighting its α -helical configuration critical for membrane interaction. (E) Molecular docking pattern showing the interaction of P17 with the $\alpha v \beta 3$ integrin receptor via multiple hydrogen bonds, enhancing tumor targeting. (F) TEM image of P17 aggregates, illustrating regular monodisperse spherical morphology with an average diameter of approximately 50 nm, suitable for drug delivery applications Adapted with modification from Ma et al. (2023).

evaluation. Future research could have focused on optimizing drug loading efficiency and extending *in vivo* studies to different cancer models to better understand the therapeutic potential and safety profile of the nanomedicine.

In Zheng's design, the recombinant proteins, incorporating RGD peptides for targeting integrin $\alpha v \beta 3$, BAK for pro-apoptotic activity, GFP for tracking, and a histidine tag for purification, were engineered to self-assemble into protein nanoparticles (Lv et al., 2020). These nanoparticles demonstrated a uniform size of approximately 23 nm and stability in human serum. *In vitro* studies revealed that these nanoparticles exhibited significantly enhanced cellular uptake and cytotoxicity in tumor cell lines, including C6, C26, and MCF-7 cells, compared to nanoparticles without the RGD peptide (Figure 2A). Confocal microscopy confirmed the targeted internalization of the nanoparticles in tumor cells, indicating the effectiveness of the RGD peptide in enhancing tumor targeting. This research underscores the potential of RGD-based self-assembling protein nanoparticles for targeted cancer therapy by improving delivery and therapeutic efficacy. Notably, this method could be improved by conducting *in vivo* experiments to confirm the efficacy and safety of the RGD-based self-assembling protein nanoparticles in animal models.

Fang et al. (2017) have reported the design and development of a novel DOX-loaded RGD-terminated poly(ethylene glycol)-block-poly(trimethylene carbonate) (RGD-PEG-PTMC) polymeric micelle for targeted osteosarcoma chemotherapy. These RGD-installed micelles demonstrated enhanced tumor targeting and cellular uptake in osteosarcoma cells, with significantly lower IC_{50} values compared to non-targeted micelles (Figure 2B). The micelles showed efficient drug loading and release properties, with *in vitro* studies confirming their potential for improved and targeted osteosarcoma treatment. However, further *in vivo* studies are necessary to confirm their efficacy and safety in animal models. Similarly, Zhang and coworkers developed linear RGD-based self-assembling nanodrugs that enhanced tumor targeting and cytotoxicity *in vitro*, and Song's group created amphiphilic camptothecin nanoparticles with glutathione-responsive and tumor-targeting abilities, demonstrating prolonged circulation and improved colorectal cancer therapy (Shi et al., 2019; Song et al., 2023). However, both studies underscored the need for further *in vivo* validation.

The study by Xie et al. (2022) reported the development of a self-assembled nanodrug composed of IR825, IRI-ATRA, and

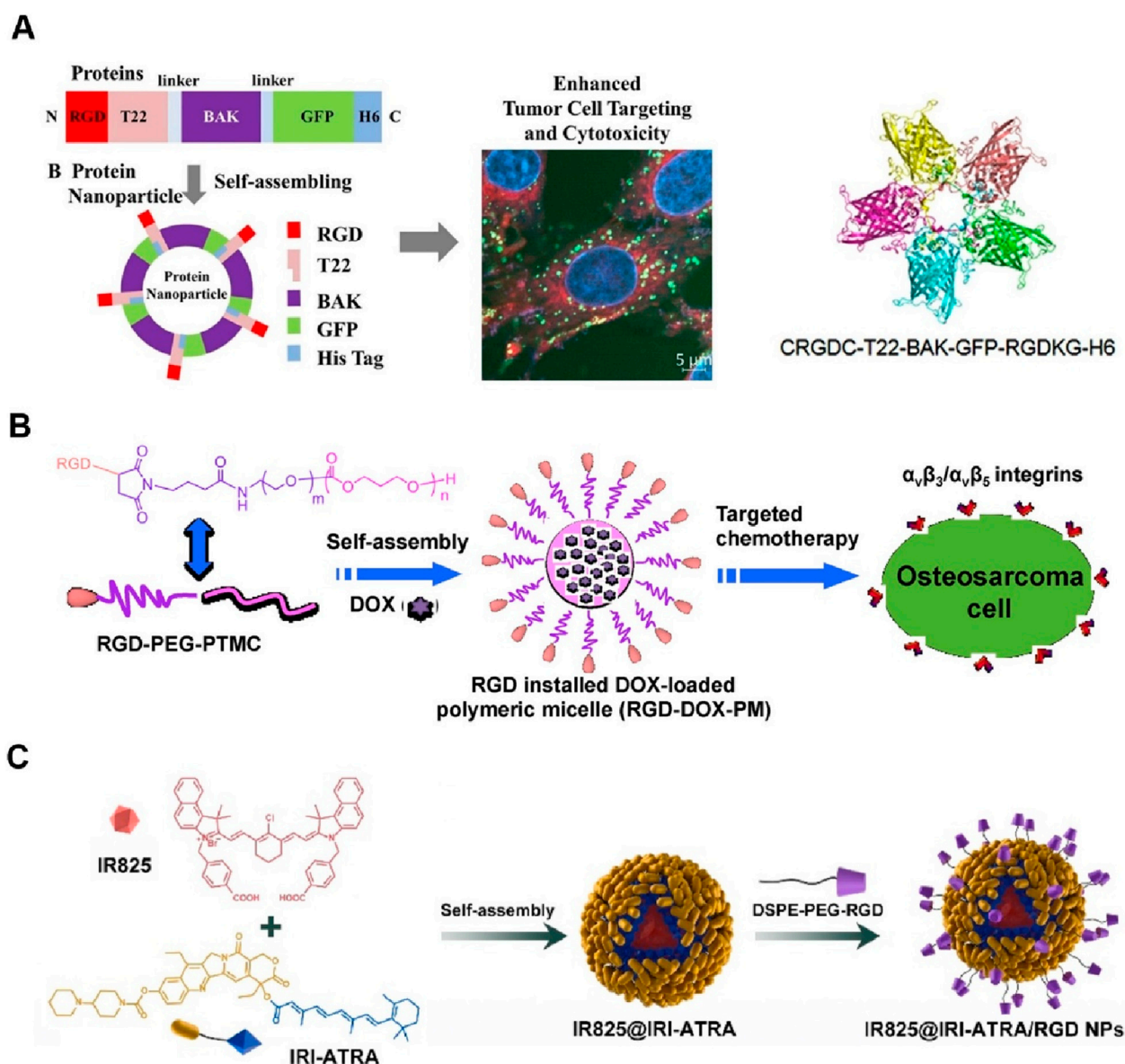


FIGURE 2

(A) Schematic illustration of the design and self-assembly of a protein nanoparticle incorporating RGD, T22, BAK, GFP, and His Tag for enhanced tumor cell targeting and cytotoxicity. The right panel shows the protein structure and a confocal microscopy image demonstrating targeted internalization in tumor cells Adapted with modification from Lv et al. (2020). (B) Diagram of the RGD-installed DOX-loaded polymeric micelle (RGD-DOX-PM) for targeted osteosarcoma chemotherapy. The RGD-PEG-PTMC micelle self-assembles and targets $\alpha_v\beta_3/\alpha_v\beta_5$ integrins on osteosarcoma cells, facilitating enhanced drug delivery Adapted with modification from Fang et al. (2017). (C) Formation process of IR825@IRI-ATRA/RGD NPs. IR825 and IRI-ATRA self-assemble into nanoparticles, which are then modified with DSPE-PEG-RGD to enhance tumor targeting and imaging capabilities Adapted with modification from Xie et al. (2022).

DSPE-PEG-RGD for the combination therapy of breast cancer stem cells. The nanodrug (IR825@IRI-ATRA/RGD NPs) exhibited self-assembly into nanoparticles, which demonstrated excellent tumor targeting and imaging capabilities. Upon cellular uptake, the nanoparticles effectively released their therapeutic agents in response to the acidic and esterase-rich tumor microenvironment (Figure 2C). The study highlighted significant cytotoxicity, enhanced cellular uptake, and superior photothermal properties, resulting in effective tumor growth inhibition and metastasis prevention. *In vivo* studies confirmed the potential of this

nanodrug for improved breast cancer therapy. The study could be improved by conducting more extensive *in vivo* experiments to evaluate long-term safety and efficacy, as well as exploring the potential for scaling up the synthesis process for clinical applications. Jia utilized ffKGRGD, a chain RGD peptide, as a tumor-targeting ligand to enhance the therapeutic efficacy of self-assembling nanodrugs. The ffKGRGD-modified nanodrugs significantly improved tumor targeting, cellular uptake, and antitumor activity in cancer models by promoting integrin receptor-mediated endocytosis (Jia et al., 2022).

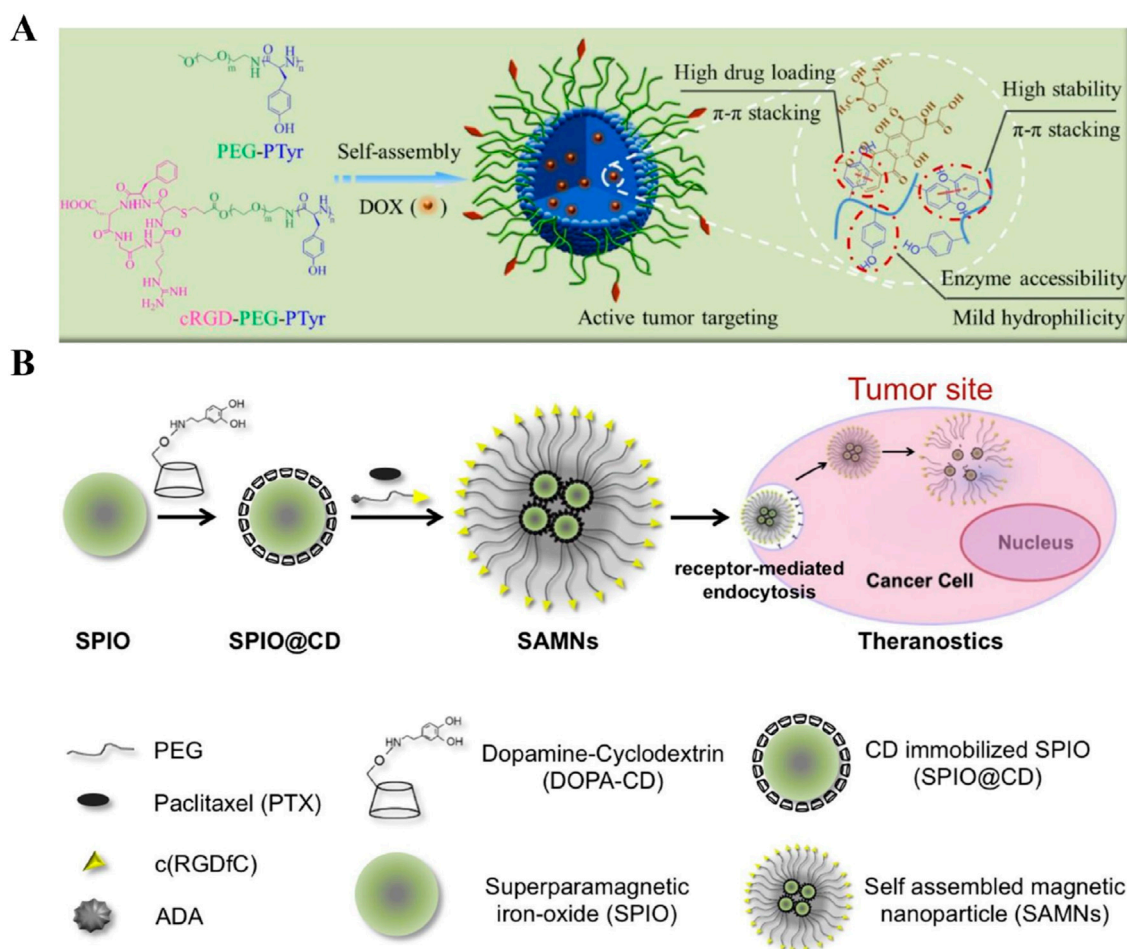


FIGURE 3
(A) Schematic of the formation of cyclo(RGDfC)-based self-assembling nanodrugs. Cyclo(RGDfC) peptides form stable nanoparticles through disulfide bonds, encapsulating chemotherapeutic agents like doxorubicin (DOX) for enhanced drug delivery Adapted with modification from [Gu et al. \(2019\)](#). **(B)** Illustration of the preparation of magnetic nanoclusters (SAMNs) coated with β-cyclodextrin (CD) and polyethylene glycol (PEG), followed by surface functionalization with cyclo(RGDfC). This functionalization improves cellular uptake, targeted delivery to integrin-rich cancer cells, and controlled release of paclitaxel (PTX), leading to better MRI contrast and therapeutic outcomes Adapted with modification from [Nguyen et al. \(2016\)](#).

3.2 Cyclo(RGDfC)-based self-assembling nanodrugs

Cyclo(RGDfC)-based self-assembling nanodrugs are innovative therapeutic agents for cancer treatment. The cyclic RGDfC peptides, stabilized by disulfide bonds, form stable nanostructures, enhancing drug delivery efficiency. These nanodrugs self-assemble into nanoparticles, encapsulating chemotherapeutic agents for controlled release, thus increasing drug concentration at the tumor site while minimizing systemic toxicity. Future research should optimize their stability, biocompatibility, and scalability for clinical applications.

[Gu et al. \(2019\)](#) have developed the cyclo(RGDfC)-based self-assembling nanodrugs for targeted cancer therapy. Cyclo(RGDfC) peptides, stabilized by disulfide bonds, form stable nanoparticles that enhanced drug delivery. These nanoparticles targeted αvβ3 and αvβ5 integrin receptors overexpressed on tumor cells, ensuring specific drug delivery. The amphiphilic peptides facilitated stable self-assembly and controlled release of doxorubicin (DOX) in the tumor microenvironment, increasing drug concentration at the

tumor site while minimizing systemic toxicity ([Figure 3A](#)). *In vitro* and *in vivo* experiments demonstrated superior antitumor activity, higher cellular uptake, and improved tumor targeting compared to non-targeted formulations. Despite promising results, challenges like optimizing biocompatibility, stability, and scalability need to be addressed for clinical translation. Such Cyclo(RGDfC)-based self-assembling nanodrugs have also been witnessed in several other studies ([Liang et al., 2016](#); [He et al., 2016](#); [Li et al., 2019](#)).

Alternatively, [Nguyen et al. \(2016\)](#) have developed self-assembling nanodrugs where Cyclo(RGDfC) was attached to the surface of pre-formed nanoparticles. Superparamagnetic iron oxide nanoparticles (SPIO) were first coated with β-cyclodextrin (CD) and polyethylene glycol (PEG) to form stable, self-assembling magnetic nanoclusters (SAMNs). The SAMNs were further functionalized with the tumor-targeting peptide Cyclo(RGDfC) ([Figure 3B](#)). This modification enhanced the uptake of nanodrugs into tumor cells by targeting integrin receptors, which are overexpressed on cancer cells. The nanoclusters showed improved drug loading efficiency and controlled release of paclitaxel (PTX), leading to enhanced magnetic

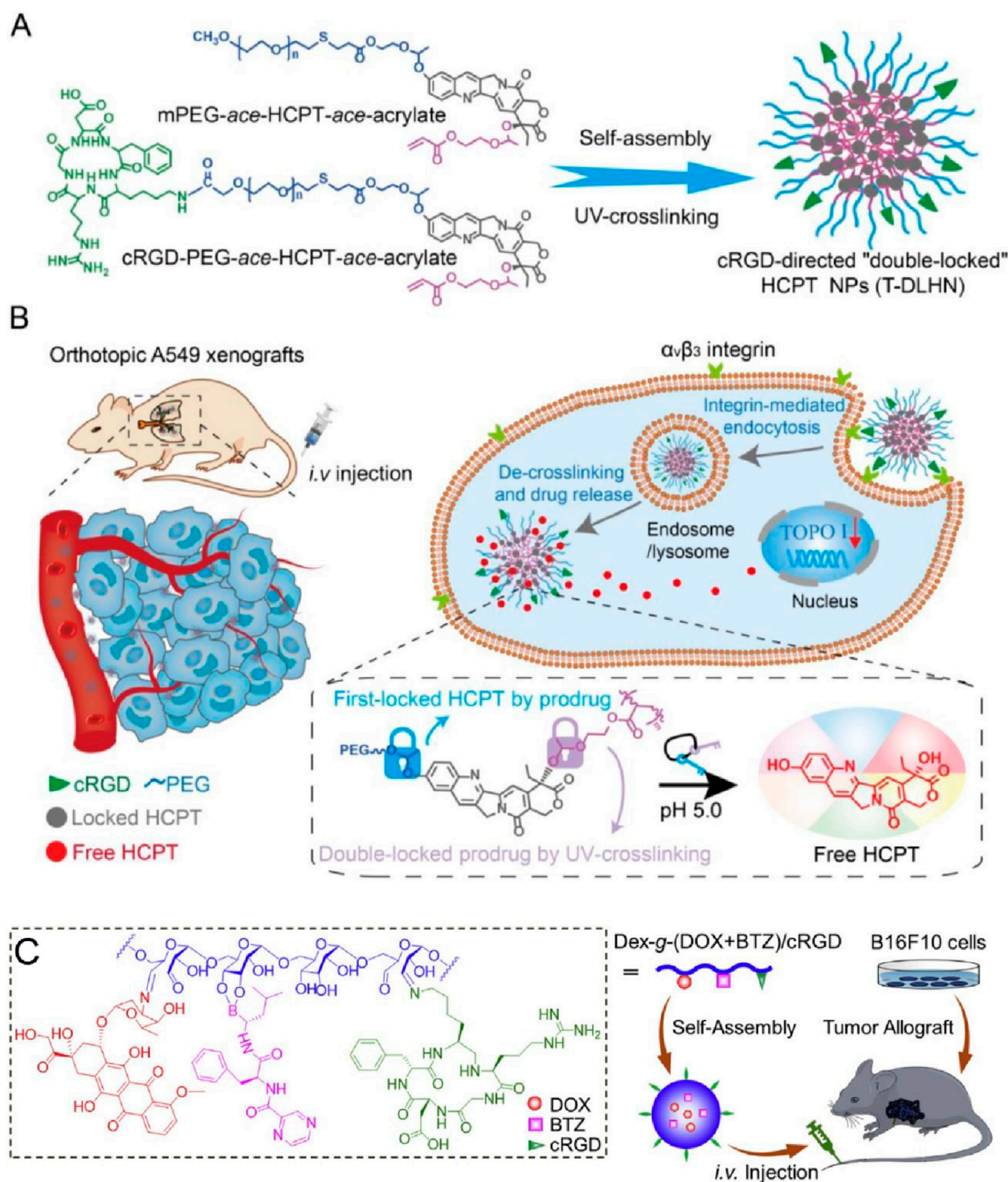


FIGURE 4

(A) Confocal images of T-24 cells with FITC-labeled micelles; left panel: non-modified micelles, right panel: RGD-modified micelles, showing higher uptake for RGD-modified micelles. (B) Flow cytometry analysis indicating increased fluorescence intensity in T-24 cells treated with RGD-modified micelles, confirming enhanced targeting. Adapted with modification from Zhang J. et al. (2023). (C) Quantitative uptake analysis over time, with RGD-modified micelles showing higher fluorescence at all-time points compared to non-modified micelles. Adapted with modification from Li et al. (2020).

resonance imaging (MRI) contrast and targeted drug delivery in cancer therapy. *In vitro* studies demonstrated that the Cyclo(RGDfC)-functionalized SAMNs had superior cellular

uptake and higher antitumor efficacy compared to non-targeted formulations. The SAMNs provided a controlled release of PTX, which was triggered by competitive guest molecules, ensuring

efficient drug delivery to tumor sites while minimizing systemic toxicity. These findings suggest that Cyclo(RGDfC)-functionalized SAMNs have significant potential for application in image-guided cancer chemotherapy. Notably, this study needs improvements in biocompatibility, scalability, drug loading and release optimization, targeting efficiency, and comprehensive *in vivo* efficacy studies to facilitate clinical translation. Additionally, aligning with regulatory guidelines is crucial for future development. Such strategy for the development of Cyclo(RGDfC)-based nanodrugs have also been utilized in Ding, Zhang, and Bao's study, respectively (Ding et al., 2021; Zhang et al., 2024; Bao et al., 2016). Ding et al. (2021)'s study demonstrated that CDDP-loaded polyamino acid nanoparticles, combined with RGD peptides, improved tumor targeting and reduced toxicity in non-small cell lung cancer models. Zhang et al. (2024)'s research focused on zwitterionic dendrimer self-assembled nanodrugs, highlighting their high drug loading and enhanced anti-tumor efficacy in acidic tumor environments. Bao et al. (2016)'s work illustrated the superior tumor targeting and antitumor activity of cRGD-modified micelles loaded with DOX, showcasing their potential in overcoming multidrug resistance.

3.3 Cyclo(RGDfK)-based self-assembling nanodrugs

Cyclo(RGDfK) is a cyclic peptide known for its high binding affinity to integrin receptors overexpressed in tumor cells. Its stability and specificity make it particularly effective for targeted drug delivery in cancer therapy. Cyclo(RGDfK)-based self-assembling nanodrugs are designed for targeted cancer therapy, enhancing tumor targeting, drug delivery efficiency, and therapeutic outcomes by leveraging integrin receptor interactions.

Zhong's group developed cyclo (RGDfK)-based self-assembling nanodrugs for targeted lung cancer therapy, focusing on a "double-lock" mechanism for enhanced stability and efficacy (Zhang J. et al., 2023). The nanodrugs were prepared by first assembling HCPT-loaded nanoparticles using mPEG-ace-HCPT-ace-acrylate and cRGD-PEG-ace-HCPT-ace-acrylate, followed by UV-crosslinking to create the stable "double-locked" nanostructures (T-DLHN) (Figure 4A). Cyclo (RGDfK) peptides on the surface of these nanoparticles facilitated targeted delivery to tumor cells. Figure 4B illustrates the therapeutic mechanism in orthotopic A549 lung cancer xenografts. The cRGD-functionalized nanoparticles targeted $\alpha v \beta 3$ integrin receptors on tumor cells, enhancing cellular uptake through integrin-mediated endocytosis. Once internalized, the nanoparticles underwent de-crosslinking and drug release in the acidic tumor microenvironment, effectively delivering HCPT to the nucleus, inhibiting topoisomerase I, and inducing cell death. *In vivo* studies demonstrated that these nanodrugs significantly prolonged circulation time, improved tumor accumulation, and enhanced antitumor efficacy while minimizing systemic toxicity, making them a promising strategy for safe and efficient chemotherapy in lung cancer. This method needs improvements in optimizing the biocompatibility and long-term safety of the nanodrugs, particularly in terms of potential immune responses. Additionally, further research is required to enhance the scalability and cost-effectiveness of the production process for clinical applications.

In another study, the authors developed a Cyclo (RGDfK)-based self-assembling nanodrug, where HCPT was conjugated with cRGD-PEG and self-assembled into nanoparticles (Li et al., 2020). The resulting "double-locked" nanoparticles (T-DLHN) exhibited enhanced stability and tumor-targeting capabilities. The nanodrug demonstrated effective integrin-mediated endocytosis and acid-triggered drug release, leading to significant tumor suppression in orthotopic A549 xenograft models with minimal systemic toxicity (Figure 4C). The method requires improvements in optimizing the drug release rate and ensuring the long-term stability of the nanoparticles in the physiological environment. Further studies are needed to evaluate the safety and efficacy of these nanodrugs in clinical settings. Additionally, Cyclo(RGDfK) have also been utilized for the synthesis of several other self-assembling nanodrugs for cancer therapy. Chen's study described a novel strategy to preserve structurally labile peptide assemblies after molecular modification by designing sheet-forming peptides that allow for staggered alignment, enabling the creation of densely functionalized nanosheets with various molecules, including c(RGDfK) (Chen et al., 2024). This study faced challenges in maintaining the structural integrity of peptide assemblies after molecular functionalization, requiring improved methods to preserve assembly morphology while achieving high functionalization density. Zhou's study developed a targeted drug delivery system using c(RGDfK)-decorated micelles for intravesical instillation chemotherapy of superficial bladder cancer, demonstrating high affinity to bladder cancer cells and significant inhibitory effects on cell proliferation (Nguyen et al., 2016). In addition to improving stability, the c(RGDfK) modification was shown to enhance the biodistribution of the micelles by promoting selective accumulation at the tumor site through specific binding to integrin receptors, thereby reducing off-target effects and systemic toxicity. This selective targeting and improved biodistribution highlight the potential of c (RGDfK)-decorated micelles to optimize drug delivery efficiency. However, the study identified the need to further enhance the stability and drug loading efficiency of c(RGDfK)-decorated micelles for effective bladder cancer treatment, suggesting further optimization of the micelle formulation (Zhou et al., 2013). Zou et al. (2016) reported on self-crosslinkable and intracellularly decrosslinkable micellar nanoparticles decorated with c (RGDfK), showing enhanced targeted delivery and therapeutic efficacy against melanoma and glioma cells, highlighting their potential for clinical translation. This research highlighted the complexity and potential safety concerns of self-crosslinkable micellar nanoparticles, indicating a need for simpler and safer design for clinical translation.

3.4 Cyclo(RGDyK)-based self-assembling nanodrugs

Cyclo(RGDyK)-based self-assembling nanodrugs offer a promising approach for targeted cancer therapy by leveraging the specific binding affinity of the cyclic peptide Cyclo(RGDyK) to integrin receptors overexpressed on tumor cells. These nanodrugs self-assemble into stable nanoparticles that encapsulate chemotherapeutic agents, ensuring controlled release and enhanced drug concentration at the tumor site. Cyclo(RGDyK) has a high binding affinity for integrin

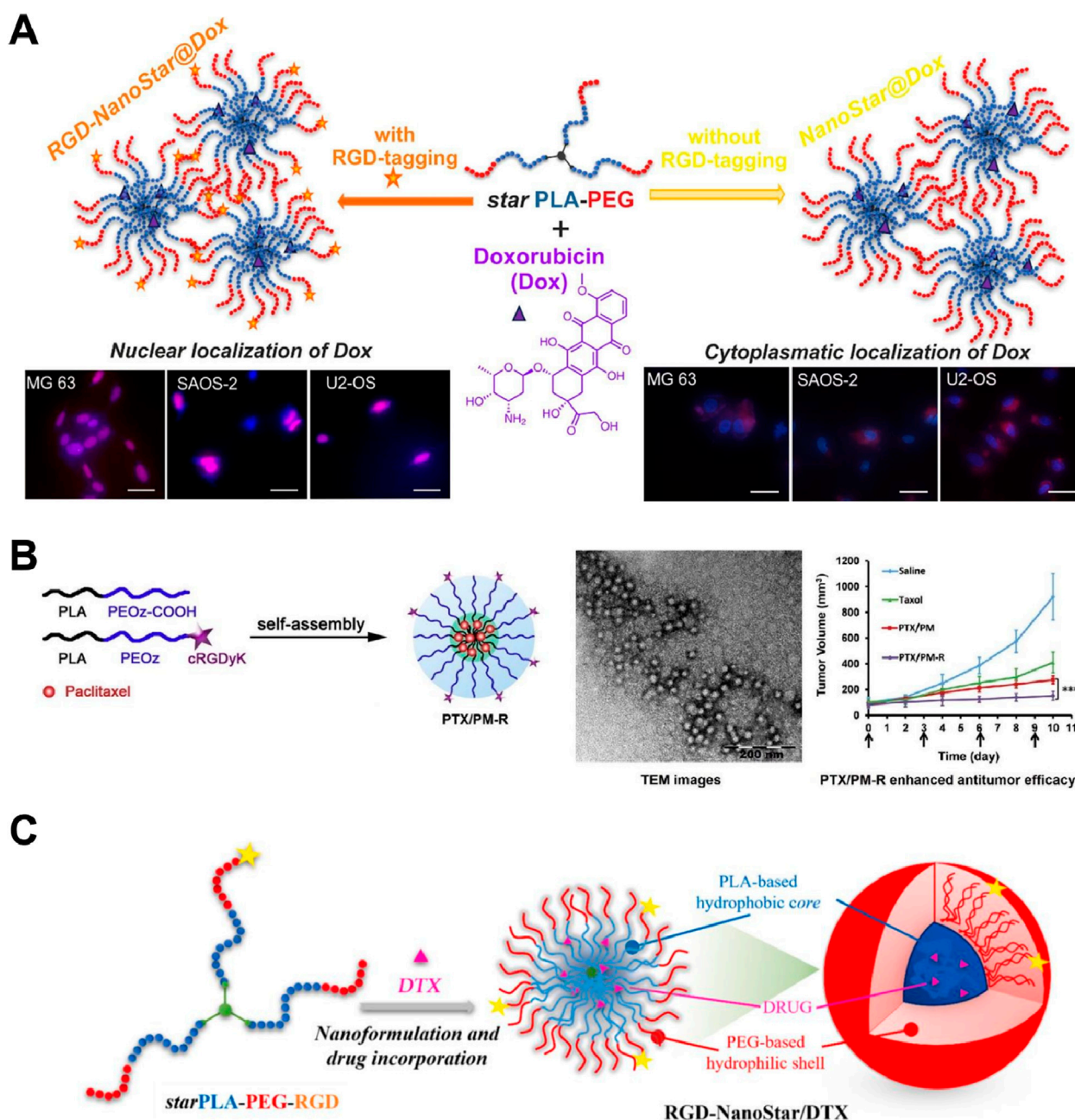


FIGURE 5

(A) Schematic and fluorescence images of RGD-tagged (RGD-NanoStar@Dox) versus non-tagged (NanoStar@Dox) micellar nanoassemblies for Dox delivery. RGD-tagged nanoassemblies showed enhanced nuclear localization of Dox in osteosarcoma cells (MG63, SAOS-2, U2-OS), indicating effective integrin-mediated endocytosis Adapted with modification from Oliva et al. (2024). (B) Schematic and TEM images of cyclic RGDyK-conjugated, paclitaxel-loaded pH-responsive polymeric micelles (PTX/PM-R). TEM images confirmed uniform spherical nanoparticles. *In vivo*, PTX/PM-R significantly suppressed tumor growth compared to PTX/PM and Taxol, due to improved tumor targeting and pH-responsive drug release Adapted with modification from Gao et al. (2015). (C) Schematic, TEM images, and tumor volume reduction graph of star-shaped PLA-PEG-RGD nanoshuttles for docetaxel (DTX) delivery. TEM confirmed nanoparticle structure. *In vivo*, RGD-NanoStar/DTX significantly reduced tumor volume compared to saline, Taxol, and untargeted formulations, due to enhanced targeting and uptake by tumor cells Adapted with modification from Torcasio et al. (2022).

receptors, particularly $\alpha v \beta 3$ and $\alpha v \beta 5$, which are overexpressed on tumor cells, enhancing specificity and targeting efficiency. Such targeted delivery system minimizes systemic toxicity and improves therapeutic efficacy. Future research should focus on optimizing the stability, biocompatibility, and clinical scalability of these nanodrugs for effective cancer treatment.

Oliva et al. (2024) developed star-shaped PLA-PEG micellar nanoassemblies tagged with cyclic RGDyK (RGD-NanoStar@Dox) for targeted delivery of doxorubicin (Dox) to osteosarcoma cells. The RGD-tagged nanoassemblies demonstrated enhanced nuclear localization of Dox in MG63, SAOS-2, and U2-OS osteosarcoma cells, as evidenced by the fluorescence images showing Dox

accumulation in the cell nuclei (Figure 5A). In contrast, untargeted NanoStar@Dox showed cytoplasmic localization of Dox, indicating ineffective delivery to the nucleus. This highlights the crucial role of the cyclic RGDyK peptide in facilitating integrin-mediated endocytosis and efficient drug delivery to the tumor cell nucleus, enhancing therapeutic efficacy while minimizing effects on healthy osteoblasts (hFOB). Notably, one limitation of this method is the potential for immune response due to the presence of RGD peptides, and there is a need to further investigate long-term safety and efficacy *in vivo*. Additionally, optimizing the drug release rate to ensure a more consistent therapeutic effect is necessary.

In another study, researchers developed cyclic RGDyK-conjugated, paclitaxel-loaded pH-responsive polymeric micelles (PTX/PM-R) for targeted cancer therapy (Gao et al., 2015). The micelles self-assembled into nanoparticles, enhancing drug delivery and tumor targeting efficiency. Transmission electron microscopy (TEM) images confirmed the uniform spherical structure of these nanoparticles. *In vivo* studies demonstrated that PTX/PM-R significantly suppressed tumor growth in comparison to non-targeted micelles (PTX/PM) and Taxol (Figure 5B). The enhanced antitumor efficacy was attributed to the improved targeting and uptake by tumor cells, facilitated by the cyclic RGDyK peptide, and the pH-responsive release of paclitaxel within the tumor microenvironment. However, one potential drawback of this method is the complexity of synthesizing and optimizing the pH-responsive polymeric micelles, which may impact large-scale production. Additionally, further investigation into the long-term stability and potential immune responses *in vivo* is necessary to ensure safety and efficacy.

Torcasio et al. (2022) developed star-shaped PLA-PEG-RGD nanoshuttles for docetaxel (DTX) delivery, forming self-assembled micelles with a hydrophobic core and hydrophilic shell. TEM confirmed the nanoparticle structure. *In vivo* tests demonstrated that RGD-NanoStar/DTX significantly reduced tumor volume compared to saline, Taxol, and untargeted formulations. The improved efficacy was attributed to the enhanced targeting and uptake by tumor cells, facilitated by the RGD peptide, leading to better drug accumulation and antitumor effects (Figure 5C). This method needs to further optimize drug loading efficiency and stability of the nanoshuttles to ensure consistent therapeutic outcomes. Such design has also been applied in Dai and Fang's study. Dai's study has demonstrated that cyclic RGDyK-conjugated nanoparticles significantly enhanced the penetration and chemotherapy efficacy of paclitaxel against advanced gliomas. These nanoparticles showed improved tumor targeting, deeper tissue penetration, and higher accumulation in glioma cells, leading to prolonged survival in mice models (Wang et al., 2021). Fang and coworkers have developed cyclic RGDyK-functionalized poly(trimethylene carbonate)-based nanoparticles for paclitaxel delivery. The results showed enhanced endocytic uptake by glioma cells, improved tumor penetration, and superior antitumor effects compared to non-targeted nanoparticles and conventional treatments, with minimal *in vivo* toxicity (Jiang et al., 2013).

3.5 Cyclo(RGDyC)-based self-assembling nanodrugs

Cyclo(RGDyC) has a unique cysteine (C) residue that can form a disulfide bond, which significantly enhances their stability and

resistance to enzymatic degradation. The presence of the cysteine residue allows for site-specific conjugation with various therapeutic agents or imaging molecules, facilitating multifunctional applications. This feature makes Cyclo(RGDyC) particularly suitable for developing targeted therapies that require stable, long-circulating nanocarriers with the ability to deliver payloads precisely to tumor sites. Additionally, Cyclo(RGDyC) exhibits a distinct affinity for certain integrin subtypes, making it particularly effective in targeting tumors with specific integrin expressions, such as gliomas and metastatic cancers, where stability and precise targeting are crucial.

The cRGD-PEOz-Hz-DOB molecules, incorporating Cyclo(RGDyC), facilitated specific binding to $\alpha v \beta 3$ receptors on tumor cells, enhancing targeted delivery and cellular uptake (Chen et al., 2023). At pH 5.0, the hydrazone bond in cPzD exhibited a 42% breakage rate within 48 h, resulting in an 82.9% cumulative release rate of DOX in acidic conditions, which was 22.5% higher than at pH 7.4. Uptake of cPzDGX in A549/ADR cells increased by 62% at 2 h, and EGFP expression was 36% higher compared to the control group, indicating that Cyclo(RGDyC) significantly improved the liposomes' targeting ability (Figure 6A). Additionally, the cell viability of the cPzDGX group decreased by 17.2% relative to the free DOX group, and the diameter reduction of 3D tumor microspheres increased by 34.4%, demonstrating enhanced anticancer efficacy due to Cyclo(RGDyC). The study's main limitation is the lack of *in vivo* testing to confirm the therapeutic efficacy and safety of cPzDGX liposomes, as well as potential immune responses over prolonged use. Future research should focus on optimizing the stability of these liposomes in biological fluids and improving large-scale manufacturing processes to ensure consistent quality and effectiveness.

Liu N. et al. (2021) demonstrated that CR780RGD-NPs enabled efficient optoacoustic imaging of brain tumors with high specificity and strong signal generation, exhibiting a 1.85 times higher optoacoustic generation efficiency compared to ICG. These nanoparticles retained a strong signal at a depth of 5 mm in brain tissue and successfully penetrated the blood-brain barrier, showing a 3.5-fold increase in optoacoustic intensity in tumor regions (Figure 6B). CR780RGD-NPs also showed effective targeting and retention in brain tumors for at least 24 h, confirming their potential for precise tumor imaging and diagnosis. A unique limitation of this study was the insufficient exploration of the long-term *in vivo* stability of CR780RGD-NPs in the brain tumor microenvironment. Li et al. (2021) designed a pH-sensitive nanocarrier, cRGD-PEG-Hyd-R6-SA (cPHRS), utilizing Cyclo(RGDyC) for enhanced tumor targeting. Cyclo(RGDyC) facilitated specific binding to $\alpha v \beta 3$ integrins on cancer cells, improving drug accumulation via receptor-mediated endocytosis. The average size of drug-loaded nanoparticles was 143.13 nm, and they exhibited stability in physiological conditions but disassembled in acidic environments, releasing GNA002 (Figure 6C). This system significantly enhanced cytotoxicity against HN6, HeLa, and A549 cells and demonstrated notable tumor growth inhibition *in vivo*, confirming its potential for precise and efficient cancer therapy. Notably, the long-term stability of these nanocarriers in the acidic tumor microenvironment is unknown. Ouyang et al. developed cRGD-PAE-PEG-DSPE nanoparticles for targeted docetaxel delivery, enhancing tumor targeting via Cyclo(RGDyC). These nanoparticles demonstrated high serum stability and acid responsiveness, leading to increased uptake by MDA-MB-231 cells

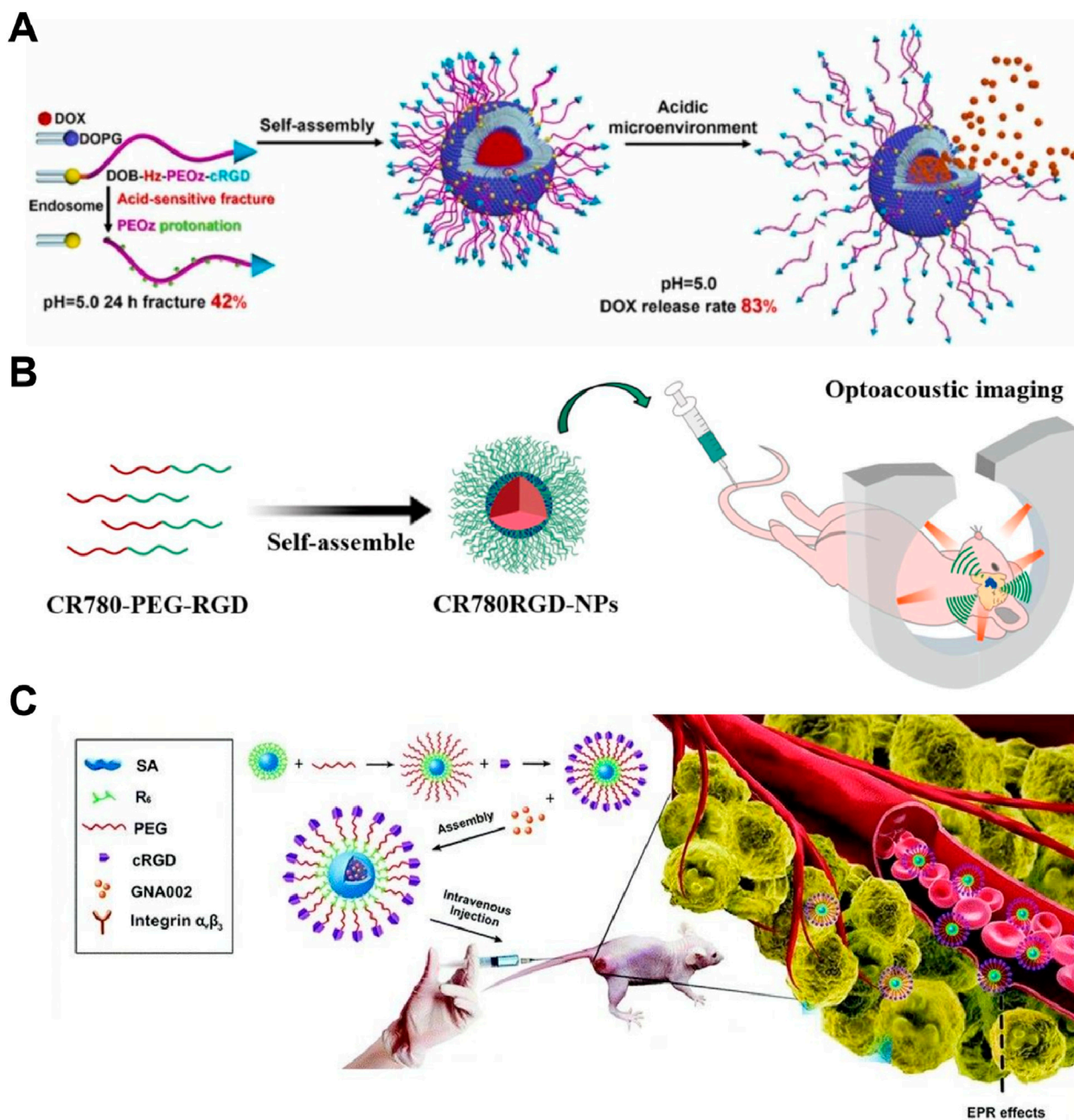


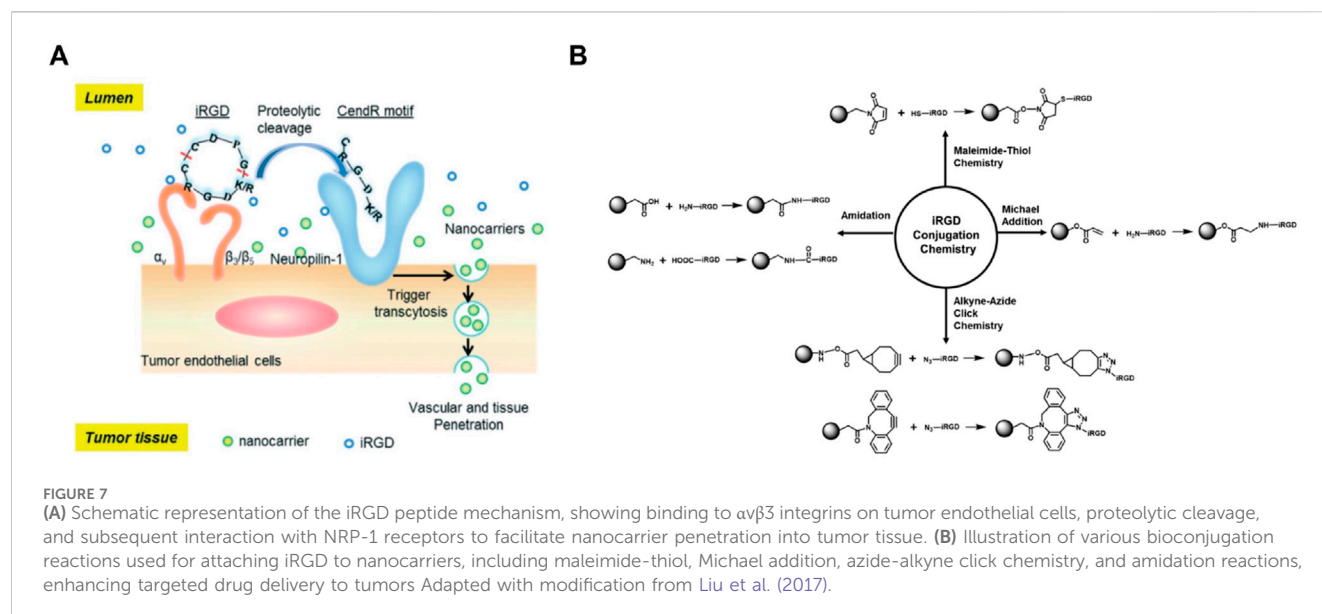
FIGURE 6
(A) cPzDGX liposomes with Cyclo (RGDyC) showed enhanced DOX release and uptake in A549/ADR cells, reducing cell viability and 3D tumor spheroid diameter Adapted with modification from [Chen et al. \(2023\)](#). **(B)** CR780RGD-NPs, utilizing Cyclo (RGDyC), demonstrated superior optoacoustic imaging efficiency and specificity for brain tumors, with significant signal retention at 5 mm depth Adapted with modification from [Liu N. et al. \(2021\)](#). **(C)** cPHRS nanocarriers with Cyclo (RGDyC) facilitated targeted drug delivery, showing increased cytotoxicity against cancer cells and notable tumor growth inhibition *in vivo* Adapted with modification from [Li et al. \(2021\)](#).

and significant tumor growth inhibition *in vivo* compared to free docetaxel, confirming their potential for efficient and targeted cancer therapy ([Ouyang et al., 2024](#)).

3.6 iRGD-based self-assembling nanodrugs

iRGD (internalizing RGD) is a tumor-penetrating peptide that combines the RGD motif for integrin binding with a CendR motif

(sequence CRGDRGPDC) that activates a tissue-penetrating pathway. This dual functionality makes iRGD particularly effective for tumor targeting and penetration. The RGD motif in iRGD binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are overexpressed on tumor cells and their vasculature ([Liu et al., 2017](#); [Sugahara et al., 2010](#)). Upon binding, iRGD undergoes proteolytic cleavage to expose the CendR motif, which interacts with neuropilin-1 (NRP-1) receptors, facilitating deep tissue penetration and enhanced drug delivery into the tumor parenchyma ([Figure 7A](#)).



This unique mechanism allows iRGD-based self-assembling nanodrugs to not only target tumor cells with high specificity but also penetrate deeply into tumor tissues, overcoming the limitations of poor drug distribution within solid tumors. Additionally, iRGD can enhance the permeability of tumor vasculature, improving the delivery of co-administered therapeutic agents (Figure 7B). These properties make iRGD-based nanodrugs highly promising for improving the efficacy of cancer treatments by ensuring that therapeutic agents reach all areas of the tumor, including those that are difficult to access with conventional drugs. The ability of iRGD to enhance both targeting and penetration significantly increases the therapeutic index of the encapsulated drugs, making it a valuable component in the design of advanced nanodrug delivery systems (Pang et al., 2014; Simón-Gracia et al., 2016).

Unlike other cyclic RGD peptides, iRGD is a tumor-penetrating peptide that combines cell-homing and cell-penetrating abilities. iRGD specifically binds to integrin receptors, particularly $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are overexpressed on tumor vasculature. Upon binding, iRGD undergoes proteolytic cleavage, exposing the CendR motif (C-end rule), which activates neuropilin-1 (NRP-1) receptors on tumor cells. This interaction triggers tissue penetration, facilitating the delivery of therapeutic agents deep into the tumor parenchyma. As a result, iRGD enhances both the targeting and penetration of drug-loaded nanoparticles, significantly improving their therapeutic index and distribution within solid tumors (Sugahara et al., 2010; Teesalu et al., 2013; Jiang et al., 2022).

Liu Y. et al. (2021) developed iRGD-PEG-HA-DOCA and CIPHD/DAS hybrid nanodrugs for enhanced tumor targeting and penetration. These nanodrugs were fabricated through a self-assembly process followed by biomineralization to improve stability against harsh blood conditions (Figure 8A). iRGD facilitated specific binding to $\alpha_v\beta_3$ integrins and neuropilin-1 receptors on tumor endothelial cells, promoting transendothelial transport and accumulation in tumors (Figure 8B). A unique limitation this study was the insufficient evaluation of the long-term effects of CAF phenotypic reversion on overall tumor progression and recurrence. Additionally, the nanodrugs modulated cancer-

associated fibroblasts (CAFs) to revert their phenotype, leading to ECM remodeling and further enhancing nanodrug penetration and tumor inhibition.

Hao et al. (2024) developed iRGD-GelAc-SS-CPT-Pa Janus prodrug nanoassemblies, combining prodrug nanoaggregates and target micelles linked via copper-free click chemistry. The iRGD peptide enhanced tumor targeting by binding to integrins and facilitating penetration through neuropilin-1 receptors. In the tumor microenvironment, the high concentration of MMP2 triggered the degradation of the prodrug nanoaggregates into smaller nanoparticles, improving cellular uptake and penetration. The prodrug released camptothecin (CPT) in response to glutathione (GSH), while photosensitizer Pa generated reactive oxygen species (ROS) under 660 nm laser irradiation, enhancing antitumor efficacy through synergistic chemotherapy and photodynamic therapy (Figure 9A). Notably, this study's main drawback was the insufficient optimization of the gelatin-based carriers' biocompatibility and stability under physiological conditions, necessitating improvements in material selection for enhanced performance. Zhang et al. (2022) developed iRGD@ZnPc + TPZ nanoparticles for targeted glioma therapy. The nanoparticles were self-assembled from SPC, ZnPc, DSPE-PEG2K-iRGD, and TPZ. iRGD facilitated the crossing of the blood-brain barrier and targeted gliomas, while ZnPc acted as a photosensitizer, and TPZ as a hypoxia-activated prodrug. This system enhanced tumor penetration and therapeutic efficacy through a combination of photodynamic therapy and hypoxia-activated chemotherapy (Figure 9B). Hu et al. (2021) developed iRGD-PEG-PLA nanoparticles for sorafenib delivery, enhancing tumor targeting and penetration through nanoprecipitation. The amphiphilic copolymers mPEG-PLA and iRGD-PEG-PLA facilitated the self-assembly of the nanoparticles, improving the solubility and therapeutic efficacy of sorafenib in hepatocellular carcinoma cells (Figure 9C). This system significantly increased apoptosis and cytotoxicity in cancer cells, demonstrating potential for enhanced cancer treatment. Ray et al. (2019) developed pH-responsive copolymer nanoparticles using mPEG-PLA and iRGD-PEG-PLA

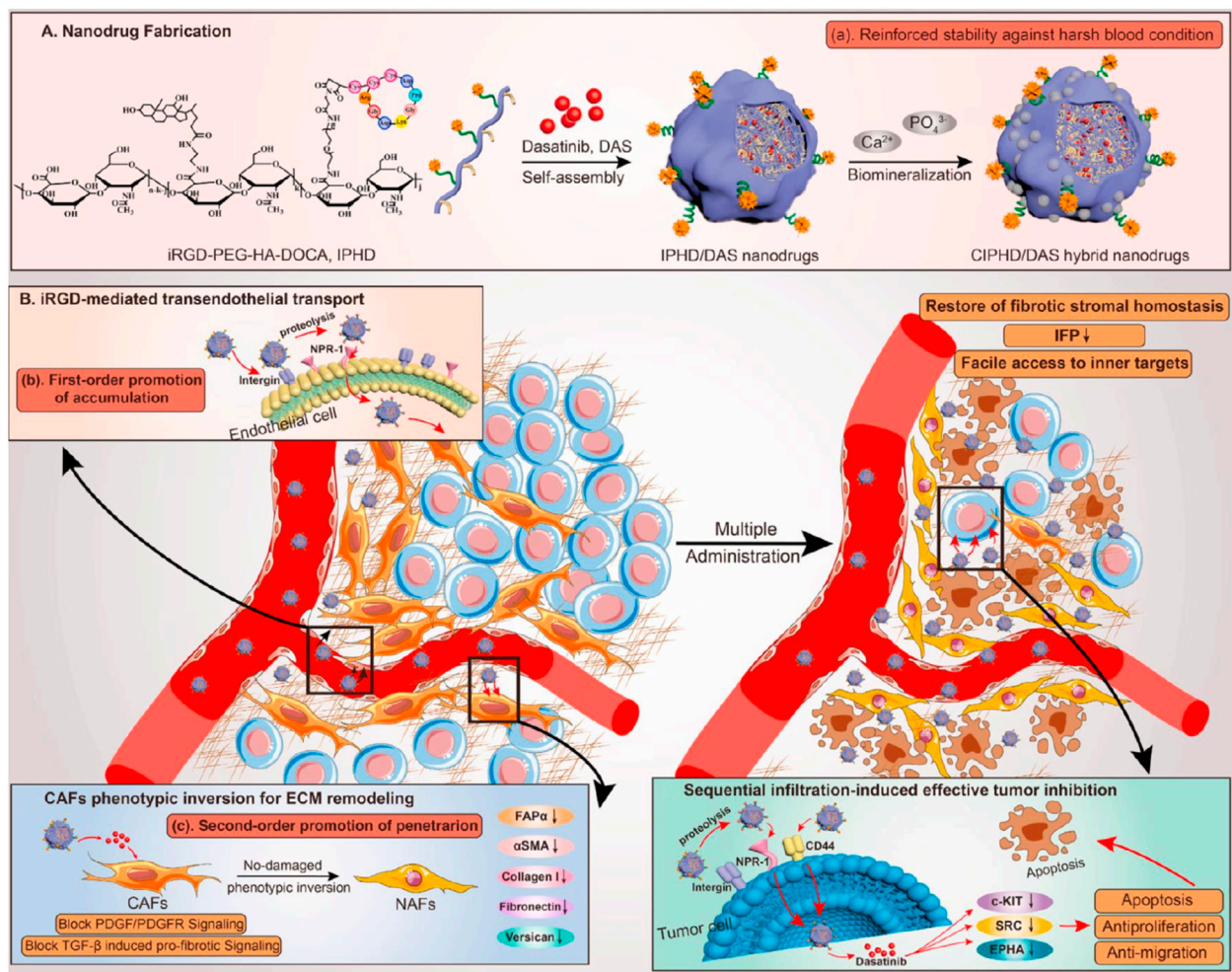


FIGURE 8

(A) iRGD-based self-assembling nanodrug fabrication, illustrating the synthesis and assembly process of iRGD-PEG-HA-DOCA and CIPHD/DAS hybrid nanodrugs, including steps for enhanced stability in harsh blood conditions through biom mineralization. (B) iRGD-mediated transendothelial transport and CAFs modulation, showcasing the first-order promotion of nanodrug accumulation in tumors via integrin and NPR-1 binding, and second-order promotion of penetration through ECM remodeling and CAF phenotypic inversion Adapted with modification from Liu Y. et al. (2021).

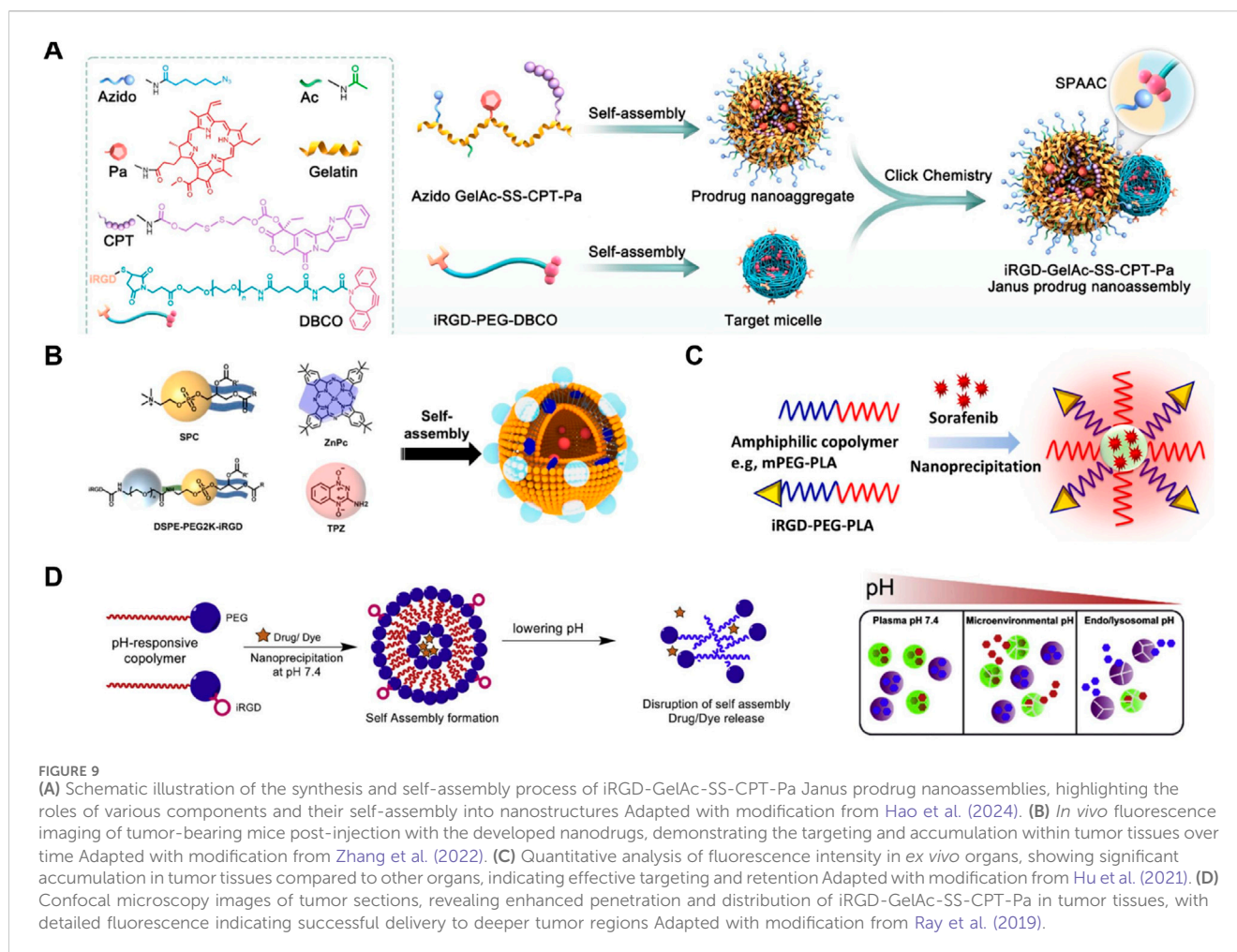
for targeted drug delivery. These nanoparticles self-assembled at pH 7.4 and disassembled under acidic conditions, releasing the encapsulated drug or dye. This system enhanced targeted drug release in the tumor microenvironment and endo/lysosomal compartments, improving therapeutic efficacy and drug accumulation (Figure 9D). iRGD has been widely utilized in this field due to its exceptional ability to enhance tumor targeting and penetration. Numerous studies have demonstrated its effectiveness in improving the delivery and therapeutic outcomes of various nanodrug systems (Pei et al., 2022; Lu et al., 2020; Le et al., 2024; Yang et al., 2020).

In conclusion, the recent advancements in RGD-based self-assembled nanodrugs have shown significant promise in enhancing the efficacy of cancer therapies. By exploiting the high affinity of RGD peptides for integrin receptors, these nanodrugs achieve targeted delivery, improved tumor penetration, and reduced systemic toxicity. Multifunctional capabilities, including combined chemotherapy, phototherapy, and imaging, offer comprehensive cancer treatment approaches. Despite the impressive progress,

challenges such as optimizing biocompatibility, stability, and large-scale production remain. Continued research and development are essential to address these issues, ensuring that these innovative nanodrugs can be effectively translated into clinical applications, offering improved therapeutic outcomes for cancer patients.

4 Challenges and future directions

While RGD-based self-assembled nanodrugs offer significant promise for targeted cancer therapy, several challenges must be addressed to fully realize their potential. One major challenge is immunogenicity and biocompatibility. The body's immune system may recognize these nanodrugs as foreign, leading to immune responses that can reduce their efficacy and cause adverse effects. Ensuring that these nanodrugs are biocompatible and minimizing their immunogenicity are critical steps to improve patient safety and treatment outcomes. Additionally, the stability of nanodrugs



in the bloodstream is a concern, as factors such as protein adsorption, aggregation, and premature drug release can compromise their effectiveness. Developing strategies to enhance their stability, such as surface modifications like PEGylation, can help prolong circulation time and improve targeting efficiency.

Another significant challenge is the heterogeneity of tumors. Variability in integrin expression among different tumor types, stages, and even within the same tumor can affect the targeting efficiency of RGD-based nanodrugs. Addressing this issue may require personalized medicine approaches and combination therapies to enhance treatment efficacy. Moreover, the scale-up and manufacturing of RGD-based self-assembled nanodrugs for clinical use present additional hurdles. The complexity of the self-assembly process and the need for precise control over nanodrug characteristics demand advanced manufacturing techniques to ensure consistency and reproducibility in large-scale production, which is crucial for regulatory approval and clinical application.

Looking forward, several future directions can enhance the development and application of RGD-based self-assembled nanodrugs. Research should focus on improving targeting capabilities by developing multifunctional nanocarriers that combine RGD peptides with other targeting ligands, such as antibodies or aptamers, to enhance specificity and efficacy.

Incorporating stimuli-responsive systems that release the drug in response to the tumor microenvironment can further improve targeted delivery. Additionally, improving the biocompatibility and reducing the immunogenicity of these nanodrugs through the use of biocompatible materials and stealth properties like PEGylation will be essential. Personalized medicine approaches that tailor nanodrug formulations to the specific characteristics of a patient's tumor can enhance treatment efficacy and reduce resistance risks.

Furthermore, combining RGD-based nanodrugs with other therapeutic modalities, such as immunotherapy, radiotherapy, or other chemotherapeutic agents, can provide synergistic effects and improve overall treatment outcomes. Advanced manufacturing techniques, such as microfluidics and nanoprecipitation, will be necessary to ensure the consistent production of high-quality nanodrugs. Finally, extensive preclinical and clinical studies are required to evaluate the safety, efficacy, and pharmacokinetics of these nanodrugs. Rigorous testing in animal models and human clinical trials will provide valuable insights into their therapeutic potential and guide their path to clinical use. By addressing these challenges and pursuing these future directions, the successful clinical translation of RGD-based self-assembled nanodrugs can be achieved, offering improved outcomes for cancer patients.

5 Conclusion

RGD-based self-assembled nanodrugs represent a significant advancement in targeted cancer therapy, combining the benefits of nanotechnology with the specificity of RGD peptides. These nanodrugs offer enhanced tumor targeting, increased cellular uptake, and reduced off-target effects, leading to improved therapeutic outcomes. The self-assembly process facilitated by RGD peptides results in stable, efficient nanostructures that protect and deliver chemotherapeutic agents effectively. Despite the promise, challenges such as immunogenicity, stability in the bloodstream, tumor heterogeneity, and manufacturing scalability remain. Addressing these issues through improved biocompatibility, advanced targeting strategies, personalized medicine approaches, and innovative manufacturing techniques is essential for successful clinical translation.

While $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins have historically been the primary focus of RGD-targeted cancer therapies, recent advances in the understanding of integrin biology suggest that other RGD-binding integrins, such as $\alpha 5\beta 1$ and $\alpha v\beta 6$, may offer more selective and efficacious targets for tumor therapy. Future research should continue to explore the diverse roles of these integrins in cancer progression and develop strategies to exploit their unique expression patterns for improved therapeutic outcomes.

Future research should focus on enhancing the multifunctionality of these nanodrugs and conducting rigorous preclinical and clinical evaluations to ensure their safety and efficacy. Incorporating stimuli-responsive systems that release the drug in response to the tumor microenvironment can further improve targeted delivery. Additionally, improving the biocompatibility and reducing the immunogenicity of these nanodrugs through the use of biocompatible materials and stealth properties like PEGylation will be essential. Personalized medicine approaches that tailor nanodrug formulations to the specific characteristics of a patient's tumor can enhance treatment efficacy and reduce resistance risks.

Combining RGD-based nanodrugs with other therapeutic modalities, such as immunotherapy, radiotherapy, or other chemotherapeutic agents, can provide synergistic effects and improve overall treatment outcomes. Advanced manufacturing techniques, such as microfluidics and nanoprecipitation, will be necessary to ensure the consistent production of high-quality nanodrugs. Finally, extensive preclinical and clinical studies are required to evaluate the safety, efficacy, and pharmacokinetics of

these nanodrugs. Rigorous testing in animal models and human clinical trials will provide valuable insights into their therapeutic potential and guide their path to clinical use.

By overcoming these challenges and leveraging the unique advantages of RGD-based self-assembled nanodrugs, we can significantly improve cancer treatment outcomes, offering a more effective and safer therapeutic option for patients. The continued development and refinement of these nanodrugs hold great promise for advancing cancer therapy and improving patient survival rates.

Author contributions

BW: Writing—original draft. DT: Writing—original draft. JC: Supervision, Writing—review and editing. HJ: Validation, Writing—review and editing. JY: Writing—review and editing. ZG: Writing—original draft, Writing—review and editing.

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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.

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From defense to offense: antimicrobial peptides as promising therapeutics for cancer

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Antimicrobial peptides (AMPs), naturally occurring components of innate immunity, are emerging as a promising new class of anticancer agents. This review explores the potential of AMPs as a novel class of anticancer agents. AMPs, naturally occurring peptides with broad-spectrum antimicrobial activity, exhibit several characteristics that make them attractive candidates for cancer therapy, including selectivity for cancer cells, broad-spectrum activity, and immunomodulatory effects. Analysis of a dataset of AMPs with anticancer activity reveals that their effectiveness is influenced by various structural properties, including net charge, length, Boman index, and hydrophobicity. These properties contribute to their ability to target and disrupt cancer cell membranes, interfere with intracellular processes, and modulate the immune response. The review highlights the promising potential of AMPs as a new frontier in cancer treatment, offering hope for more effective and less toxic therapies. AMPs demonstrate promising potential in cancer therapy through multiple mechanisms, including direct cytotoxicity, immune response modulation, and targeting of the tumor microenvironment, as evidenced by extensive preclinical studies in animal models showing tumor regression, metastasis inhibition, and improved survival rates. AMPs show significant potential as cancer therapeutics through their direct cytotoxicity, immune response modulation, and tumor microenvironment targeting, with promising results from preclinical studies and early-phase clinical trials. Future research should focus on optimizing AMP properties, developing novel delivery strategies, and exploring synergistic combination therapies to fully realize their potential as effective cancer treatments, while addressing challenges related to stability, delivery, and potential toxicity.

KEYWORDS

antimicrobial peptides, cancer therapy, immunomodulation, cytotoxicity, tumor

1 Introduction

Cancer, a leading cause of death worldwide, continues to pose a significant challenge to global health (1). Despite advancements in conventional therapies such as surgery, chemotherapy, and radiotherapy, the treatment of cancer remains fraught with limitations (2–4). These modalities often come with debilitating side effects, lack selectivity for cancer cells, and struggle to combat drug resistance, leading to a persistent need for innovative therapeutic approaches (5–7). In this context, antimicrobial peptides (AMPs) emerge as a promising frontier in cancer therapy, offering a novel and multifaceted strategy to combat this formidable disease (8, 9). AMPs are naturally occurring, short-chain peptides found throughout the biological world, playing a crucial role in innate immunity (10). They act as the first line of defense against invading pathogens, exhibiting broad-spectrum antimicrobial activity against bacteria, fungi, viruses, and parasites (11, 12). Their diverse range of structures, with variations in amino acid sequence, length, and charge distribution, contributes to their potent antimicrobial effects (13). AMPs exert their antimicrobial activity through various mechanisms, often targeting the cell membrane of pathogens, disrupting its integrity, and leading to cell lysis (14). While their primary function lies in host defense, recent research has unveiled the remarkable potential of AMPs in cancer therapy. This emerging field holds significant promise due to the unique properties of AMPs, offering distinct advantages over conventional therapies (15). AMPs exhibit a remarkable ability to selectively target and kill cancer cells while sparing healthy cells. This selectivity stems from the inherent differences between the cell membranes of cancer cells and normal cells. AMPs preferentially bind to and disrupt the altered membranes of cancer cells, leading to their selective destruction (16). Furthermore, AMPs often exhibit broad-spectrum activity, effectively targeting a wide range of cancer types. This makes them particularly attractive for treating complex or multi-drug resistant cancers, where conventional therapies may struggle to achieve efficacy (17, 18). AMPs can also modulate the immune response, stimulating the immune system to recognize and eliminate cancer cells. They can directly activate immune cells, enhance cytokine production, and promote antigen presentation, leading to a robust anti-tumor immune response. Beyond their direct cytotoxic and immunomodulatory effects, AMPs can also disrupt the tumor microenvironment, targeting processes that contribute to tumor growth and metastasis (19, 20). They can inhibit angiogenesis, the formation of new blood vessels that supply tumors with nutrients and oxygen, effectively starving them. AMPs can also suppress metastasis by interfering with cell adhesion, migration, and extravasation, preventing the spread of cancer cells to distant sites (21). The unique properties of AMPs, including their selectivity, broad-spectrum activity, immunomodulatory effects, and ability to target the tumor microenvironment, make them a highly promising frontier in cancer therapy. Preclinical studies in animal models have demonstrated their efficacy against various cancer types, including solid tumors and hematologic malignancies (22, 23). The encouraging results from these studies have led to the initiation of

clinical trials in humans, exploring the safety and efficacy of AMPs for cancer treatment, especially for reduction of side effects of chemotherapy (24). While AMPs hold significant promise, several challenges remain in translating them into effective cancer therapies. These challenges include improving their stability, optimizing delivery strategies, and addressing potential toxicity. Overcoming these hurdles requires focused research and development efforts, including the development of novel AMP candidates, the optimization of delivery systems, and the exploration of synergistic combination therapies. With continued research and development, AMPs have the potential to revolutionize cancer treatment, offering patients new hope and improving outcomes. This review delves into the mechanisms of action of AMPs in cancer, explores preclinical studies and clinical trials, and discusses the challenges and future directions for this exciting field.

2 The global burden of cancer

Cancer represents a major global health challenge, with rising incidence and mortality rates worldwide. It is estimated that by 2030, the world will see approximately 26 million new cancer diagnoses and 17 million cancer-related fatalities annually (25). This alarming trend underscores the urgent need for new and effective cancer therapies. While significant progress has been made in cancer treatment, current modalities often face limitations. Surgery, while effective for localized tumors, may not be feasible for advanced or metastatic cancers. Chemotherapy, a mainstay of cancer treatment, often suffers from severe side effects, including nausea, hair loss, and immunosuppression. Moreover, chemotherapy can be ineffective against certain types of cancer, and resistance to these drugs is a growing concern. Radiotherapy, though effective in targeting specific areas, can also damage healthy tissues and is not always suitable for all types of cancer. These limitations highlight the need for alternative therapeutic approaches that are more targeted, have fewer side effects, and are effective against a broader range of cancers (25, 26).

3 Antimicrobial peptides: a promising class of therapeutics

AMPs are naturally occurring, short-chain peptides with broad-spectrum antimicrobial activity. Found throughout the biological world, from bacteria to humans, AMPs play a crucial role in innate immunity, forming the first line of defense against invading pathogens. These peptides are characterized by their diverse range of structures, with variations in amino acid sequence, length, and charge distribution. This structural diversity contributes to their broad-spectrum activity, enabling them to target a wide range of microbes, including bacteria, fungi, viruses, and parasites. While AMPs are well-known for their antimicrobial properties, recent research has also highlighted their potential as therapeutic agents for cancer treatment. The anticancer activity of AMPs has been described in the literature for some time, suggesting that this is not a novel activity. AMPs can exert their anticancer effects through various mechanisms, including direct cytotoxicity towards cancer

cells, modulation of the immune response, and induction of apoptosis. Their ability to selectively target cancer cells while sparing normal cells makes them attractive candidates for cancer therapy. The diverse nature of AMPs, with their unique structures and mechanisms of action, presents a vast reservoir of potential therapeutic agents for various applications, including cancer treatment. Ongoing research continues to explore and optimize the use of AMPs as novel anticancer agents, with the aim of developing effective and safe therapies for patients (27–29).

4 AMPs in cancer therapy: a promising frontier

The exploration of AMPs in cancer therapy represents a promising frontier in the field of oncology (30). Traditionally recognized for their role in defending against microbial infections, AMPs are now being investigated for their potential anticancer properties. This emerging area of research is driven by the unique characteristics of AMPs that may offer significant advantages over conventional cancer therapies (31). AMPs exhibit several features that make them attractive candidates for cancer treatment. One of the key advantages is their selectivity. Unlike traditional chemotherapeutics, which often lack specificity and can cause severe side effects by damaging healthy cells, AMPs have the potential to target cancer cells more selectively. This selectivity can be attributed to differences in the membrane composition and physiology of cancer cells compared to normal cells (32). For instance, cancer cell membranes often have altered lipid compositions and higher levels of specific receptors, which can be exploited by certain AMPs to enhance their binding and internalization. Another significant advantage of AMPs is their broad-spectrum activity. While conventional cancer therapies are typically designed to target specific molecular pathways or cellular processes, AMPs can exert their effects through multiple mechanisms (33–35). This includes direct cytotoxicity through membrane

disruption, induction of apoptosis, and modulation of the immune response. The ability of AMPs to engage multiple pathways simultaneously can enhance their efficacy and potentially overcome resistance mechanisms that cancer cells may develop against single-target therapies. Furthermore, AMPs have shown potential for synergistic effects when combined with other therapeutic modalities. For example, the immunomodulatory properties of some AMPs can enhance the body's natural antitumor immune response, making them ideal candidates for combination therapies with immunotherapies. Additionally, the direct cytotoxic effects of AMPs can complement traditional chemotherapy and radiotherapy, potentially improving treatment outcomes by targeting both the tumor and its microenvironment. In summary, the emerging potential of AMPs in cancer therapy is supported by their selectivity, broad-spectrum activity, and ability to synergize with other treatments. These advantages position AMPs as a promising frontier in the development of novel anticancer agents, offering hope for more effective and less toxic therapies in the fight against cancer (35–37). All reported AMPs with anticancer activity were summarized in [Supplementary Table 1](#). This table summarizes the key characteristics and anti-cancer activities of antimicrobial peptides identified through a comprehensive review of published literature. The table includes information on peptide source, sequence, structure, charge, hydrophobicity, and Boman index.

The table provided offers a comprehensive overview of the sources of AMPs with anticancer effects. This diverse array of sources highlights the ubiquitous nature of these peptides and their potential as a rich resource for novel anticancer therapeutics ([Figure 1](#)).

4.1 Most frequent sources

1. Amphibians: Frogs and toads are the most abundant sources of AMPs in this dataset. Species from genera

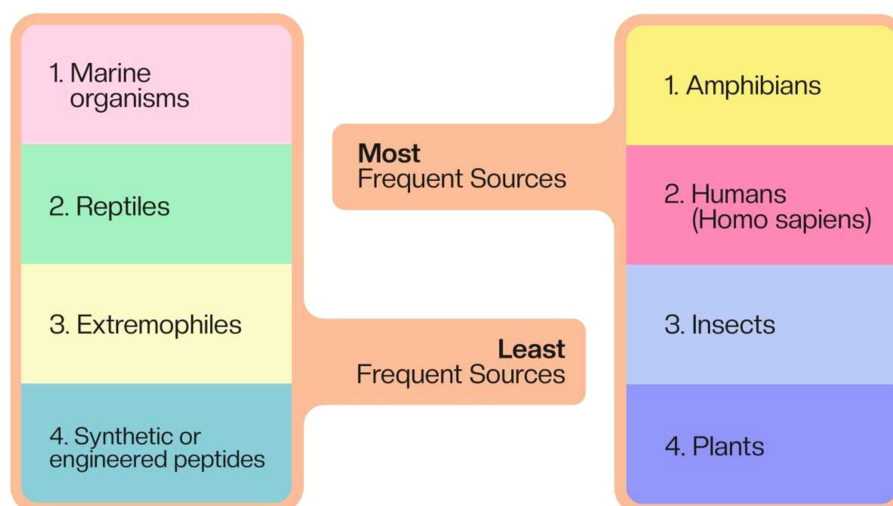


FIGURE 1
The sources of antimicrobial peptides with anticancer effects.

such as *Litoria*, *Rana*, *Bombina*, and *Phyllomedusa* are particularly well-represented. This prevalence is likely due to the rich diversity of peptides in amphibian skin secretions, which serve as a defense mechanism against pathogens in their environment.

2. Humans (*Homo sapiens*): A significant number of AMPs are derived from various human tissues and fluids, including neutrophils, skin, saliva, and colonic mucosa. This highlights the potential for developing endogenous human peptides as therapeutics, potentially reducing immunogenicity issues.
3. Insects: Bees, wasps, and various fly species contribute a notable number of AMPs. Venom-derived peptides from these insects often exhibit potent antimicrobial and anticancer properties.
4. Plants: Several plant species, particularly from genera like *Viola*, *Clitoria*, and *Viscum*, are sources of AMPs. This demonstrates the potential of plant-derived peptides in cancer therapy.

4.2 Least frequent sources

1. Marine organisms: While present, AMPs from marine sources such as fish and marine invertebrates are less represented compared to terrestrial sources.
2. Reptiles: Only a few entries are from reptilian sources, such as crocodiles and snakes.
3. Extremophiles: There are limited entries from extremophile organisms, such as alkalophilic bacteria.
4. Synthetic or engineered peptides: While present, purely synthetic or engineered peptides are less common than naturally derived ones.

This distribution reflects both the natural abundance of AMPs in certain organisms and the research focus in the field. Amphibians, particularly frogs, have been extensively studied due to their rich repertoire of bioactive peptides. The significant presence of human-derived AMPs suggests a growing interest in developing endogenous peptides as therapeutics.

The diversity of sources underscores the vast potential for discovering novel anticancer peptides across different kingdoms of life. It also highlights opportunities for exploring less-studied sources, such as marine organisms and extremophiles, which might yield unique peptides with advantageous properties.

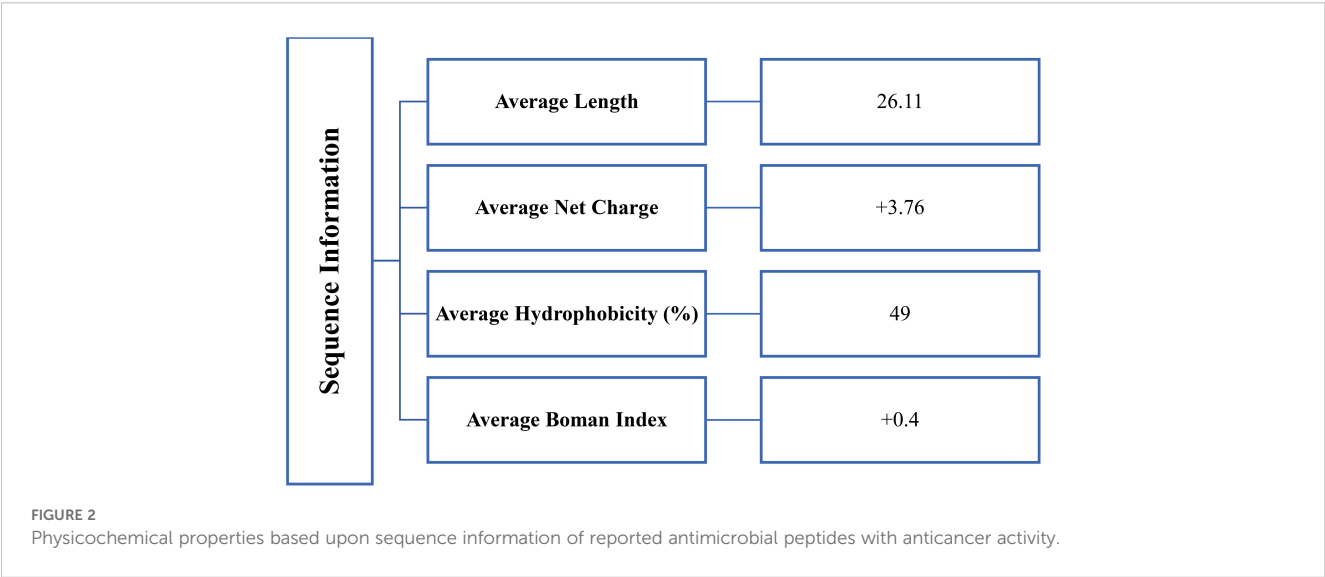
Future research could benefit from a more balanced approach, investigating underrepresented sources while continuing to explore the rich diversity of well-established sources like amphibians and insects. Additionally, the development of synthetic and engineered peptides based on natural templates offers promising avenues for optimizing anticancer efficacy and reducing potential side effects.

In conclusion, this dataset demonstrates the wide distribution of anticancer AMPs in nature and emphasizes the importance of biodiversity in drug discovery efforts. It also underscores the potential for developing novel anticancer therapies from both natural and engineered peptide sources.

The sequence information of reported anticancer peptides was shown in Figure 2. The data presented on antimicrobial peptides with anti-cancer effects provides valuable insights into how specific structural characteristics influence their anticancer activities. The impact of net charge, peptide length, Boman index, and hydrophobicity on the anti-cancer properties of these peptides can be considered as below:

Net Charge: The net charge of peptides plays a crucial role in their anti-cancer activities. Positively charged peptides are generally more effective against cancer cells due to:

- Enhanced electrostatic interaction with the negatively charged cancer cell membranes.



- Increased ability to disrupt cancer cell membranes, leading to cell death.
- Potential to target mitochondria in cancer cells, which often have a more negative membrane potential.

A higher positive charge may lead to greater selectivity for cancer cells over normal cells, improving the therapeutic index.

Peptide Length: The length of anticancer peptides can significantly impact their efficacy.

- Shorter peptides (typically 10-30 amino acids) often demonstrate better penetration into tumor tissues and cancer cells.
- They may have reduced immunogenicity compared to longer peptides.
- Shorter lengths can facilitate easier synthesis and modification.

However, the optimal length can vary depending on the specific cancer target and mechanism of action. Some longer peptides might offer more complex interactions with cellular targets.

Boman Index: The Boman index, indicating the potential for protein-protein interactions, is relevant for anti-cancer activities:

- A higher Boman index suggests greater potential for intracellular interactions in cancer cells.
- This could lead to interference with key cellular processes specific to cancer cells, such as signal transduction or gene expression.
- Peptides with a higher Boman index might exhibit multiple mechanisms of action against cancer cells, potentially reducing the likelihood of resistance development.

On the other hand, a Boman index value of 1 or below suggests that the protein possesses a reduced risk of causing side effects and toxicity. The Boman index, with values less than one, can indicate relatively favorable interactions with cancer cells and, at the same time, exhibit lower toxicity by having weaker interactions with normal cells (38).

Hydrophobicity: The hydrophobicity of peptides is critical for their anti-cancer effects.

- Moderate hydrophobicity is often optimal, allowing for:
 - Efficient interaction with and penetration of cancer cell membranes.
 - Better discrimination between normal and cancer cell membranes.
- Highly hydrophobic peptides might show increased cytotoxicity but could lose selectivity for cancer cells.
- The right balance of hydrophobicity can enhance cellular uptake and intracellular distribution in cancer cells.

The prevalence of helical structures in the data suggests that this conformation is particularly important for anti-cancer activities. Helical structures can:

- Facilitate interaction with and disruption of cancer cell membranes.

- Potentially form pores in cancer cell membranes, leading to cell death.
- Enable better penetration into solid tumors.

In conclusion, the anti-cancer properties of these peptides are intricately linked to their structural characteristics. The optimal combination of positive net charge, appropriate length, balanced Boman index, and moderate hydrophobicity can lead to peptides with enhanced selectivity and efficacy against cancer cells. Future research focusing on fine-tuning these parameters could lead to the development of more potent and selective anti-cancer peptides, potentially offering new avenues for cancer therapy with reduced side effects compared to traditional chemotherapeutics.

The comprehensive analysis of these antimicrobial peptides reveals that their various characteristics - including length, charge, structure, Boman index, and hydrophobicity - are all well-suited for their anti-cancer activities. The peptide lengths observed are generally optimal for penetrating tumor tissues and cancer cells, while maintaining selectivity. Their net charges, predominantly positive, facilitate strong interactions with cancer cell membranes. The prevalence of helical structures enhances their ability to disrupt cancer cell membranes effectively. The Boman indices of these peptides suggest a good balance between membrane interaction and potential intracellular activities, which is crucial for diverse anti-cancer mechanisms. Additionally, their hydrophobicity levels appear to be in a range that allows for effective membrane penetration without excessive toxicity to normal cells. Collectively, these characteristics contribute to the peptides' ability to target and eliminate cancer cells through various mechanisms, including membrane disruption, intracellular targeting, and potential signaling pathway interference. The combination of these properties makes these antimicrobial peptides particularly promising candidates for anti-cancer therapies, offering a multifaceted approach to combating cancer cells while potentially minimizing harm to normal tissues (37, 39–41).

5 Mechanisms of action of AMPs in cancer

AMPs exert their anti-cancer effects through a multifaceted approach, targeting both cancer cells directly and the tumor microenvironment.

5.1 Direct cytotoxicity

AMPs exhibit direct cytotoxicity towards cancer cells through several mechanisms, primarily involving interactions with the cell membrane and subsequent induction of apoptosis. The membrane-disrupting activity of AMPs is a key factor in their anticancer effects. AMPs can bind to and insert into the lipid bilayer of cancer cell membranes, leading to membrane destabilization and eventual rupture. This disruption results in the leakage of intracellular contents and cell death. The cationic nature of many AMPs allows

them to interact preferentially with the negatively charged phospholipids found in higher concentrations on the surface of cancer cells, enhancing their selectivity and efficacy. In addition to membrane disruption, AMPs can induce apoptosis in cancer cells. This process involves the activation of caspase pathways and the release of pro-apoptotic factors. Some AMPs can trigger the formation of membrane pores, leading to the influx of calcium ions and activation of calpain proteases, which in turn activate caspases and initiate apoptosis. Other AMPs may directly interact with intracellular targets, such as mitochondria, leading to the release of cytochrome c and the activation of the intrinsic apoptotic pathway. The specific structural features of AMPs play a crucial role in their cytotoxic activity. For instance, the amphipathic nature of many AMPs, characterized by a combination of hydrophobic and hydrophilic regions, allows them to interact effectively with the lipid bilayer. The length, charge distribution, and secondary structure of AMPs also influence their ability to disrupt membranes and induce apoptosis. Alpha-helical AMPs, for example, are particularly effective at penetrating and disrupting membranes due to their ability to adopt a helical conformation in the lipid environment (42–45).

5.2 Modulation of immune response

AMPs can stimulate the immune system to recognize and eliminate cancer cells, making them valuable agents in cancer immunotherapy. By modulating the immune response, AMPs can enhance the body's natural defenses against cancer. One mechanism involves the activation of pattern recognition receptors (PRRs) on immune cells, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). These receptors recognize AMPs and trigger signaling pathways that lead to the production of pro-inflammatory cytokines and the activation of immune cells. AMPs can also modulate the function of various immune cells, including dendritic cells, macrophages, and T cells. For example, AMPs can enhance the maturation and antigen-presenting capabilities of dendritic cells, leading to increased activation of T cells and improved immune surveillance of cancer cells. Additionally, AMPs can stimulate the production of cytokines such as interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α), which have potent antitumor effects. The ability of AMPs to modulate immune cell function and cytokine production makes them valuable tools in enhancing the immune response against cancer. By promoting a more robust and effective immune reaction, AMPs can help eliminate cancer cells and prevent tumor recurrence (32, 46, 47).

5.3 Targeting the tumor microenvironment

The tumor microenvironment plays a critical role in cancer progression, including processes such as angiogenesis, metastasis, and interactions with stromal cells. AMPs can impact the tumor microenvironment in several ways, disrupting these processes and

enhancing therapeutic efficacy. One significant effect of AMPs on the tumor microenvironment is their ability to inhibit angiogenesis, the formation of new blood vessels that supply nutrients and oxygen to the tumor. AMPs can target endothelial cells lining blood vessels, disrupting their function and reducing vascular supply to the tumor. This vascular disruption can lead to tumor hypoxia and necrosis, impairing tumor growth and survival. AMPs can also influence metastasis, the spread of cancer cells to distant organs. By disrupting the interactions between cancer cells and the extracellular matrix, AMPs can inhibit the migration and invasion of cancer cells. Additionally, AMPs can target cancer-associated fibroblasts and other stromal cells, reducing their supportive functions and limiting tumor growth. Furthermore, AMPs can modulate the immune cells within the tumor microenvironment, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which often suppress the immune response and promote tumor growth. By reducing the activity of these suppressive cells, AMPs can enhance the antitumor immune response and improve the efficacy of other therapeutic modalities (42, 44, 48).

In summary, the mechanisms of action of AMPs in cancer involve direct cytotoxicity, modulation of the immune response, and targeting of the tumor microenvironment. These multifaceted effects make AMPs promising agents in the development of novel anticancer therapies, offering potential for more effective and targeted treatment strategies.

6 *In vitro* assays for anticancer peptides

The efficacy of AMPs as anticancer agents has been extensively evaluated *in vitro*, providing foundational evidence for their potential in cancer therapy (49). In [Supplementary Table 1](#), we summarized all reported AMPs with anticancer activity. Most of these reported peptides have been evaluated in *in vitro*. These *in vitro* assays have demonstrated the ability of AMPs to inhibit cancer cell proliferation, induce apoptosis, and disrupt cellular membranes across a variety of cancer cell lines. Here, we summarize key findings from *in vitro* studies involving several notable AMPs from prepared list. *In vitro* studies have shown that cathelicidin family, including LL-37, can effectively reduce the viability of breast cancer and ovarian cancer cells by inducing apoptosis and inhibiting cell migration. This peptide family disrupt the cancer cell membrane, leading to cell death, and has been observed to modulate the expression of genes involved in cancer progression (50). Magainins, as class of AMPs found in the amphibians, have been tested on colon cancer and gastric cancer cell lines, where it has demonstrated significant cytotoxicity. The mechanism involves pore formation in the cancer cell membrane, leading to increased permeability and subsequent cell lysis (51, 52). *In vitro* experiments with lactoferricin, an amphipathic, cationic peptide with dual biological activity (anti-microbial and anti-cancer), have shown inhibition of melanoma and leukemia cell lines. The peptide induces apoptosis through mitochondrial pathways and has been

observed to inhibit angiogenesis by affecting endothelial cells (53). Studies have reported that Protegrin PG-1, a cysteine-rich peptide, is effective against prostate cancer and other carcinoma cell lines. It disrupts the membrane integrity of cancer cells, leading to rapid cell death, and has shown synergistic effects when used in combination with conventional chemotherapeutics (54). Dermaseptins, especially Dermaseptins -B2, has shown significant anticancer activity against a variety of cancer cell lines, including melanoma and breast cancer cells. *In vitro* studies reveal that this peptide exerts its effects primarily through membrane disruption and the induction of apoptosis, effectively reducing cancer cell viability and proliferation (15, 55–57). Cecropins family have been tested on bladder cancer and breast cancer cell lines, showing significant reduction in cell viability. The mechanism primarily involves disruption of the cancer cell membrane and induction of apoptosis (37, 58). *In vitro* studies with melittin have demonstrated its efficacy against lung and liver cancer cell lines. It acts by disrupting cellular membranes and inducing apoptotic pathways, and it has been utilized in nanoparticle delivery systems to enhance its selectivity for cancer cells (59). The aurein peptides, derived from frog skin, have demonstrated notable cytotoxic effects against cancer cell lines, such as prostate and breast cancer cells. These peptides function by disrupting cancer cell membranes and initiating apoptotic pathways, leading to cell death (16). Alloferons, originally isolated from insects, have been investigated for their anticancer properties. *In vitro* assays have shown that these peptides can induce apoptosis in leukemia and gastric cancer cells. They activate immune responses and enhance the cytotoxicity of immune cells against cancer cells (60). Synthetic hybrid peptide CE-MA has been shown to possess potent anticancer activity against a range of cancer cell lines, including lung and bladder cancers. It acts by disrupting cell membranes and enhancing apoptosis, demonstrating greater efficacy compared to its parent peptides alone (61). Maximin peptides, isolated from amphibians, have been studied for their effects on cancer cells such as melanoma and gastric cancer. *In vitro*, they disrupt cancer cell membranes and induce cell death, showing promise as potential anticancer agents (62). Temporin A and Temporin L, isolated from frog skin, have shown *in vitro* efficacy against breast and prostate cancer cell lines. They exert their anticancer effects through membrane disruption and the induction of apoptotic pathways, highlighting their potential in cancer therapy (63). Known for its broad-spectrum antimicrobial activity, indolicidin has also shown *in vitro* efficacy against leukemia and breast cancer cells. It induces apoptosis by triggering mitochondrial dysfunction and inhibiting cell cycle progression. Human Neutrophil Peptides (HNP-1, HNP-2, HNP-3) have demonstrated potent anticancer effects *in vitro* against lung and colorectal cancer cells. They act by disrupting cellular membranes and modulating inflammatory responses, which contribute to their anticancer activity (64). Synthetic, lysine-rich mutant of CRAMP-18 has been engineered to enhance its anticancer properties. *In vitro* studies have demonstrated its ability to disrupt cancer cell membranes and induce apoptosis in various cancer cell lines, including breast and colon cancer. The lysine-rich composition enhances its interaction with negatively charged cancer cell

membranes, leading to increased cytotoxicity (65). Brevinins and their derivatives have shown potent anticancer activity *in vitro*. They have demonstrated efficacy against melanoma and breast cancer cell lines by inducing membrane disruption and apoptosis. The presence of disulfide bonds (1S=S) contributes to its structural stability and anticancer activity. Synthetic mutant of Brevinin-1-AW, with a Q15K substitution (B1AW-K), exhibits enhanced anticancer activity compared to its parent peptide. *In vitro* assays have shown increased cytotoxic effects on prostate and pancreatic cancer cells, attributed to improved membrane interaction and apoptotic induction (66–68). A leucine-rich natural AMP from amphibians, Figainin 2BN has been effective in reducing viability in colon and lung cancer cell lines. Its mechanism involves membrane permeabilization and induction of apoptosis, highlighting its potential as a therapeutic agent (69). Picturin 1BN and Picturin 2BN, natural AMPs from amphibians, have shown promising *in vitro* anticancer activity against leukemia and glioblastoma cells. They function by disrupting cellular membranes and triggering apoptotic pathways, leading to significant reductions in cancer cell proliferation (70). Dermaseptins, derived from amphibians, have exhibited strong anticancer effects *in vitro*. They target a wide range of cancer cell lines, including skin and cervical cancers, by causing membrane disruption and subsequent apoptosis (71, 72). MPC-A5K, an analog of Mastoparan C, is lysine- and leucine-rich and has shown enhanced *in vitro* anticancer activity against gastric and prostate cancer cells. It achieves its effects through improved membrane interaction and increased induction of apoptotic pathways (73). Ranatuerins have been designed to improve anticancer activity. *In vitro* studies indicate their efficacy in inducing apoptosis and reducing proliferation in breast and liver cancer cells through enhanced membrane disruption (74). Raniseptin PL from amphibians has shown *in vitro* effectiveness against ovarian and lung cancer cell lines. It operates by disrupting cancer cell membranes and activating apoptotic mechanisms, leading to reduced tumor cell survival (75). Figainin 2PL and Hylin PL, derived from amphibian sources, demonstrate strong *in vitro* anticancer activity against a variety of cancer cell lines, including leukemia and colon cancer. Their mode of action involves membrane permeabilization and apoptosis induction (75). StigA6 and StigA16, synthetic analogs of Stigmurin, have been optimized for enhanced anticancer activity *in vitro*. They exhibit potent effects against colorectal and pancreatic cancer cells by disrupting membranes and inducing apoptosis (41). AP-64, identified as C5orf46, has shown promise *in vitro* against hematological and solid tumors. It exerts its effects through membrane disruption and the activation of apoptotic pathways, making it a candidate for further anticancer research (40). AaeAP1a and AaeAP2a, lysine-rich synthetic analogs, have been developed to enhance anticancer activity. *In vitro* studies show their effectiveness in reducing viability in breast and colon cancer cells by disrupting membranes and inducing apoptosis (39). The *in vitro* studies of these AMPs provide compelling evidence of their direct cytotoxic effects on cancer cells and support their potential as novel anticancer agents. These findings lay the groundwork for further exploration in preclinical animal models and clinical trials, with the aim of optimizing their use in cancer therapy.

7 Preclinical studies and clinical trials

The therapeutic potential of AMPs in cancer treatment has been extensively explored in preclinical studies, with promising results paving the way for clinical trials.

7.1 Preclinical studies in animal models

Preclinical studies in animal models have provided substantial evidence supporting the efficacy of AMPs against various types of cancer. These studies have investigated the effects of AMPs on a wide range of cancers, including solid tumors and hematologic malignancies, and have explored different treatment regimens and delivery strategies.

Solid Tumors: AMPs have demonstrated significant antitumor activity against various solid tumors in preclinical models. LL-37 has shown efficacy against breast cancer in mice, reducing tumor growth and metastasis (76). Another study investigated the effects of magainin II on colon cancer in a rat model, reporting significant tumor regression and improved survival (77). Lactoferricin B has also exhibited potent antitumor activity against melanoma in mice, inhibiting tumor growth and angiogenesis (78). Furthermore, Protegrin PG-1 has demonstrated efficacy against prostate cancer in a mouse model, reducing tumor volume and improving survival (54).

Hematologic Malignancies: AMPs have also shown promise in the treatment of hematologic malignancies in preclinical studies. The LTX-315 has demonstrated potent antileukemic activity *in vitro* and *in vivo*, inducing apoptosis in leukemia cells and reducing tumor burden in mouse models (79). Another study investigated the effects of PFR-1 on lymphoma in mice, reporting significant tumor regression and improved survival (80). Additionally, Cyclic AMP has shown efficacy against multiple myeloma in a mouse model, reducing tumor growth and enhancing the effects of conventional chemotherapy (81).

Treatment Regimens and Outcomes: Preclinical studies have explored various treatment regimens and delivery strategies for AMPs in cancer therapy. Single-agent therapy with AMPs has demonstrated significant antitumor activity in several studies (82–85). However, combination therapy approaches have also shown promise. Targeted delivery strategies have also been explored to enhance the efficacy and specificity of AMPs in cancer treatment (86, 87). One study investigated the use of a tumor-targeting peptide conjugated to the AMP cecropin A, demonstrating improved tumor localization and antitumor activity in a mouse model of breast cancer (86). Another study utilized a nanoparticle delivery system to target the AMP melittin to lung cancer cells, resulting in enhanced tumor regression and reduced systemic toxicity in mice (55).

The therapeutic outcomes observed in preclinical studies have been promising, with AMPs demonstrating significant antitumor effects across various cancer types. Tumor regression, metastasis inhibition, and improved survival have been consistently reported in animal models treated with AMPs (76, 77, 81). These findings highlight the potential of AMPs as novel therapeutic agents for cancer treatment and provide a strong foundation for further clinical development.

In conclusion, preclinical studies in animal models have provided compelling evidence supporting the efficacy of AMPs in cancer treatment. These studies have demonstrated the antitumor activity of AMPs against a wide range of solid tumors and hematologic malignancies, explored various treatment regimens and delivery strategies, and reported promising therapeutic outcomes. However, further research is needed to optimize AMP-based therapies, elucidate their mechanisms of action, and address potential challenges in translating these findings to human clinical trials.

7.2 Clinical trials in humans

The promising preclinical results have led to the initiation of clinical trials in humans to evaluate the safety and efficacy of AMPs for cancer therapy. Several clinical trials are currently ongoing, investigating the use of AMPs for various cancer types. These trials span different phases, each with specific objectives and patient populations (82, 88, 89). Clinical trials are targeting a range of patient populations, including those with advanced cancers who have exhausted conventional therapies, such as in the LTX-315 trial focusing on patients with metastatic solid tumors. AMPs are also being explored as potential treatments for patients with drug-resistant cancers. Additionally, some trials are exploring the use of AMPs as adjuvant therapies in early-stage cancers, such as the bovine lactoferrin trial in head and neck cancer patients aiming to prevent recurrence in those who have undergone primary treatment. Preliminary results from early-phase clinical trials are encouraging. In the Phase I trial of LTX-315, 50% of evaluable patients showed stable disease, and one patient metastatic soft tissue sarcoma experienced a partial response (90). These results suggest that AMPs may have significant therapeutic potential, particularly when combined with other immunotherapies. However, it's important to note that larger-scale, late-stage clinical trials are needed to confirm these findings and establish the true efficacy of AMPs in cancer treatment. The field also faces challenges in optimizing delivery methods, reducing potential systemic toxicity, and overcoming regulatory hurdles. In conclusion, while the clinical development of AMPs for cancer therapy is still in its early stages, the ongoing trials and preliminary results offer promising avenues for future research and potential new treatment options for cancer patients. The diverse range of AMPs being investigated, from naturally derived peptides like LL-37 to synthetic variants like LTX-315, highlights the versatility of this approach. As research progresses, it will be crucial to address challenges such as peptide stability, targeted delivery, and potential immunogenicity to fully realize the therapeutic potential of AMPs in cancer treatment.

7.3 Challenges and future directions

The development of antimicrobial peptides (AMPs) for clinical application in cancer therapy faces several challenges that must be addressed to translate these promising agents into effective treatments. Key issues include stability, delivery, and potential

toxicity, each of which poses significant hurdles for the successful clinical use of AMPs.

7.3.1 Challenges in AMP development

Stability: One of the primary challenges in AMP development is ensuring their stability in biological environments. AMPs can be susceptible to degradation by enzymes such as proteases, which can limit their efficacy and duration of action. Additionally, the chemical stability of AMPs in the presence of physiological conditions, such as changes in pH and temperature, must be considered to maintain their structural integrity and functional activity.

Delivery: Effective delivery of AMPs to the target site within the body is another major challenge. Systemic administration of AMPs can lead to off-target effects and toxicity due to their interaction with healthy tissues. Localized delivery methods, such as direct injection into tumors, can improve specificity but may not be feasible for disseminated cancers. Novel delivery systems, including nanoparticles, liposomes, and polymeric carriers, are being explored to enhance the targeting and efficacy of AMPs while reducing systemic exposure.

Potential Toxicity: While AMPs have shown selectivity for cancer cells, concerns remain about their potential toxicity to healthy tissues. Some AMPs can induce inflammation and immune reactions, particularly at higher doses, which can lead to adverse effects. Understanding the mechanisms of AMP-induced toxicity and developing strategies to minimize these effects are crucial for their safe and effective use in cancer therapy (21, 86, 91).

7.3.2 Future directions for research and development

To overcome these challenges and translate AMPs into effective cancer therapies, several future directions for research and development are being pursued.

Structural Modifications: One approach is to modify the structure of AMPs to enhance their stability and reduce toxicity. This can involve altering the peptide sequence, introducing chemical modifications, or engineering fusion proteins that combine the anticancer activity of AMPs with other functional domains. These modifications can improve the resistance of AMPs to proteolytic degradation and enhance their selectivity for cancer cells.

Delivery Systems: Developing advanced delivery systems is another key area of research. Nanotechnology-based approaches, such as encapsulating AMPs within nanoparticles or conjugating them to targeting ligands, can improve their delivery to tumors and reduce systemic exposure. These delivery systems can also be designed to release AMPs in a controlled manner, enhancing their therapeutic window and minimizing side effects.

Combination Therapies: Exploring the use of AMPs in combination with other therapeutic modalities is a promising strategy to enhance their efficacy. Combining AMPs with traditional chemotherapy, radiotherapy, or immunotherapy can exploit synergistic effects and improve treatment outcomes. This approach can also help overcome resistance mechanisms and target multiple pathways involved in cancer progression.

Clinical Trials and Regulatory Approval: Advancing AMPs through clinical trials and obtaining regulatory approval are critical steps for their translation into clinical practice. Future clinical trials should focus on optimizing dosing regimens, assessing long-term safety and efficacy, and demonstrating the clinical benefit of AMPs in specific cancer types. Collaborations between academia, industry, and regulatory agencies will be essential to navigate the complex process of drug development and approval (21, 32, 92).

In summary, while the development of AMPs for clinical application in cancer therapy faces significant challenges, future research and development efforts are focused on overcoming these hurdles. By addressing issues related to stability, delivery, and potential toxicity, and by exploring novel strategies such as structural modifications, advanced delivery systems, and combination therapies, AMPs have the potential to become effective and safe anticancer agents.

8 Applications of AMPs in cancer therapy

8.1 Single-agent therapy

Single-Agent Therapy involves the use of a single therapeutic agent, in this case, antimicrobial peptides (AMPs), as standalone agents for cancer treatment. This approach leverages the unique properties of AMPs, such as their direct cytotoxicity and ability to modulate the immune response, to target and eliminate cancer cells.

The potential of different AMP classes for specific cancer types is an area of active research. For instance, certain cationic AMPs, known for their membrane-disrupting activity, have shown efficacy in treating solid tumors with high levels of negatively charged phospholipids on their cell surfaces. Examples include α -helical peptides like melittin and magainin, which have been investigated for their anticancer effects in breast, prostate, and colon cancers.

Other AMP classes, such as defensins and cathelicidins, exhibit additional mechanisms of action beyond membrane disruption. These peptides can induce apoptosis, modulate the immune response, and interact with intracellular targets, making them suitable for a broader range of cancer types. For example, α -defensins have been studied for their potential in treating hematological malignancies, while LL-37, a cathelicidin peptide, has shown promise in melanoma and glioblastoma.

The use of AMPs as Single-Agent Therapy offers several advantages, including their broad-spectrum activity, potential for reduced resistance, and ability to target multiple pathways within cancer cells. However, challenges such as stability, delivery, and potential toxicity must be addressed to optimize their efficacy and safety as standalone agents (93–96).

8.2 Combination therapy

Combination therapy involves the use of AMPs in conjunction with other cancer therapies, such as chemotherapy, radiotherapy,

and immunotherapy. This approach aims to exploit synergistic effects and enhance the overall efficacy of treatment regimens.

The rationale for combining AMPs with other therapies is based on their complementary mechanisms of action. For example, AMPs can enhance the cytotoxic effects of chemotherapy by disrupting tumor cell membranes and facilitating the entry of chemotherapeutic agents into cancer cells. Similarly, AMPs can potentiate the effects of radiotherapy by inducing apoptosis and modulating the tumor microenvironment, thereby improving the response to radiation.

In the context of immunotherapy, AMPs can stimulate the immune system to recognize and eliminate cancer cells. By activating pattern recognition receptors and modulating immune cell function, AMPs can enhance the efficacy of checkpoint inhibitors and other immunotherapeutic agents. Preclinical studies have demonstrated synergistic effects when AMPs are combined with immunotherapy, leading to improved tumor regression and survival rates.

Clinical evidence supporting the benefits of AMP combination therapies is emerging. Early-phase clinical trials have shown promising results, with some patients experiencing enhanced tumor responses and prolonged survival when AMPs are used in combination with conventional therapies. These findings highlight the potential of AMP combination therapies to improve treatment outcomes and overcome resistance mechanisms in cancer (97–100).

8.3 Targeted drug delivery

Targeted drug delivery involves the use of AMPs as delivery vehicles to specifically target cancer cells and enhance the efficiency of drug delivery. This approach leverages the natural properties of AMPs, such as their ability to bind to and penetrate cancer cell membranes, to deliver therapeutic payloads directly to tumor cells.

AMPs can be engineered to carry cytotoxic agents, imaging contrast agents, or therapeutic genes, allowing for targeted and controlled release within cancer cells. For example, AMPs can be conjugated with chemotherapeutic drugs, such as doxorubicin or paclitaxel, to create peptide-drug conjugates that selectively deliver the drugs to tumor cells. These conjugates can reduce systemic exposure to the drugs, minimizing side effects and improving the therapeutic index.

Additionally, AMPs can be engineered to target specific receptors or surface markers overexpressed on cancer cells. By incorporating targeting ligands, such as monoclonal antibodies or small molecules, into the AMP structure, the peptide can be directed to bind preferentially to cancer cells, enhancing its specificity and efficacy. This targeted approach can also reduce off-target effects and improve the overall safety profile of the therapy.

In summary, the applications of AMPs in cancer therapy encompass Single-Agent Therapy, combination therapy, and targeted drug delivery. Each approach leverages the unique properties of AMPs to enhance their anticancer effects and improve treatment outcomes. By addressing challenges related to stability, delivery, and potential toxicity, and by exploring innovative strategies such as combination therapies and targeted

drug delivery, AMPs have the potential to become valuable agents in the fight against cancer (101–105).

9 Conclusion and future perspectives

9.1 Summary of key findings

This review has highlighted the promising potential of antimicrobial peptides (AMPs) as cancer therapeutics. Key findings from preclinical studies and early-phase clinical trials indicate that AMPs exhibit direct cytotoxicity, modulate the immune response, and can target the tumor microenvironment, making them valuable agents in the fight against cancer. The diverse mechanisms of action of AMPs, coupled with their broad-spectrum activity and potential for reduced resistance, position them as a novel class of anticancer therapeutics with significant advantages over conventional treatments.

9.2 Future directions and research needs

Several critical areas for future research in AMP development have been identified to fully realize their potential as effective cancer therapies. These include:

Optimization of AMP Properties: Further research is needed to optimize the structural and functional properties of AMPs to enhance their stability, selectivity, and efficacy. This can involve structural modifications, such as altering peptide sequences, introducing chemical modifications, or engineering fusion proteins, to improve their resistance to proteolytic degradation and reduce potential toxicity.

Development of Novel Delivery Strategies: The development of advanced delivery systems is crucial for improving the targeting and efficacy of AMPs while minimizing systemic exposure. Novel delivery strategies, such as nanotechnology-based approaches, liposomes, and polymeric carriers, can enhance the delivery of AMPs to tumors and reduce off-target effects. Additionally, targeted delivery systems that incorporate specific ligands to preferentially bind to cancer cells can improve the specificity and efficacy of AMP therapy.

Exploration of Synergistic Combination Therapies: The use of AMPs in combination with other therapeutic modalities, such as chemotherapy, radiotherapy, and immunotherapy, offers a promising strategy to enhance their efficacy. Future research should focus on identifying optimal combination regimens and exploring the synergistic effects of these therapies. Preclinical and clinical studies should be conducted to assess the safety, efficacy, and clinical benefit of AMP combination therapies.

9.3 Conclusion

In conclusion, the significant potential of AMPs to revolutionize cancer treatment and improve patient outcomes cannot be overstated. With ongoing research and development efforts focused on optimizing AMP properties, developing novel delivery strategies, and exploring synergistic combination therapies, AMPs have the potential to become valuable agents in the fight against

cancer. By addressing the challenges associated with AMP development and leveraging their unique properties, we can advance the field of cancer therapy and provide new hope for patients in need of effective and targeted treatments.

Author contributions

HZ: Project administration, Supervision, Writing – original draft. ES: Investigation, Methodology, Writing – original draft. AJ (3rd author): Investigation, Methodology, Validation, Writing – review & editing. RG: Investigation, Methodology, Writing – original draft. AJ (5th author): Data curation, Visualization, Writing – review & editing. AP: Methodology, Investigation, Visualization, Writing – review & editing. JJ: Methodology, Investigation, Resources, Writing – review & editing.

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Supplementary material

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Recent advances in cell membrane-coated porphyrin-based nanoscale MOFs for enhanced photodynamic therapy

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Porphyrins-based nanoscale metal-organic frameworks (nMOFs) has been widely utilized to kills tumor cells by generating cytotoxic reactive oxygen species (ROS). However, porphyrin based nMOFs (por-nMOFs) still face challenges such as rapid immune clearance and weak tumor targeting. Researchers have discovered that using a top-down biomimetic strategy, where nMOFs are coated with cell membranes, can promote long blood circulation, evade the reticuloendothelial system, and improve cancer cell targeting, thereby significantly enhancing the photodynamic therapy (PDT) effect of nMOFs. This review summarizes the recent work on different cell membranes-coated por-nMOFs for enhanced tumor PDT. This review details the changes in physicochemical properties, enhanced homotypic cancer cell-selective endocytosis, improved tumor tissue targeting, and increased cytotoxicity and effective *in vivo* tumor suppression after the nMOFs are wrapped with cell membranes. Additionally, this review compares the biological functions of various types of cell membranes, including cancer cell membranes, red blood cell membranes, aptamer-modified red blood cell membranes, and hybrid membranes from the fusion of cancer and immune cells. The review highlights the enhanced immunogenic cell death function when using hybrid membranes derived from the fusion of cancer and immune cell membranes. By summarizing the augmented PDT effects and the combined antitumor outcomes with other therapeutic modalities, this review aims to provide new insights into the biomedical applications of por-nMOFs and offer more references for the preclinical application of porphyrin-based photosensitizers.

KEYWORDS

photodynamic therapy, porphyrin, metal-organic frameworks, cancer cell membranes, red blood cell membranes

1 Introduction

Malignant tumors are a type of disease characterized by extremely high mortality rates and difficulty in treatment (Ferlay et al., 2021). The relevant cancer research has prompted significant investment, including human resources, materials, and finances (Cao et al., 2021). As nanotechnology and biomedical science advance, an increasing number of treatment options are becoming available (Brigger et al., 2002; Peer et al., 2007). Compared to traditional radiotherapy, chemotherapy, and surgical treatments, photodynamic therapy is a relatively new but not yet mainstream approach (Lan et al., 2019; Chen et al., 2022; Crous and Abrahamse, 2022). However, PDT offers several advantages, such as spatiotemporal controllability, low invasiveness, low toxicity, and cost-effectiveness, making it a promising therapeutic method (Zhao et al., 2021; Di Bartolomeo et al., 2022). In PDT, light-activated photosensitizers transition from the ground state to an excited state, followed by intersystem crossing to a triplet state (Zhang et al., 2018). The triplet-state photosensitizer can then transfer energy to oxygen in tissues, generating highly reactive $^1\text{O}_2$ that kills tumor cells (Zhao et al., 2021; Ma et al., 2022). Among the widely studied organic photosensitizers, porphyrins, bodipy dyes, methylene blue, and Cy dyes are notable, with porphyrins being particularly favored due to their excellent biocompatibility (Ethirajan et al., 2011; Ormond and Freeman, 2013; Taldiev et al., 2023). However, the hydrophobic nature and large π -conjugated system of porphyrins lead to aggregation and precipitation in aqueous environments, which necessitates the development of carrier materials to prevent this issue (Zheng et al., 2020). While micelles and dendrimers can partially mitigate aggregation, the integration of porphyrins with porous materials like MOFs has shown distinct advantages for PDT applications (Chen J. et al., 2021).

MOFs are periodic, ordered, and porous structures formed by the coordination of inorganic metal clusters with organic molecules (Horcajada et al., 2010; Wu and Yang, 2017). The modifiable structure of porphyrins allows them to be designed as ligands with four carboxyl groups, coordinating with metals such as Zr, Hf, and Fe to form MOF materials (Furukawa et al., 2013). In these MOFs, porphyrin molecules are arranged periodically and separated, which prevents intermolecular stacking and the associated decrease in $^1\text{O}_2$ quantum yield (Chen J. et al., 2021). In comparison with conventional systems where PEG-based polymers are conjugated with porphyrin molecules to form micelles for phototherapy, it can be observed that the hydrophobic porphyrin molecules are located in the hydrophobic core and may still aggregate, leading to a decrease in the singlet oxygen quantum yield. Additionally, the conjugation process involves complex organic synthesis. On the other hand, using non-covalent interactions for encapsulation often results in low loading efficiency of the molecules. However, the high loading efficiency of porphyrin photosensitizers within the MOF matrix can be achieved (Wu and Yang, 2017). The porous nature of these materials facilitates the entry of O_2 and the release of short-lived ROS (Park et al., 2016). This enhances the therapeutic efficacy. Research has also shown that under mildly acidic conditions, particularly in the presence of phosphates, the stability of MOFs can be compromised (Rojas et al., 2019). This will allow for the controlled release of other functional drug molecules loaded within the framework (Wang et al., 2020). This feature can be exploited for

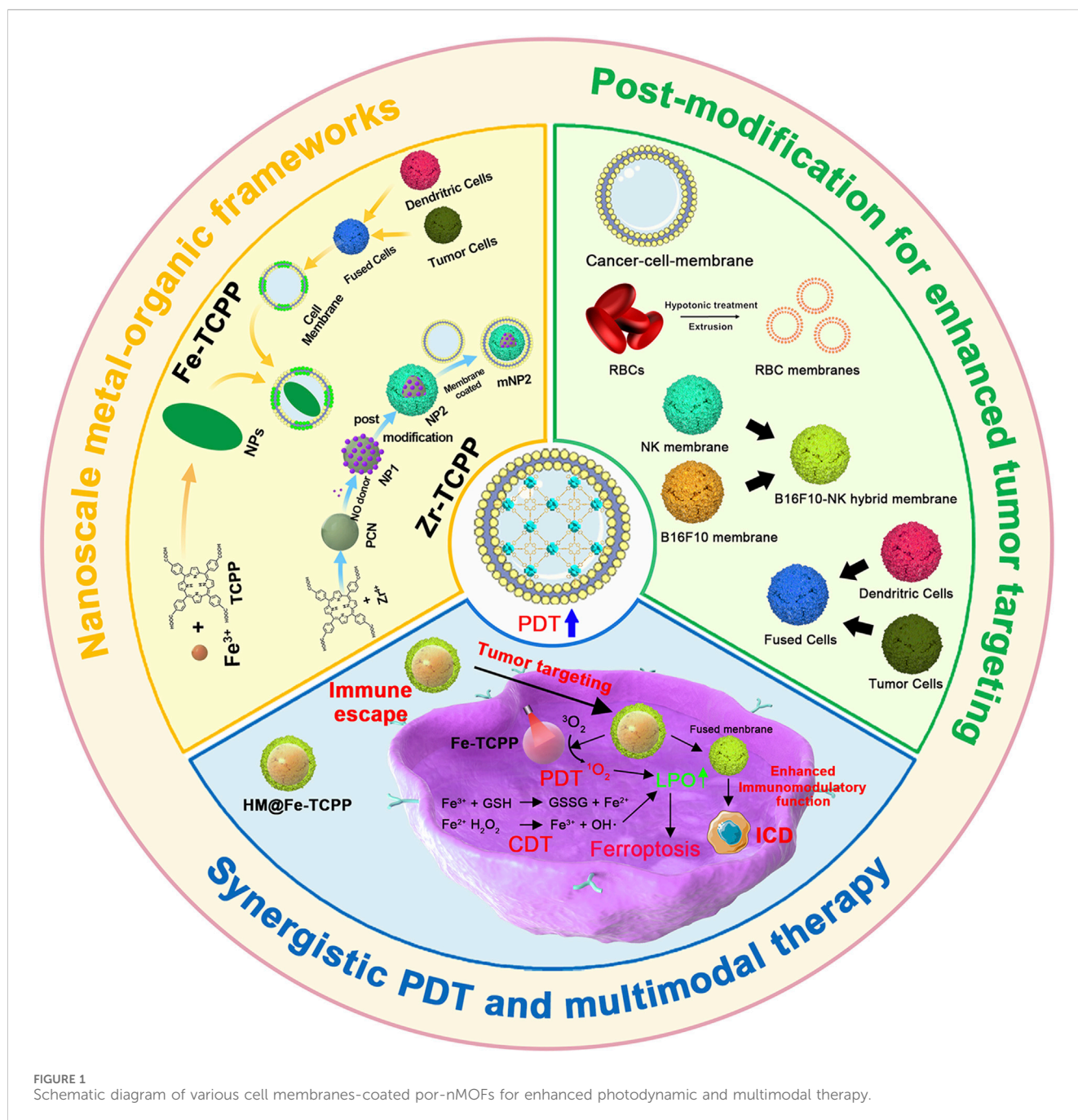
combination therapies, such as the release of L-arginine for nitric oxide (NO)-mediated gas therapy (Yang et al., 2022), TPZ for hypoxia-activated chemotherapy (Li et al., 2017a), or mitochondrial respiration inhibitors to enhance PDT by limiting oxygen consumption (Yu et al., 2023). Additionally, the surface of MOFs can be modified through non-covalent interactions to load enzymes like glucose oxidase and catalase for starvation therapy combined with PDT (Yang et al., 2022), further broadening the biomedical functions of por-nMOFs.

The growing interest in por-nMOFs as a nanoplatform is driven by these application advantages (Park et al., 2016). To improve the circulatory stability and cancer cell targeting of these nMOFs, while reducing clearance by endothelial cells, various surface modifications have been explored (Fu et al., 2022). For example, coating MOFs with polymers such as pegylation and hyaluronic acid enhances stability and promotes accumulation in tumor tissue via the enhanced permeability and retention effect (Sun et al., 2019; Fu et al., 2022). Wrapping MOFs with proteins like HSA can increase biocompatibility and reduce immune clearance (Kato et al., 2019). These modifications have shown some success in improving the therapeutic outcomes of por-nMOFs in PDT. However, to achieve even better results, researchers have turned to cell membrane coatings (Fang et al., 2018). Cancer cell membranes, red blood cell membranes, and hybrid membranes have all been used (Fang et al., 2017). Cancer cell membranes not only enhance the dispersion and circulatory stability of por-nMOFs but also facilitate active homotypic targeting and evasion of phagocytosis (Krishnan et al., 2023). Red blood cell membranes provide similar benefits, with additional aptamer modification to enhance cancer cell targeting (Luk and Zhang, 2015). Hybrid membranes, which are created by fusing cancer and immune cell membranes, can improve circulation and targeting. These membranes possess immunomodulatory functions and significantly enhance the immunogenic cell death (ICD) that is induced by photodynamic therapy (Cai et al., 2024).

This review summarizes the advances in using various cell membrane coatings to enhance the PDT efficacy of por-nMOFs (Figure 1). We highlight the immune-evasive properties provided by these coatings, which allow for effective treatment at lower concentrations, thereby minimizing side effects. We also discuss the homotypic targeting capabilities conferred by the complex antigen profile on cancer cell membranes and the synergistic benefits of hybrid membranes in promoting ICD. By comparing the functional characteristics of different cell membranes, we aim to provide a comprehensive analysis of their respective advantages. This review compiles recent work on the synergistic mechanisms of cell membrane-coated, por-nMOFs for enhanced PDT and combination therapies against tumors (Figure 1). Through these insights, we hope to promote the further application of por-nMOFs in oncology and offer new design perspectives and reference value for the preclinical application of porphyrin photosensitizers.

2 PCN MOF and cell membrane acquisition procedures and mixing strategies

To highlight the specific information of cell membrane-coated MOF (CM@MOF) obtained by combining por-nMOFs with cell

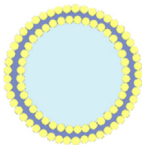
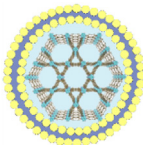
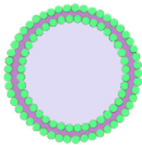
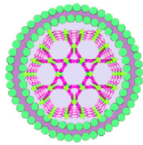
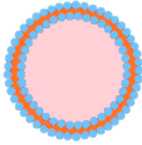
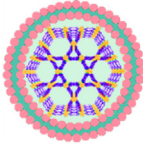
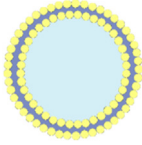
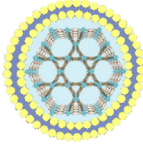
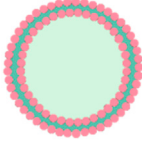
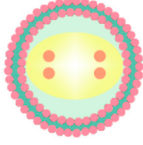
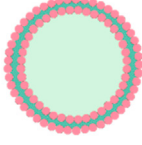
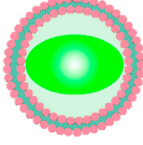
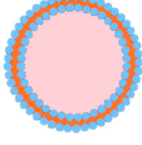
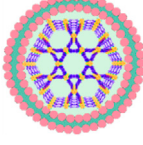
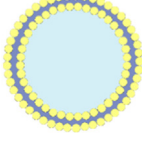
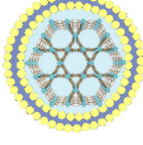
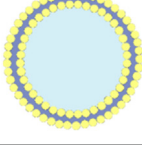
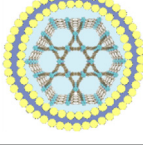


membranes, Table 1 details the various sizes and therapeutic mechanisms of CM@MOF materials for enhanced PDT. In this review, the designed porphyrins and MOFs are primarily PCN-structured frameworks (Table 1). Therefore, a brief description of the preparation steps for PCN MOFs, based on the literature, is provided as follows: $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$ (300 mg), TCPP (100 mg), and benzoic acid (2.8 g) are dissolved in DMF, and the mixture is stirred (100 mL, 90°C, 5 h). Subsequently, PCN-224 is collected via centrifugation at 12,000 rpm for 30 min and washed 3 times with DMF to obtain uniform-sized PCN MOF material (Li et al., 2017a).

The types of cell membranes involved in this review are numerous. Different cell membranes have different sources and

characteristics. For example, the 4T1 cell membrane is derived from mouse breast cancer cells, and the HeLa cell membrane is from human cervical cancer cells. The different sources of these two leads to certain differences in the components of the cell membranes, including the content of membrane proteins and the types of phospholipids on the cell membranes. Among them, different membrane proteins can affect cell-to-cell signal transduction and recognition. The phospholipid content can affect the charge distribution after the combination of the cell membrane and MOFs, thereby influencing the dispersion stability of MOFs. In addition, according to the latest data released by the International Agency for Research on Cancer, breast cancer is the most common cancer among women and has a significant impact on women's

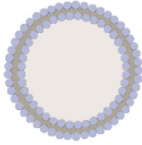
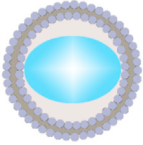
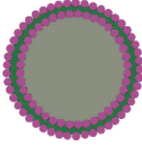
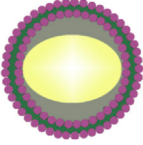
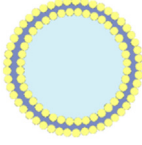
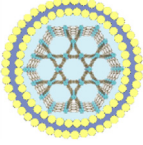
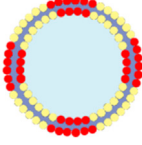
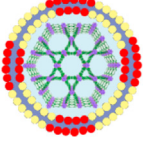
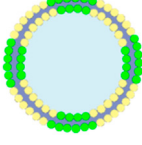
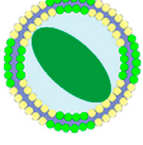
TABLE 1 Cell membrane-coated porphyrin nMOFs for enhanced PDT Applications.

Material	Structure		Function/Therapeutic advantages	Ref.
	Cell membrane	CM@MOF		
4T1 CM Zr-PtTCPP			150.5 nm, −28.5 mV real time O2 sensing, homotypic targeting, immune escape, PDT	Li et al. (2018)
HeLa CM Zr-TCPP			160 nm, −63.41 mV dual-mode imaging (MRI/FL), homotypic targeting, IO2-evolving PDT	Zhang et al. (2019)
CT26 CM Zr-TCPP			200 nm, −24 mV homotypic targeting, block ROS elimination pathway for enhanced PDT	Cheng et al. (2019b)
4T1 CM Zr-TCPP			105 nm, ~−23 mV, 660 nm, homotypic targeting, ROS-induced high NO generation, PDT + gas therapy	Wan et al. (2018)
RBCMA Cu-TCPP			220 nm, −6.5 mV, 630 nm longer circulation, faster liver clearance, higher tumor accumulation, PDT + Chemo	Falsafi et al. (2021)
RBCMA Fe-TCPP			~210 nm, −40.7 mV, longer circulation and tissue residence time, tumor-targeted, high enrichment, PDT + CDT	Zhao et al. (2020)
CT26 CM Zr-TCPP			265 nm, −19.7 mV, 808 nm, 660 nm, good immune evasion, homotypic targeting, O2 generation, PDT + PTT	Cheng et al. (2019a)
4T1 CM Zr-TCPP			227.5 nm, −20.9 mV, 660 nm homotypic targeting, immune escape, O2 generation, PDT + ST	Li et al. (2017b)
4T1 CM Zr-TCPP			154 nm, −37.1 mV, immune escape, homotypic targeting, PDT + TPZ (hypoxia-activated bioreductive therapy)	Li et al. (2017a)

(Continued on following page)

health (Bray et al., 2024). Therefore, research and treatment of breast cancer are urgent. Given the unique homotypic targeting of 4T1 cell membranes to 4T1 cancer cells, here we take the acquisition of 4T1 cell membranes as an example. To obtain the 4T1 cell membrane, researchers first need to culture and passage 4T1 cells. The cells are then collected and resuspended in

TABLE 1 (Continued) Cell membrane-coated porphyrin nMOFs for enhanced PDT Applications.

Material	Structure		Function/Therapeutic advantages	Ref.
	Cell membrane	CM@MOF		
MDA-MB-231 CM Fe-TCPP			201 nm, −14.22 mV, immune escape, homotypic targeting, O ₂ generation, PDT + TPZ + Ferroptosis	Pan et al. (2022)
RBCM Fe-TCPP			223 ± 24 nm in length, long circulation, enhanced tumor accumulation, PDT + CDT + immunotherapy	Li et al. (2022)
4T1 CM Zr-TCPP			<200 nm, −36.9 mV, homotypic targeting, immune escape, NO generation, PDT + gas therapy + ST	Yang et al. (2022)
FCM1 Zr-TCPP			~100 nm, −14.2 mV, homotypic targeting, immune escape, immunomodulatory functions, PDT + ICD	Gao et al. (2024)
FCM2 Fe-TCPP			~25 nm, ~−7.5 mV, homotypic targeting, immune escape, immunomodulator, PDT + CDT + Ferroptosis + immunotherapy	Cai et al. (2024)

Abbreviated specification: CM, cell membrane; RBCM, red blood cell membrane + aptamer; FCM1, hybrid membrane from NK cell and tumor cell; FCM2, hybrid membrane from DC cell and tumor cell; PDT, photodynamic therapy; chemo, chemotherapy; CDT, chemodynamic therapy; PTT, photothermal therapy; ST, starvation therapy; ICD, immunogenic cell death.

hypotonic lysis buffer containing membrane protein extraction reagents and phenylmethylsulfonyl fluoride. The cell suspension is incubated on ice for about 10–15 min. Following this, 4T1 cells undergo freeze-thaw cycles to further disrupt the cellular structure. After this process, the disrupted cells are centrifuged at 700 g for 10 min at 4°C to remove unbroken cells and large debris. The supernatant from the first centrifugation is then subjected to a second centrifugation at 14,000 g for 30 min to isolate the plasma membrane components. The final step involves collecting the white precipitate, which is rich in plasma membrane, and lyophilizing it for subsequent combination with nMOFs (Cheng et al., 2016; Li et al., 2017a).

To prepare CM materials, researchers can mix PCN with the cell membrane in a 1:1 mass ratio. The mixture is then extruded through a 400 nm polycarbonate porous membrane to obtain CM@PCN nanomaterials encapsulated with 4T1 cell membrane. By adjusting post-modification or preparation conditions, PCN MOF materials of varying sizes (100–300 nm) can be achieved (Li et al., 2017a). Although there are some differences in the methods of extracting different cell membranes, all extracted cell membranes exhibit negative surface potentials. After combining with MOFs, the resulting CM@MOF also displays a negative potential similar to that of the cell membranes (Table 1).

3 Biomimetic por-nMOFs for PDT and phosphorescence imaging of O₂

Due to an uneven vascular and O₂ supply, solid tumors are always hypoxic, which can reduce the efficacy of PDT of por-nMOFs (Zhang et al., 2019). Therefore, monitoring O₂ concentration during PDT is crucial for predicting its therapeutic effectiveness (Li et al., 2018). Based on this concept, Li et al. designed a MOF material that can detect changes in O₂ concentration, formed by the coordination of Zr⁴⁺ ion with tetracarboxylic platinum porphyrin (Li et al., 2018). The authors then coated the prepared nMOFs with cancer cell membranes to create the mPPt nanosystem (Figure 2). The presence of the cancer cell membrane provided the material with good biocompatibility, enhanced immune evasion, and improved homotypic cancer cell targeting. Moreover, the authors ingeniously utilized the phosphorescence emitted during the transition of the mPPt photosensitizer from the triplet state back to the ground state, which is quenched at high oxygen concentrations, to monitor hypoxia during PDT (Figure 2). This system cleverly integrates the PDT functionality of the cell membrane-coated por-nMOFs with real-time O₂ concentration detection. It both elucidates the energy transfer process of the porphyrin nMOFs photosensitizer in the triplet state and

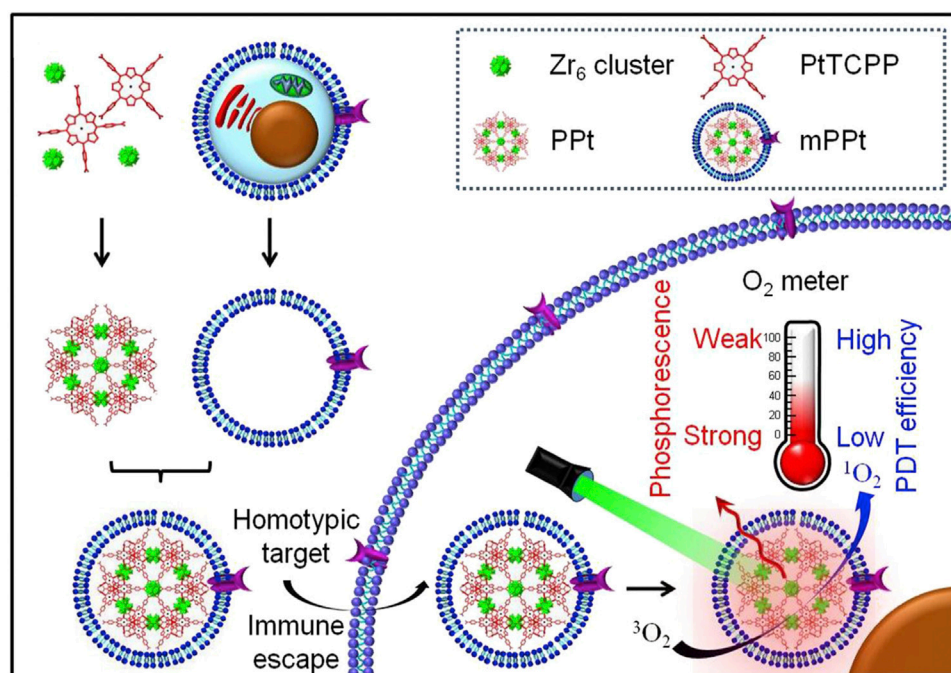


FIGURE 2
Preparation and the hypothesized mechanism of mPPt in cancer targeting and phosphorescence-guided PDT. Reproduced with permission from (Li et al., 2018). Copyright (2018), Elsevier.

highlights the critical role of hypoxia in affecting PDT outcomes. By incorporating cancer cell membranes, the authors have improved the stability of the por-nMOFs and endowed them with active homotypic targeting and immune evasion. This innovation opens new avenues and application prospects for the PDT of por-nMOFs. During PDT, monitoring oxygen concentration changes is necessary. Conventional methods like the Clark electrode method and fluorescence quenching technology have their own pros and cons (Zhang et al., 2021). The Clark electrode method is invasive and has limitations in measuring complex or moving samples for large-area and dynamic monitoring despite its high accuracy (Liebisch et al., 2020). The fluorescence quenching technology is highly sensitive, miniaturizable, portable and suitable for on-site rapid detections, but may be interfered by factors like fluorescent substance stability and environmental impurities, causing measurement result deviations (Werner et al., 2021). The phosphorescence imaging technology in this system has unique advantages, overcoming the shortcomings of the other two methods and performing well in hypoxia monitoring and for studying oxygen distribution in complex biological situations (Li et al., 2018).

4 Biomimetic por-nMOFs for O₂-Evolving PDT

After recognizing that hypoxia can limit the efficacy of PDT using por-nMOFs, researchers have proposed strategies to alter the tumor's hypoxic microenvironment. This may effectively enhance the performance of oxygen-dependent porphyrin-based nMOF photosensitizers. One common approach is to use inorganic

materials that react with excess H₂O₂ within cancer cells to generate oxygen (Zhang et al., 2019). For instance, Xiao et al. developed a Zr-TCPP nMOF by coordinating tetracarboxylic porphyrin (TCPP) with metal Zr, followed by surface modification with MnO₂ nanosheets (Zhang et al., 2019). The resulting core-shell nanostructure was then encapsulated with cell membranes to form the CM-MMNPs nanosystem. Experimental results showed that the MnO₂ in the nanosystem could react with H₂O₂ under mildly acidic conditions to produce oxygen, thereby alleviating the hypoxic environment and the limitations imposed by the oxygen consumption of the por-nMOFs during PDT. Furthermore, the cell membrane coating provided the nMOFs with good dispersibility and enhanced homotypic targeting to cancer cells. This self-oxygen-generating nMOFs system presents a new strategy for treating hypoxic solid tumors with porphyrin-based photosensitizers (Figure 3A).

The diminished fluorescence of the MMNPs confirms the formation of the MnO₂/MOF composite. Furthermore, the recovery of fluorescence upon the addition of H₂O₂ demonstrates the interaction mechanism between MnO₂ and H₂O₂ under mildly acidic conditions (Figure 3B). To demonstrate the oxygen-generating capability of the MnO₂, the authors used ABDA as the ¹O₂ scavenger to test the ability of the material to produce ¹O₂ under different conditions. The results showed that under mildly acidic conditions, the capacity of the por-nMOFs to generate ¹O₂ increased significantly with increasing H₂O₂ concentration (Figure 3C). Moreover, this enhancement was closely related to the weakly acidic environment (Figure 3D). In neutral conditions, the nanosystem lacks self-oxygen generation and photoreactivity, ensuring good biocompatibility. However, it shows

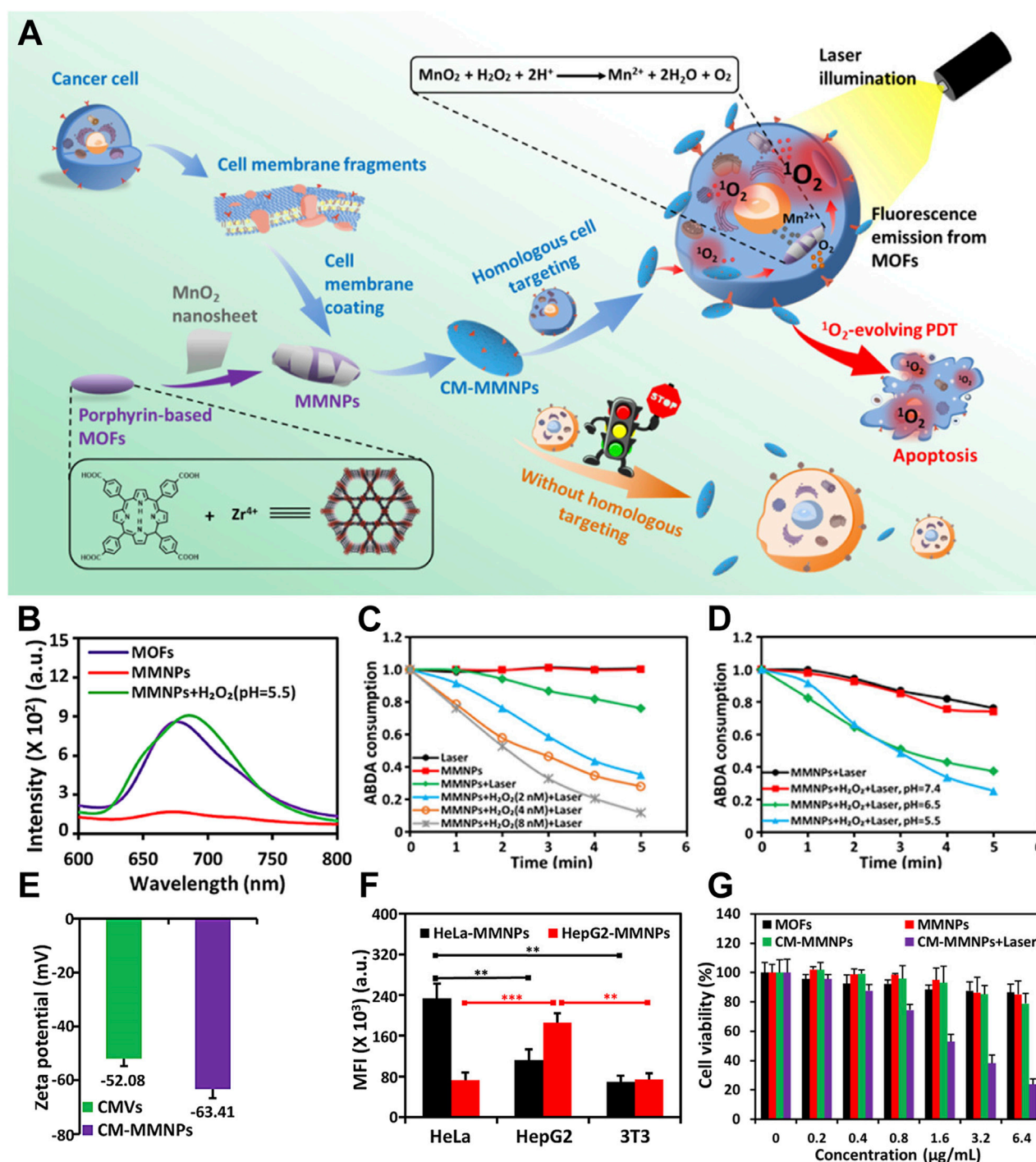


FIGURE 3

(A) A schematic representation of CM-MMNPs for homologous targeting, as well as for dual-mode MRI/fluorescence imaging and PDT. (B) Fluorescence spectra of MOFs, MMNPs, and MMNPs in the presence of H_2O_2 . (C) The time-dependent consumption of ABDA under different conditions. (D) The time-dependent consumption of ABDA when MMNPs are exposed to laser light, with and without H_2O_2 , at various pH levels. (E) Zeta potential measurements of cancer membrane vesicles (CMVs) and cancer membrane-coated magnetic multifunctional nanoparticles (CM-MMNPs). (F) The corresponding mean intensity of red fluorescence from HeLa, HepG2, and 3T3 cells after co-incubation with HeLa cell membrane-coated CM-MMNPs. (G) Cytotoxicity assessment of MOFs, MMNPs, and CM-MMNPs. Reproduced with permission from (Zhang et al., 2019). Copyright (2019), American Chemical Society.

significantly increased photoreactivity in the mildly acidic cancer cell environment. To enhance dispersibility and cancer cell targeting, the authors coated the por-nMOFs with cancer cell membranes. The zeta potential of the cancer cell membrane-

coated NPs was -63.41 mV, close to the zeta potential of the cell membrane itself, confirming the successful application of the cell membrane in the system (Figure 3E). Quantitative analysis of the confocal images further supported the homotypic targeting effect of

the cancer cell membranes (Figure 3F). The authors then demonstrated that the HeLa cell membrane-coated por-nMOFs system exhibited significantly higher phototoxicity under 409 nm light illumination compared to the uncoated material (Figure 3G). This system utilizes the reaction between MnO_2 and H_2O_2 to provide an O_2 source for the PDT process of por-nMOFs. Moreover, the cancer cell membrane coating significantly enhances the therapeutic effects of the por-nMOFs. This approach brings new insights into the biomedical applications of porphyrin photosensitizers.

5 Enhancing PDT efficacy of por-nMOFs by redox interference

In addition to the direct impact of hypoxic microenvironments on PDT efficacy, cancer cells also possess intrinsic protective mechanisms that can counteract the oxidative damage caused by ROS generated during PDT (Cheng et al., 2019b). These include antioxidants such as glutathione, small vitamin molecules, and enzymes like superoxide dismutase, catalase, peroxidases, and thioredoxin reductase. These antioxidants effectively reduce the oxidative stress induced by ROS, maintaining cellular redox balance (Cheng et al., 2019b). Since PDT relies on the generation of ROS to induce cancer cell apoptosis, its effectiveness can be significantly inhibited by these intracellular reducing agents. Therefore, disrupting the synthesis pathways of these reducing agents or inhibiting their function can enhance the efficacy of PDT. Researchers have found that the alkaloid piperlongumine (PL) increases intracellular ROS levels by inhibiting thioredoxin reductase, thereby altering the redox balance. Based on this, it was hypothesized that combining PL with por-nMOFs could enhance PDT outcomes (Cheng et al., 2019b).

Cheng et al. developed a PCN nMOFs by coordinating TCPP with metal Zr, which was then co-stirred and centrifuged with PL to form the PCN-PL nanosystem (Cheng et al., 2019b). This system was further coated with cell membranes through co-extrusion to create the PCN-PL@CM nanosystem. Under mildly acidic intracellular conditions, the Zr-O bonds become unstable, leading to the release of PL. Additionally, the protonation of the amine groups on PL facilitates its efficient release. Upon light exposure, the PCN-PL@CM system generates ROS, while the released PL inhibits thioredoxin reductase, thus preventing the elimination of ROS. This results in a strong oxidative stress and causes cell death (Figure 4). Cytotoxicity experiments confirmed that both PCN-PL and PCN-PL@CM effectively inhibited cell survival, exhibiting excellent phototoxicity. Subsequent animal studies showed that the PCN-PL@CM with light exposure achieved the best tumor suppression, due to the enhanced circulatory stability and homotypic cancer cell targeting provided by the cancer cell membrane coating. This system's design shows that inhibiting intracellular antioxidants enhances the phototherapeutic function of por-nMOFs. The nMOFs' porous structure and surface allow for loading inhibitors and coating with cancer cell membranes, improving targeting and efficacy. This approach provides valuable insights for developing advanced por-nMOFs.

6 Biomimetic por-nMOFs for combined PDT + gas therapy

While self-generated oxygen can effectively enhance PDT efficacy, the short-lived and limited diffusion radius of the generated ROS still pose challenges for optimal therapeutic outcomes (Cheng et al., 2016). Research has shown that certain radical species, similar to ROS, exhibit strong reactivity but have a longer half-life (~5 s) and are more capable of diffusing within cells (40–200 μm) (Wan et al., 2018). These radicals can also react with intracellular substrates, potentially offering unexpected benefits in tumor therapy. Radicals like NO, which contain unpaired electrons, are typical examples of such biologically active species (Wan et al., 2018). In recent years, gas therapies, including CO, H_2S , and NO, have become a focus of research for some groups (Fan et al., 2017; Zheng et al., 2021). As a green treatment with low toxicity and side effects, gas therapy has shown potential to enhance various therapeutic approaches (Zheng et al., 2021). The integration of PDT with gas therapy in nanosystems has garnered significant attention from researchers.

NO exhibits dose-dependent biological effects; at higher concentrations, it acts as a tumor suppressor, inducing cancer cell apoptosis (Wan et al., 2018). At lower concentrations, it modulates P-glycoprotein activity, helping to overcome multidrug resistance in cancer cells. Therefore, the design and development of nano-drug systems that combine NO gas with PDT may provide new avenues for enhancing PDT efficacy (Wan et al., 2018). The porous structure of por-nMOFs offers excellent drug-loading capabilities, enabling the incorporation of NO donor molecules, such as L-arginine. Based on this principle, Wan et al. prepared a PCN MOF by coordinating TCPP with Zr. They then loaded the MOF with L-arginine and subsequently encapsulated it with cancer cell membranes, resulting in the L-Arg@PCN@Mem nanoplateform (Figure 5) (Wan et al., 2018). Under light exposure, this nanoplateform generates $^1\text{O}_2$, which oxidizes L-arginine to produce large amounts of NO. High concentrations of NO can induce cytotoxicity, thereby achieving a combined therapeutic effect of PDT and NO-mediated gas therapy (Figure 5). The homotypic targeting capability of the cancer cell membrane enhanced the efficient inhibition of L-Arg@PCN@Mem + L on 4T1 cell proliferation. Based on the analysis of the experimental results, L-Arg alone under light exposure barely causes cell death, indicating the good biocompatibility of L-Arg and its advantage as a NO donor. Compared to PCN under light exposure, the cytotoxicity of the L-Arg-loaded L-Arg@PCN nanosystem is further increased (cell viability decreases from approximately 70% to around 62%). Notably, when encapsulated with a cell membrane, L-Arg@PCN@MeM under light exposure leads to a cell viability of approximately 12.5%, while PCN@MeM without L-Arg results in a cell viability of 37.5%. These comparative experimental results suggest that cell membrane encapsulation is more conducive to enhancing the cytotoxic effect of NO. This is because the enhanced endocytosis promoted by the cell membrane facilitates an increase in the intracellular concentration of L-Arg, leading to a higher release of NO, which is more effective in exerting cytotoxic effects and synergizing with PDT. This system uses 4T1 cell membranes to encapsulate por-nMOFs, enhancing stability and targeting of cancer

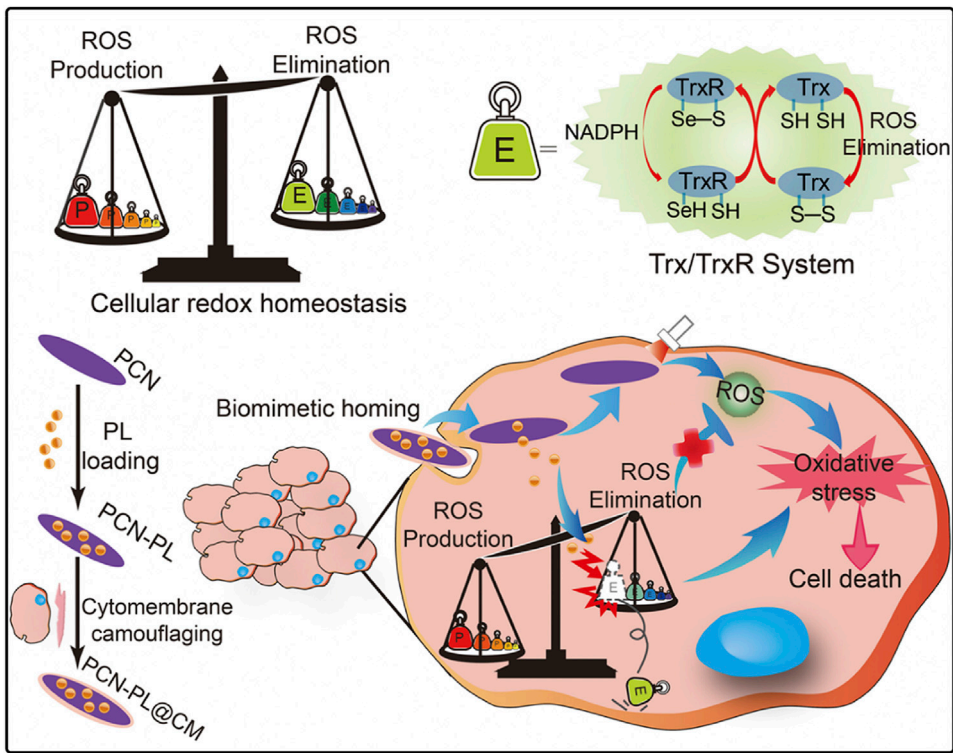


FIGURE 4
A diagrammatic representation of the strategy for disrupting redox homeostasis in cancer cells to enhance PDT. Reproduced with permission from (Cheng et al., 2019b). Copyright (2019), Elsevier.

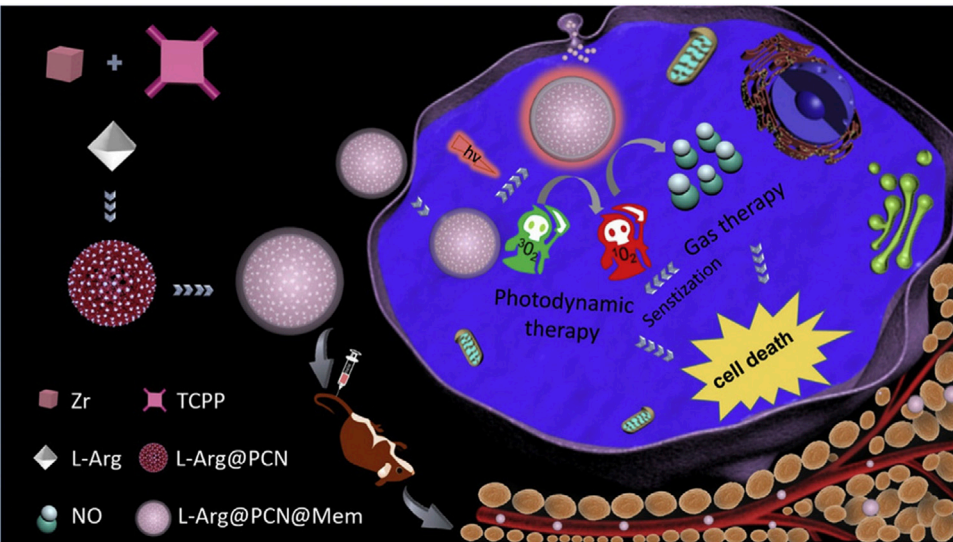


FIGURE 5
A schematic representation of the preparation of L-Arg@PCN@Mem and the lethal mechanism of gas therapy combined with sensitized PDT. Reproduced with permission from (Wan et al., 2018). Copyright (2018), Elsevier.

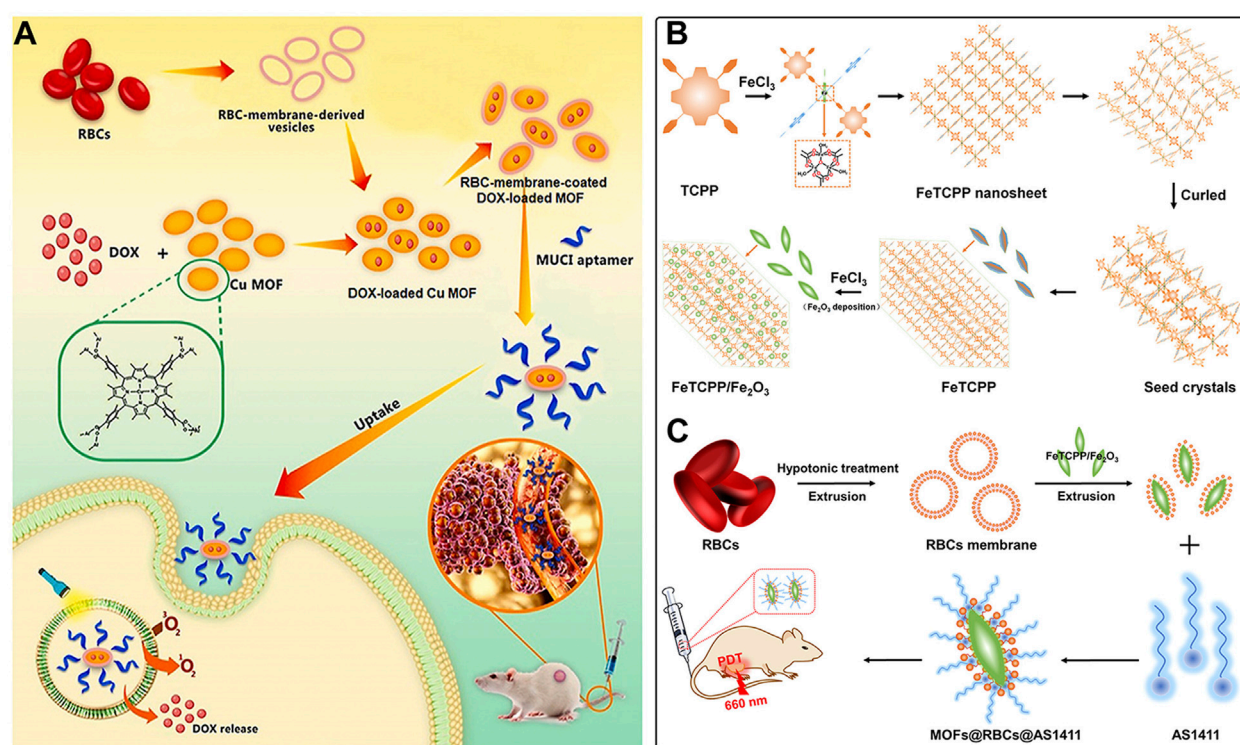


FIGURE 6
(A) The preparation of RBC-membrane-coated DOX-loaded MOF for PDT and chemotherapy. Reproduced with permission from (Falsafi et al., 2021). Copyright (2021), Elsevier. **(B)** Synthesis process of FeTCPP and the FeTCPP/Fe₂O₃ MOF nanorice; **(C)** Illustration of the MOFs@RBCs@AS1411 nanorice for targeted PDT. Reproduced with permission from (Zhao et al., 2020). Copyright (2020), American Chemical Society.

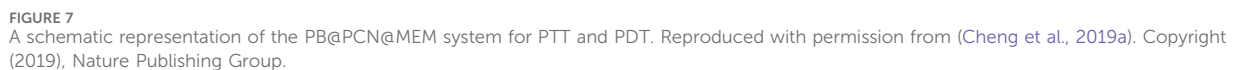
cells. This approach advances antitumor applications of por-nMOFs.

7 RBC membrane-coated por-nMOFs for enhanced PDT

Apart from cancer cell membranes, researchers have found that using aptamer-modified red blood cell membranes (RBCM) for surface modification are same effective (Falsafi et al., 2021). The RBCM encapsulation not only improves the stability of the materials, prolongs their circulation time in the bloodstream, and reduces phagocytosis by macrophages, but also confers selectivity towards a variety of cancer cells (Falsafi et al., 2021). Based on this concept, Monireh Falsafi et al. extracted RBC membranes and used them to encapsulate MOF materials pre-loaded with the chemotherapeutic drug DOX (Falsafi et al., 2021). The MOF was prepared by coordinating a porphyrin photosensitizer with metal Cu, resulting in the RBC-MOF@DOX nanosystem. The RBCM was then functionalized with carboxylated aptamers (MUC1), which have the specific ability to recognize tumor cells. Subsequent research demonstrated that the fabricated nanosystem could simultaneously target 4T1 and MCF7 breast cancer cells, achieving a combined therapy of chemotherapy and PDT, effectively inhibiting cancer cell proliferation (Figure 6A).

In addition, Zhao et al. used water and chloroform as immiscible phases and employed a liquid-liquid diffusion method to synthesize FeTCPP nMOFs and an iron oxide (Fe₂O₃) loaded nanosystem

(FeTCPP/Fe₂O₃) at the interface between the two phases (Zhao et al., 2020). The authors then isolated RBCs and modified their surface with the AS1411 aptamer. Subsequently, they combined the RBC membrane with the nMOFs material to create the MOFs@RBCs@AS1411 nanosystem, which exhibited enhanced PDT effects under 660 nm laser irradiation (Figures 6B, C). In this system, the authors controlled the ratio of porphyrin molecules to ferric ions to produce a Fe-TCPP MOF and Fe₂O₃ composite, enabling a combined CDT and PDT mechanism. Encapsulation with aptamer-modified RBC membranes significantly enhanced the circulation time and tumor accumulation of the nanomaterial in the bloodstream. This system, through the encapsulation of nMOFs with RBCM and their surface modification with cancer-specific aptamers, has shown a significant enhancement in the phototherapeutic function of por-nMOFs. The results indicate the potential of cell membrane-coated por-nMOFs for preclinical research applications. It should be noted that although the nMOFs coated with red blood cell membranes have shown significant advantages in phototherapy, the potential safety hazards that some foreign RBCM may bring to the body still cannot be ignored (Villa et al., 2016; Liu et al., 2020). Before using xenogeneic RBCM to coat nanomedicines, the surface proteins of RBCM should be detected to prevent variation (Zheng and Xiao, 2022). Meanwhile, for blood donors, blood compatibility tests and infectious disease screenings are also required, which are of great significance for preventing potential immune responses (Zheng and Xiao, 2022).



For example, Cheng et al. first synthesized Prussian Blue (PB) NPs with the assistance of polyvinylpyrrolidone (PVP) (Cheng et al., 2019a). They then added different concentrations of TCPP and zirconium ions to react and form a series of PB/PCN nanocomposites with varying shell thicknesses. The authors selected the optimal PB/PCN composite and demonstrated its ability to effectively inhibit the proliferation of CT26 cancer cells in both cellular and animal models (Figure 7). Through rational design, this system integrates por-nMOFs onto the surface of Prussian blue. It leverages the catalase-like activity of PB to decompose H_2O_2 and generate O_2 , thereby improving the hypoxic microenvironment and enhancing the efficacy of PDT. On the other

The application of nMOFs in the biomedical field is extremely broad. In addition to using their porous structure to load small molecules for

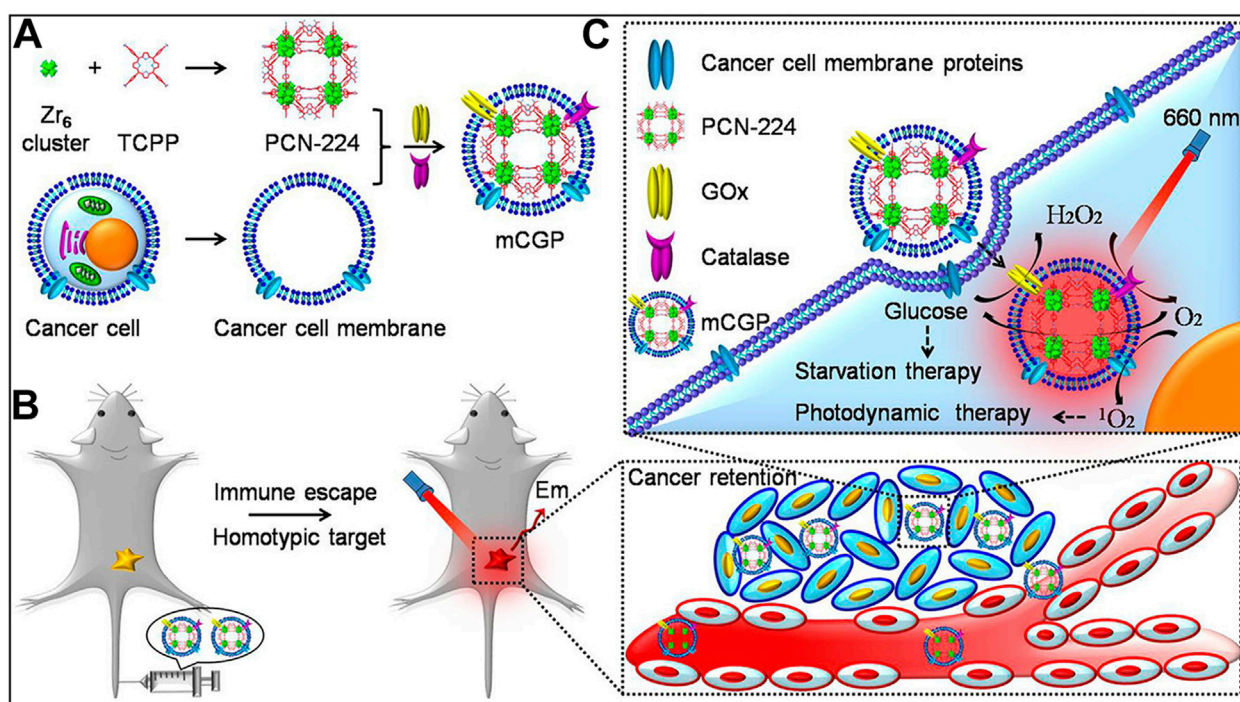


FIGURE 8

(A) The preparation steps for mCGP. (B) The immune evasion and homotypic targeting capabilities of mCGP. (C) Cascade reactions that enhance the synergistic effects of mCGP, cutting off the glucose supply to cancer cells for starvation therapy and promoting $^1\text{O}_2$ generation for PDT. Reproduced with permission from (Li et al., 2017b). Copyright (2017), American Chemical Society.

combined gas therapy, chemotherapy, and other treatments, nMOFs can also interact electrostatically with larger biomolecules, such as proteins, due to their high specific surface area (Li et al., 2017b). This allows for the integration of por-nMOFs-mediated PDT with various biological enzymes for combined therapeutic effects.

Based on this concept, Li et al. used a conventional dissolution method to synthesize PCN-224 MOF materials from TCPP and zirconium salts (Li et al., 2017b). They then loaded glucose oxidase (GOx) and catalase (CAT) onto the MOF surface through electrostatic interactions, achieving a loading efficiency of up to 13.5%. The material was subsequently encapsulated with 4T1 cancer cell membranes to form the mCGP nanocomposite system. Subsequent cellular and animal experiments demonstrated that mCGP could achieve enhanced PDT through the action of catalase and starvation therapy via glucose oxidase (Figures 8A–C). The successful design of this system further demonstrates the multifunctionality of por-nMOFs. The large specific surface area of these nMOFs enables the integration of multiple therapeutic functions through the combination with various enzymes. Additionally, the encapsulation with 4T1 cancer cell membranes significantly enhances the homotypic targeting of the por-nMOFs towards 4T1 cancer cells. The excellent antitumor efficacy of this system provides new insights for the preclinical application of por-nMOFs.

10 Biomimetic por-nMOFs for PDT and TPZ chemotherapy

In addition to using catalase to generate O_2 from H_2O_2 to improve the hypoxic microenvironment and enhance PDT

efficacy, another approach involves incorporating hypoxia-sensitive chemotherapeutic drugs to achieve a combined PDT and chemotherapy mechanism mediated by por-nMOFs (Zhang et al., 2024). For example, Li et al. synthesized PCN-224 MOF using zirconium ions and TCPP. They then co-stirred the MOF with tirapazamine (TPZ) molecules, followed by centrifugation to obtain TPZ-loaded TPZ@PCN composites (Li et al., 2017a). To enhance the circulation stability, immune evasion, and cancer cell targeting of the MOF material, the authors encapsulated the TPZ@PCN composites with 4T1 cancer cell membranes, resulting in the TPZ@PCN@Mem nanocomposite system (Figure 9A). Subsequently, at both cellular and animal levels, the authors demonstrated that the TPZ@PCN@Mem nanoplateform could exert excellent PDT effects under light exposure. The PDT process exacerbated the hypoxic microenvironment, and intracellular phosphates promoted the release of TPZ molecules from the nMOF. In the hypoxic microenvironment, TPZ is reduced to highly toxic free radicals, thereby exerting its chemotherapeutic function. The results showed that the 4T1 cancer cell membrane-encapsulated por-nMOFs could achieve a combined effect of PDT and hypoxia-activated chemotherapy (Figure 9B). The successful design of this system brings new hope for the phototherapeutic application in hypoxic solid tumors and aids in the precise and effective eradication of hypoxic solid tumors by por-nMOFs.

In addition to the common PCN framework MOFs formed by the coordination of Zr^{4+} with TCPP, Fe^{3+} can also coordinate with TCPP to form a spindle-shaped nMOF material. Researchers have found that the Fe^{3+} in Fe-TCPP MOFs can interact with excess GSH within cancer cells (Pan et al., 2022). This interaction not only

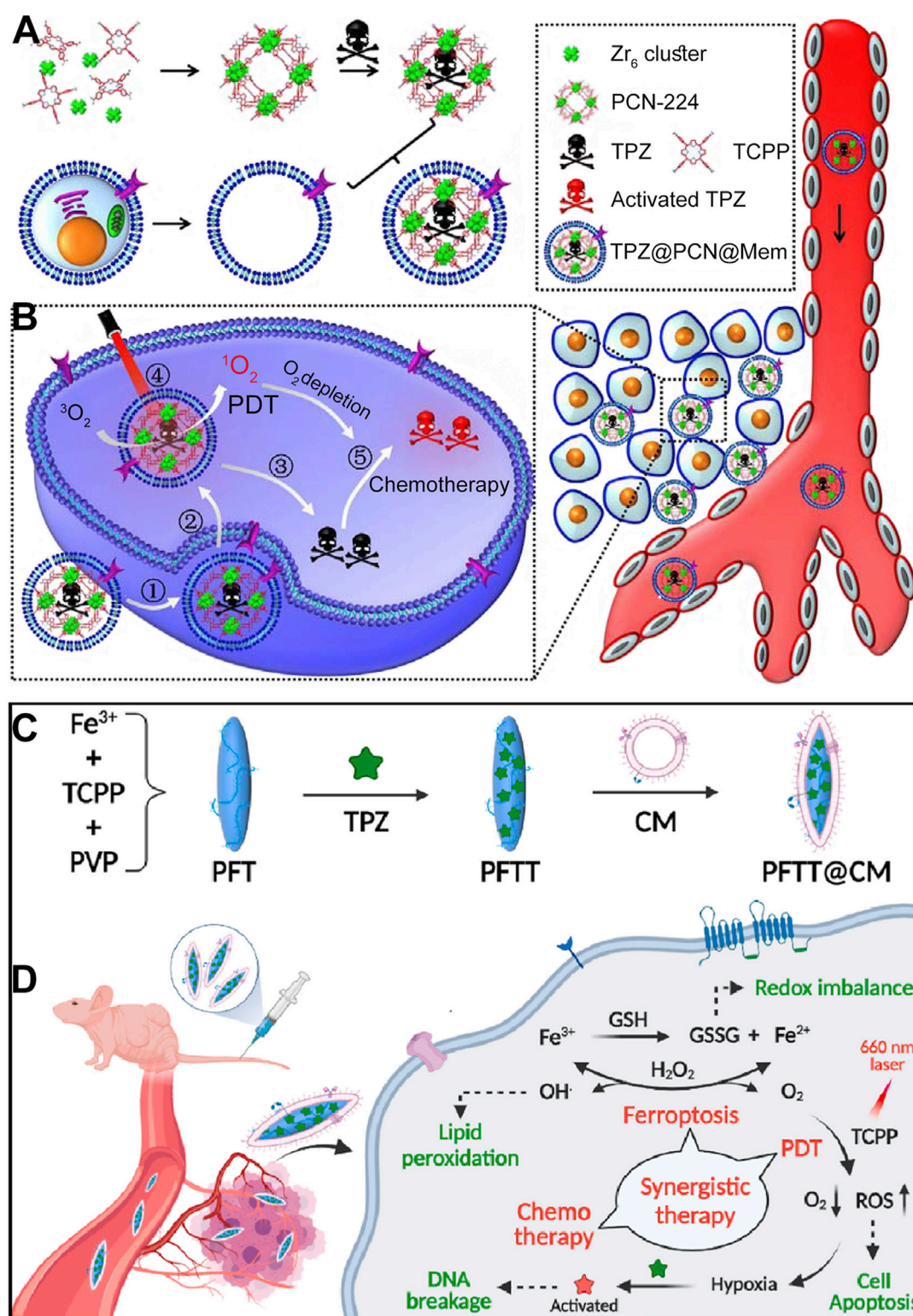


FIGURE 9

(A) Step-by-step assembly process of the cancer cell membrane-coated biomimetic nanoplatform TPZ@PCN@Mem. (B) Diagrammatic representation of the multifunctional nanoplatform, which exhibits enhanced tumor accumulation for targeted PDT and hypoxia-activated bioreductive therapy. Reproduced with permission from (Li et al., 2017a). Copyright (2017), Elsevier. (C) Fabrication process of the cell membrane-coated nanocomposites PFTT@CM. (D) Illustration of the synergistic therapeutic effects of PFTT@CM, which integrates ferroptosis, PDT and hypoxia-activated chemotherapy. Reproduced with permission from (Pan et al., 2022). Copyright (2022), Elsevier.

reduces GSH levels, which is beneficial for ROS-mediated cancer therapy, but also generates Fe^{2+} ions. Under mildly acidic conditions, Fe^{2+} ions could react with H_2O_2 to form highly oxidative hydroxyl radicals (Pan et al., 2022). These radicals oxidize lipids, producing more lipid peroxidation products and inducing ferroptosis (Zou et al., 2024). Ferroptosis is a widely-studied approach for disease treatment (Wang et al., 2022; Li Z. et al., 2023; Mao et al., 2023), and the combination of ferroptosis and phototherapy may lead to unexpected results.

For example, Pan et al. synthesized PFT NPs (Fe-TCPP MOF) under the assistance of PVP (Pan et al., 2022). They then added TPZ molecules, stirred, and centrifuged the mixture to obtain PFTT NPs loaded with the chemotherapeutic drug TPZ. To further enhance the targeting of the nanomaterials towards cancer cells, the authors coated the PFTT NPs with breast cancer cell (MDA-MB-231) membranes, resulting in the PFTT@CM nanosystem (Figure 9C). Subsequently, at the cellular level, the authors demonstrated the multifunctional combined therapeutic effects of the PFTT@CM nanosystem. The breast cancer cell membrane coating facilitated the targeted endocytosis of the nanomaterials by breast cancer cells. Upon internalization, the presence of phosphates led to the release of TPZ. Under light exposure, the porphyrin photosensitizer generated $^1\text{O}_2$, exerting PDT effects. This process further exacerbated the hypoxic microenvironment, promoting the conversion of TPZ into its radical form, which significantly enhanced cytotoxicity. Additionally, the Fe^{3+} ions in the MOF structure could interact with excess intracellular GSH and H_2O_2 , generating highly oxidative hydroxyl radicals and forming a significant amount of lipid peroxidation products. This ultimately achieved a combined therapeutic effect involving porphyrin-mediated PDT, TPZ-mediated chemotherapy, and Fe-mediated ferroptosis (Figure 9D). The encapsulation with breast cancer cell membranes enhanced the circulation stability of the porphyrin-based nMOFs materials and their homotypic targeting towards cancer cells, providing a basis and possibility for improved therapeutic outcomes. This successful design demonstrates the potential of combining multiple therapeutic modalities using a single nanoplatform, and it highlights the importance of biomimetic strategies for enhancing the selectivity and efficacy of cancer treatments. The use of cancer cell membranes for surface modification not only improves the biocompatibility and targeting of the nanomaterials but also provides a promising approach for the precise and effective treatment of solid tumors, particularly those with a hypoxic microenvironment.

11 Biomimetic por-nMOFs for combined PDT + CDT + ICD

nMOFs can not only load organic small molecules but also inorganic materials, with different strategies employed for each. For small molecules like TPZ, the porous structure of nMOFs can be utilized for loading after the nMOFs is formed. In addition, some inorganic metal NPs can be doped into the nMOFs during its synthesis, typically requiring the metal NPs to be of a smaller size (Li et al., 2022). For example, Yao et al. used a solvothermal one-pot method to synthesize Fe-TCPP MOFs, doping them with 3 nm Pt NPs to create FTP NPs, a nanocomposite material loaded with inorganic metal (Li et al., 2022). The undoped MOF material

was labeled as FT NPs. To enhance the cancer cell targeting of the material, the authors extracted red blood cell membranes (RBCM) to encapsulate the FTP, resulting in the FTP@RBCM nanosystem (Figure 10A). At the cellular level, the authors demonstrated that the Pt NPs in the FTP@RBCM nanosystem had catalase-like activity. They could catalyze the excess H_2O_2 within cancer cells to produce O_2 . As a result, this relieved the hypoxic microenvironment and enhanced the PDT efficacy. Additionally, the iron within the MOFs is capable of interacting with the excessive intracellular GSH, thereby reducing the levels of reducing agents. The generated Fe^{2+} ions could then react with intracellular H_2O_2 , initiating a chemodynamic process to form highly oxidative hydroxyl radicals. The combination of a large number of $\cdot\text{OH}$ and the $^1\text{O}_2$ produced under light exposure induced severe oxidative stress, leading to apoptosis. Furthermore, the authors found that high concentrations of ROS also triggered acute local inflammation and immunogenic cancer cell death (Figure 10B).

TEM results confirmed the spindle-shaped structure of the synthesized Fe-TCPP (Figure 10C) and showed a clear core-shell structure after RBCM encapsulation (Figure 10D). To demonstrate the catalase-like function of the Pt NPs, the authors mixed FT NPs and FTP NPs with H_2O_2 and used SOSG as a $^1\text{O}_2$ sensor to detect the photosensitizer's ability to generate $^1\text{O}_2$ under light exposure. The results showed that the FTP material containing Pt NPs had the strongest $^1\text{O}_2$ generation capability, confirming that the Pt NPs effectively catalyzed H_2O_2 to produce O_2 , thus enhancing the PDT process (Figure 10E). Additionally, comparative experiments showed that FTP NPs could utilize Fe^{3+} to reduce the concentration of intracellular GSH (Figure 10F). Using terephthalic acid (TPA) as a probe, which becomes hydroxylated and exhibits enhanced fluorescence upon reaction with $\cdot\text{OH}$, the authors detected changes in $\cdot\text{OH}$ concentration by measuring fluorescence intensity. The results indicated that Fe^{2+} generated from the interaction between Fe^{3+} and GSH could effectively react with intracellular H_2O_2 to produce $\cdot\text{OH}$ (Figure 10G). Intracellularly, the authors also observed that FTP@RBCM could effectively reduce GSH levels, facilitating enhanced ROS-mediated therapy (Figure 10H). Cell toxicity experiments showed that the RBCM-coated nanosystem could recognize normal cells and avoid being endocytosed. However, it was efficiently taken up by Hepa1-6 cancer cells. Under light exposure, it significantly inhibited the proliferation of these cancer cells (Figure 10I). To verify that the large amount of ROS generated by the nanomaterials under light exposure could trigger an immune response and induce ICD, the authors measured intracellular immune factors, and the results were consistent with expectations. Subsequently, the authors assessed several ICD-related factors. The experimental results showed that, compared to other control groups, the FTP@RBCM + L group had the least HMGB1 signal in the cell nucleus, indicating that many HMGB1 molecules were released, with the highest extracellular HMGB1 concentration detected (Figure 10J). Additionally, as a key feature of ICD known for recruiting immune cells and triggering inflammatory effects, ATP was most prominently secreted by cells treated with FTP@RBCM + L (Figure 10K). The high ATP levels detected indicated a significant ICD effect. At the animal level, the authors also demonstrated that the FTP@RBCM + L treatment group had the best inhibitory effect on Hepa1-6 tumor proliferation (Figure 10L).

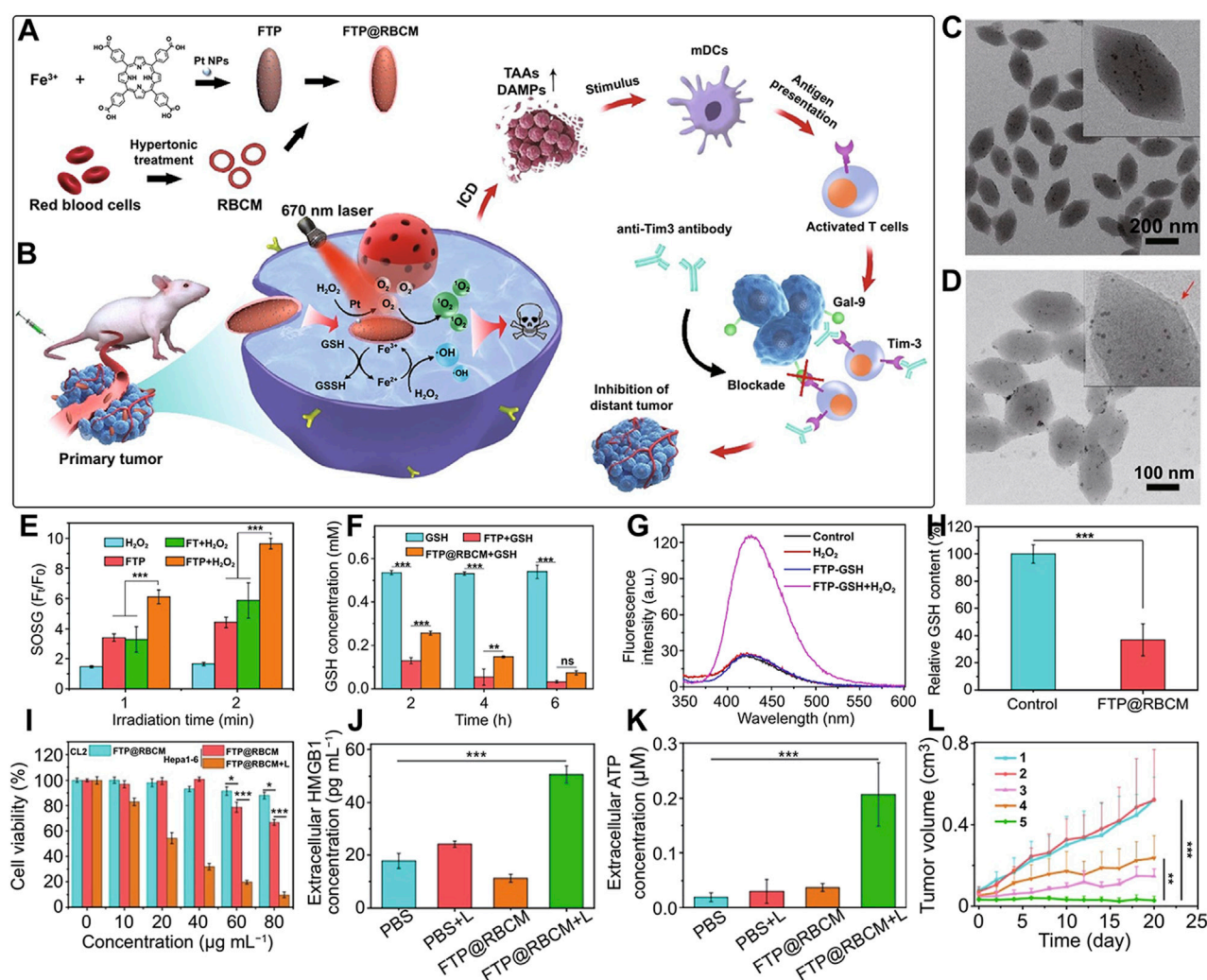


FIGURE 10

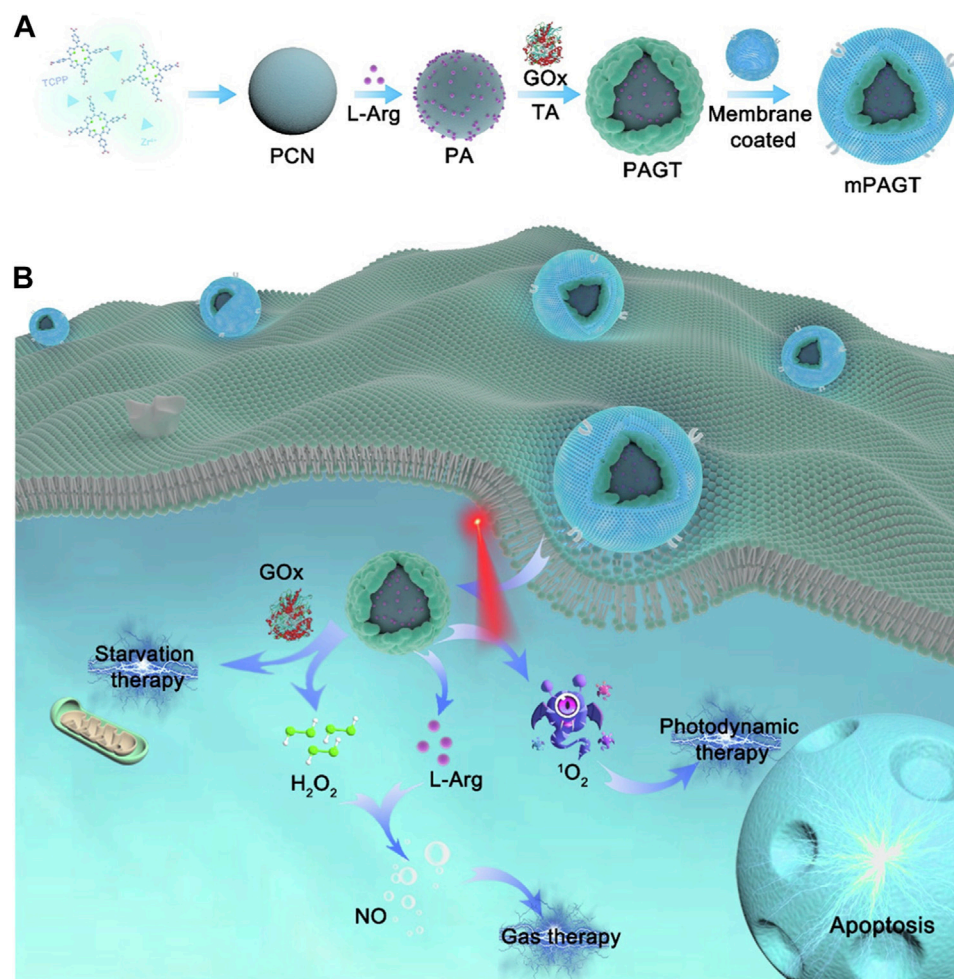
(A) Synthesis of FTP@RBCM. (B) Mechanisms of the combined immunotherapy involving radical therapy and Tim-3 checkpoint blockade. (C) TEM image of FTP. (D) TEM image of FTP@RBCM. (E) Relative fluorescence intensity of ROSG. (F) GSH concentration following various treatments. (G) Fluorescence intensity of the solution containing TPA under different treatment conditions. (H) Relative GSH content in Hep3B cells after various treatments. (I) Viability of CL2 and Hepa1-6 cells. (J) Extracellular levels of high-mobility group box 1 (HMGB1). (K) Extracellular HMGB1 levels. (L) Tumor growth curves in mice. Group 1: PBS; Group 2: PBS + Light; Group 3: FTP@RBCM + Light; Group 4: FTP@RBCM; Group 5: FTP@RBCM + Light. Reproduced with permission from (Li et al., 2022). Copyright (2022), Springer Nature.

This system utilizes por-nMOFs to load inorganic Pt NPs, and enhances cancer cell targeting through RBCM encapsulation. The system achieves enhanced PDT efficacy due to the catalase-like activity of Pt NPs, which generates O₂, and the chemodynamic mechanism mediated by Fe, which produces ·OH. The combined strong oxidative stress from ¹O₂ and ·OH triggers an ICD effect. The successful design of this nanosystem provides a new reference for the antitumor applications of por-nMOFs in conjunction with cell membranes. In this system, utilizing oxidative stress to induce the ICD effect for cancer cell inhibition is a highly effective and innovative idea. However, immunosuppression within the tumor microenvironment may affect the outcome of immunogenic cell death. Appropriately adopting other combined treatment modalities might further enhance the ICD effect. For example, using immune checkpoint inhibitors may strengthen the ability of the immune system to kill tumor cells, thereby improving the effect of ICD (Duan

et al., 2019). Alternatively, modifying CAR-T cells can overcome the immunosuppression in the tumor microenvironment and enhance the effect of its combination with ICD-inducing therapies (Li H. et al., 2023). These are all topics worthy of further research.

12 Biomimetic por-nMOFs for combined PDT + gas therapy + starvation therapy

Exploiting the porous nature and surface modifiability of nMOFs, it is possible to simultaneously load both small molecules and biomacromolecules (Yang et al., 2022). For example, Yang et al. used Zr and TCPP as precursors to synthesize PCN MOF via a solvothermal method (Yang et al., 2022). They then loaded L-arginine, which can be oxidized to



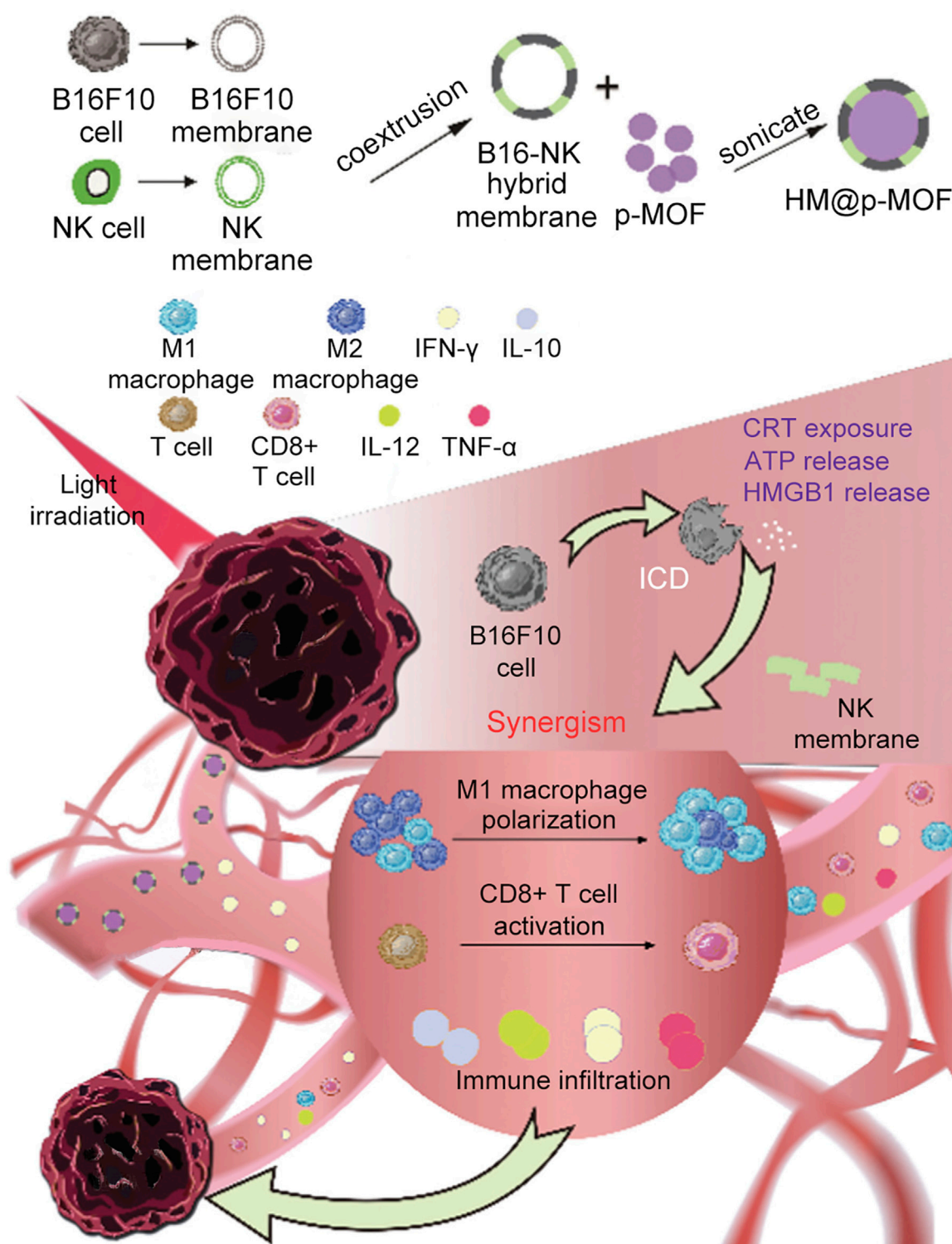


FIGURE 12
Schematic representation of the HM@p-MOF nanoplatform for immuno-enhanced tumor phototherapy. Reproduced with permission from (Gao et al., 2024). Copyright (2024), Elsevier.

various inflammatory environments (Gao et al., 2024). Numerous studies have shown that NK cells effectively activate the immune system *in vivo* (Shimasaki et al., 2020). The surface proteins on NK cell membranes can recognize malignant tumor cells and activate

phagocytes and other immune cells, thereby exerting immunomodulatory functions and eliminating tumor cells (Myers and Miller, 2021). Therefore, researchers hypothesized that encapsulating nanomaterials with NK cell membranes might

stimulate the immunomodulatory functions of the prepared nanosystems.

For example, Yu et al. synthesized PCN MOF (p-MOF) using Zr and TCPP. They then coated the p-MOF with a hybrid membrane obtained by co-extruding B16F10 cancer cell and NK immune cell membranes, resulting in the HM@p-MOF nanosystem (Figure 12) (Gao et al., 2024). Subsequent cellular experiments demonstrated that the HM@p-MOF nanosystem, assisted by the hybrid membrane, exhibited strong cancer cell targeting. Moreover, the inherent immune-activating function of the immune cell membrane enhanced the PDT effect induced by the porphyrin photosensitizer under light exposure, leading to immunogenic cell death (Figure 12). *In vivo* experimental results showed that the HM@p-MOF + L group had the most potent antitumor effect on B16F10 cancer cells compared to various control groups. Subsequently, they conducted bilateral tumor model experiments and measured the volume changes of metastatic tumors and the survival rates of mice in different treatment groups. Results showed that the HM@p-MOF + L group had the smallest metastatic tumor volumes and the longest mouse survival times. These experimental results are consistent with the expected outcomes, indicating that the enhanced ICD effect from the hybrid membrane structure can trigger systemic immune responses in mice.

14 Biomimetic por-nMOFs for combined PDT + CDT + ferroptosis + immunotherapy

In addition to the co-extrusion of NK cell membranes with cancer cell membranes to create hybrid membranes that possess both immunomodulatory and cancer-targeting properties, researchers have found that dendritic cells (DCs) can also be fused with cancer cells (Cai et al., 2024). The resulting fusion cell membranes not only effectively encapsulate and deliver nanomaterials but also simultaneously target cancer cells and activate immune responses (Cai et al., 2024). DC membranes can enable the expression of complete antigens on the surface of the hybrid membranes, thereby achieving optimal immunotherapeutic effects. Immunotherapy is extensively studied and holds significant potential in cancer treatment (Chen and Wang, 2020; Hu et al., 2020). DC membranes-enhanced immune function has been a particularly promising approach for activating the immune system.

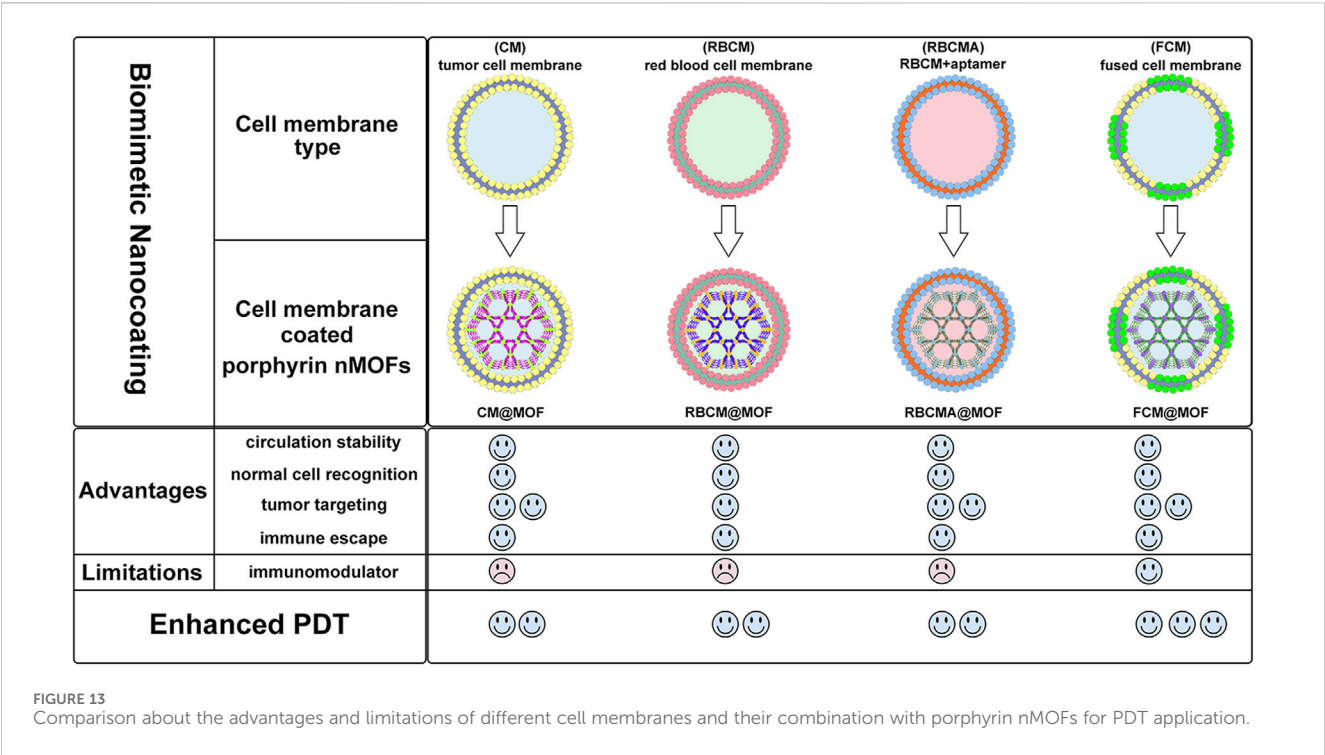
From this, Cai et al. used a self-templating strategy to mix an ethanol solution containing TCPP, oridonin (ORI), and CaO₂ with an ethanol solution of Fe³⁺ ions (Cai et al., 2024). After stirring and centrifugation, they obtained por-nMOFs NPs loaded with oridonin and CaO₂. Subsequently, the authors prepared a fusion membrane from DC and B16F10 cancer cell membranes and mixed it with the NPs via ultrasonication, resulting in the fusion membrane-coated nanosystem FM@NPs. At the cellular level, the authors demonstrated the multifunctional therapeutic effects of the prepared nanosystem. The fusion membrane coating facilitated the internalization of the nanomedicine by cancer cells. Inside the cells, the Fe³⁺ in the NPs interacted with excess intracellular GSH, forming Fe²⁺ and reducing the levels of reductive substances (Wu et al., 2024). Meanwhile, under mildly acidic conditions, CaO₂ generated H₂O₂ and oxygen. The produced O₂ alleviated tumor

hypoxia, which is beneficial for PDT. Additionally, the excess Ca²⁺ made cancer cells more sensitive. Furthermore, the H₂O₂ was generated. Along with the existing intracellular H₂O₂, it reacted with the formed Fe²⁺ through a chemodynamic mechanism and produced highly oxidative ·OH. This led to significant lipid peroxidation, and the reduction in GSH concentration ultimately induced ferroptosis. On the other hand, the acidic environment caused the degradation of the nMOFs framework, and the interaction between Fe³⁺ ions and GSH promoted the release of ORI molecules. The released ORI molecules effectively inhibited both the HSPB1/PCBP1/IREB2 and FSP1/COQ10 pathways, further inducing ferroptosis. Moreover, the immunomodulatory effect of the fusion membrane enabled the nanomedicine to effectively inhibit melanoma proliferation. This system achieved a multifunctional combined therapeutic effect, enhanced by the cancer-targeting properties of the fusion membrane. These combined mechanisms included the PDT function of the porphyrin photosensitizer, Fe³⁺/Fe²⁺-mediated CDT, ORI-mediated ferroptosis, and the immune-stimulating effect promoted by the fusion membrane. The synergistic interactions among these therapeutic effects significantly suppressed melanoma proliferation. The rational design of this system promotes the antitumor application of por-nMOFs and provides a new strategy for the treatment of highly malignant tumors such as melanoma.

15 Comparison of por-nMOFs encapsulated with different cell membranes for enhanced PDT

The framework structure of nMOFs endows porphyrin photosensitizers with unique advantages, such as inhibiting molecular aggregation and enhancing the quantum yield of ¹O₂ (Lu et al., 2014). However, for por-nMOFs to exert their therapeutic functions after intravenous injection, they must undergo several processes common to nanomedicines (Sun et al., 2014). These processes include circulation in the bloodstream, accumulation within the tumor, penetration into the tumor tissue, and subsequent internalization by cancer cells, followed by the generation of ¹O₂ under light exposure for PDT. Although nMOFs generally exhibit good biocompatibility, most nMOFs carry a positive surface charge in physiological solutions, which is not conducive to long-term circulation in the blood. Researchers have found that the zeta potential of most cell membranes is below zero (Gao et al., 2013; Kroll et al., 2017a). When combined with nMOFs, this results in a negative surface charge on the prepared nanosystems, which is favorable for prolonged circulation in the blood. Both cancer cell membranes and red blood cell membranes can enhance the dispersibility and stability of nMOFs (Figure 13).

Furthermore, summarizing the relevant experimental results, it is evident that despite the strong affinity between proliferating cancer cells. This allows them to aggregate and form solid tumors, and this unique characteristic is due to the complex antigen profile on the cancer cell surface (Fang et al., 2017). Cancer cell membrane-coated MOFs (CM@MOFs) inherit these properties. Through the recognition of surface proteins, CM@MOFs exhibit significant homotypic targeting capabilities. In contrast, red blood cell membranes do not contain cancer-specific targeting



molecules and thus cannot actively target tumors (Figure 13) (Gao et al., 2013).

Additionally, cancer cells not only possess robust survival and dissemination abilities but can also mimic certain signaling pathways using their complex membrane components, thereby evading immune surveillance and achieving immune tolerance (Fang et al., 2018). Cell membrane-coated nanosystems can effectively reduce phagocytic clearance, leading to better long-term circulation and ultimately maximizing accumulation in tumor cells (Kroll et al., 2017b). Red blood cell membranes also contain CD47 receptors, which inhibit macrophage phagocytosis of red blood cells, further aiding in the long-term circulation of the nanosystem in the blood (Gao et al., 2013). Experimental results show that both cancer cell and red blood cell membrane-coated nanoparticles result in similarly low phagocytic uptake, indicating comparable immune evasion capabilities (Figure 13). Over the past few decades, extensive research has been focused on utilizing nanomedicines, aiming to evade the immune clearance within organisms and prolong the blood circulation time (Rao et al., 2015). However, it is widely recognized that PEG polymers can trigger skin allergies. Additionally, the literature indicates that upon the second injection, PEG-modified nanoplatforms might be rapidly eliminated *in vivo* (Rao et al., 2015). To enable nanomedicines to more effectively evade immune clearance and achieve a safer and longer blood circulation duration, researchers have identified that membrane materials like red blood cell membranes are highly promising alternatives (Miao et al., 2022). A large number of preclinical studies have verified the outstanding properties of nanoplatforms encapsulated by red blood cell membranes. For instance, Rao et al. have shown that Fe₃O₄ enwrapped with red blood cell membranes exhibits a significantly prolonged blood

circulation time and can effectively resist phagocytosis by phagocytes (Rao et al., 2015). Bidkar et al. have utilized red blood cell membranes to encapsulate doxorubicin and methylene blue, capitalizing on the extended circulation time of the cell membranes to achieve more effective combined chemotherapy and photodynamic therapy (Bidkar et al., 2020). Zhang et al. have also demonstrated that the nanosystem coated with red blood cell membranes can effectively circumvent the immune clearance of organisms, thereby prolonging the blood circulation time (Miao et al., 2022). Collectively, these studies have convincingly demonstrated that cell membrane coating holds great significance for enhancing the blood circulation stability of nanomedicines and realizing the long-term circulation drug-delivery mechanism.

Beyond the use of individual cancer cell and red blood cell membranes, some researchers have combined cancer cell membranes with immune cell membranes to impart immunomodulatory functions to the hybrid membranes (Yu et al., 2024). Immune cell membranes, including those from natural killer (NK) cells and dendritic cells (DCs), are rich in antigens that can recognize tumor cells and activate phagocytes (Cai et al., 2024; Gao et al., 2024). NK cells can also secrete interferons and tumor necrosis factors, promoting the maturation and activation of DCs and modulating immune responses (Gao et al., 2024). DC membranes, when fused with cancer cell membranes, can enhance the immunomodulatory function of the hybrid membranes (Cai et al., 2024). This hybrid fusion approach leverages the complete tumor antigen presentation by immune cell membranes, significantly enhancing the therapeutic effects of PDT-induced immunogenic cell death and suppressing metastatic tumors through systemic immune activation (Figure 13). Combining cell membranes with nMOFs represents a top-down strategy. This approach not only improves the dispersibility and stability of

nMOFs, promotes immune evasion, and achieves cancer cell targeting, but also enhances the therapeutic efficacy of PDT-induced ICD by incorporating immune cell membranes (Gao et al., 2024). This integrated design provides an effective solution for the multifunctional application of nMOFs and points to new directions for the phototherapeutic applications of porphyrin-based photosensitizers.

16 Conclusion and perspectives

The advancement of nanotechnology has witnessed rapid development in the application of por-nMOFs for phototherapy and other combined therapeutic approaches against tumors (Liu et al., 2016). This review provides a comprehensive summary of the phototherapeutic applications of por-nMOFs encapsulated by cancer cell membranes, red blood cell membranes, aptamer-modified red blood cell membranes, and hybrid membranes derived from the fusion of cancer and immune cell membranes. By carefully controlling the feed ratios, catalyst amounts, and reaction times, nMOFs with large specific surface areas can be synthesized, providing a foundation for various functional applications that require surface modifications (Park et al., 2016). The effective co-extrusion strategy allows for the integration of cell membranes with nMOFs (Li et al., 2017a). These biological structures, evolved over millions of years, possess unique advantages that cannot be replicated by synthetic materials. Cancer cell-derived membranes retain a diverse range of antigens and biological functions of the original cancer cells. When combined with por-nMOFs nanosystems, these membranes confer significantly enhanced performance, including improved dispersion stability, circulatory stability, evasion of phagocytic clearance, and selective homotypic targeting of cancer cells while sparing normal cells. Moreover, if the hybrid membranes are formed from the fusion of immune and cancer cells, they inherently carry immunomodulatory properties that can augment the immunogenic cell death induced by PDT, activate systemic immunity, and inhibit cancer metastasis (Yu et al., 2024). The inspiration and application of biomimetic membranes highlight the vast potential of nMOFs when functionalized for antitumor applications. Cell membrane-coated por-nMOFs represent a nearly ideal nanomedicine, offering new hope for the deeper exploration of porphyrin photosensitizers in cancer therapy.

Despite the evident advantages of cell membrane-assisted nanomedicines, several challenges must be addressed to facilitate their widespread application (Liu et al., 2020). First, the extraction of cell membranes is time-consuming and costly, and simple extraction methods do not always ensure that all extracted membranes contain the desired functional surface antigens. Second, there is a need to explore novel cell membrane preparation techniques. For instance, various membrane extraction methods and optimization of conditions should be explored to obtain a greater quantity of high-quality cell membranes for large-scale production. Third, as delicate biological structures, the long-term storage stability of cell membranes may limit their use as a scalable material for nanomedicine preparation. Fourth, sophisticated instruments are required to closely monitor structural changes after the cell membrane coating process, as the orientation of the inner and outer leaflets of the membrane can influence its biological function. Fifth, the safety of biologics is paramount, and it is crucial to

thoroughly verify that genetic material from cancer cells is completely removed during the membrane extraction process to avoid introducing new pathogenic factors. Sixth, to comprehensively assess the safety of nanomedicines coated with cell membranes, a broader range of cancer types should be considered, including various subtypes of solid tumors (such as pancreatic cancer and liver cancer) and hematological malignancies (such as leukemia). By conducting experiments in these diverse cancer models, the therapeutic effects and potential side effects of the system can be evaluated more thoroughly, and its applicability and advantages in different cancer environments can be further identified. Seventh, to address the issue of membrane stability, it is necessary to conduct in-depth research on the factors affecting cell membrane stability, such as the complex physiological environment *in vivo* (changes in pH value, the action of enzymes, etc.). On the one hand, physical or chemical modifications of cell membranes should be attempted, such as cross-linking treatment or the addition of specific stabilizers. On the other hand, the combination method of nanocarriers and cell membranes should be optimized to develop more stable connection strategies, ensuring that cell membranes maintain a stable structure and function throughout the treatment process, thereby guaranteeing the therapeutic potential of the system. Concurrently, the biological effects of nMOFs with varying particle sizes after their interaction with cell membranes should be investigated. This is not only because the size of the nanoparticles critically influences their transport behavior *in vivo*, but also because the surface modification that facilitates this interaction is affected by the specific surface area (Xu et al., 2023). Ultimately, further attempts to combine cell membranes with nMOFs will contribute to a deeper understanding of the advantages and potential drawbacks associated with employing this biomimetic strategy to enhance the properties of nMOFs materials (Krishnan et al., 2023). Addressing these issues will accelerate the development of cell membrane-based nanomedicines. Overall, the combination of biomimetic membranes and por-nMOFs represents a highly promising research direction, which can promote the multifunctional applications of por-nMOFs and lay a solid foundation for their biomedical applications.

Author contributions

YZ: Writing—original draft, Investigation, Formal Analysis. JW: Writing—original draft, Investigation, Formal Analysis. QZ: Writing—original draft, Investigation, Formal Analysis. JC: Writing—original draft, Investigation, Formal Analysis. XL: Writing—original draft, Investigation, Formal Analysis. YQ: Writing—original draft, Investigation, Formal Analysis. RX: Writing—original draft, Investigation, Formal Analysis. WW: Writing—original draft, Investigation, Formal Analysis. XZ: Writing—review and editing, Methodology, Investigation, Funding acquisition.

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During the preparation of this work, the authors used [tongyi.ai] in order to check for word choice and grammatical errors, and revisions were made according to the modification suggestions proposed by the AI assistant. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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.

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A review on intrathecal administration of medications for leptomeningeal metastases in solid tumors

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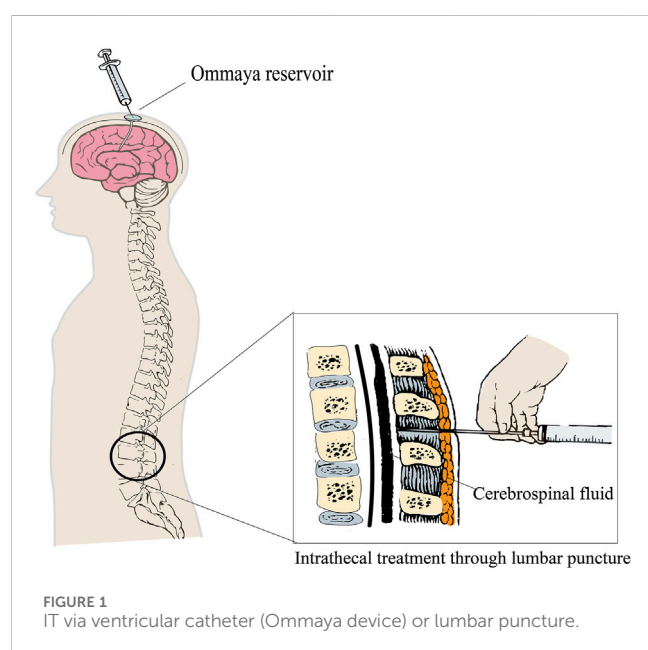
Leptomeningeal disease (LMD) is a particular mode of central metastasis in malignant tumors. It occurs when tumor cells infiltrate the subarachnoid space and cerebrospinal fluid (CSF), spreading throughout the central nervous system (CNS). LMD is a rare but devastating complication of malignant tumors. It can occur in various types of cancers, with lung and breast cancer being the most frequently associated. The treatment approach for LMD includes a combination of supportive care, surgery, chemotherapy, radiotherapy, targeted therapy, immunotherapy, and intrathecal (IT) therapy, among other modalities. Despite the challenges in determining the optimal treatment for LMD, IT therapy remains one of the primary therapeutic strategies. This therapy can directly circumvent the blood–brain barrier. Moreover, a low-dose medication can achieve a higher drug concentration in the CSF, resulting in better cytotoxic effects. Chemotherapy drugs such as methotrexate, cytarabine, and thiopeta have been widely studied as traditional IT therapies. In recent years, the advent of novel anti-tumor drugs has led to a growing number of agents being employed for IT administration in the treatment of malignant tumors with LMD. This article presents a comprehensive review of the current advancements in IT administration of chemotherapy, targeted, and immunotherapy drugs for the treatment of LMD in solid tumors. In addition, we also discuss the safety issues associated with IT therapy, summarize the advantages of IT administration of different types of anti-tumor drugs, and put forward some suggestions for reducing adverse reactions. It is hoped that future research will focus on exploring more potentially effective anti-tumor drugs for IT treatment, conducting in-depth pharmacokinetic studies, and developing long-acting and low-toxic IT administration regimens for the treatment of meningeal metastases.

KEYWORDS

solid tumors, leptomeningeal disease, intrathecal therapy, chemotherapy drugs, immunotherapy drugs, targeted drugs, anti-tumor drugs

1 Introduction

Leptomeningeal disease (LMD) of solid tumors is characterized by tumor cells invading the subarachnoid space and cerebrospinal fluid (CSF), leading to central metastasis of malignant tumors spreading throughout the central nervous system (CNS). It is a rare but fatal complication of malignant tumors. LMD can manifest across all types of malignant tumors. It is most prevalently observed in lung cancer, breast cancer, and melanoma (Thakkar et al., 2020). Approximately 5%–8% of patients with solid tumors can be associated with meningeal carcinomatosis (Carasu et al., 2021). The autopsy report shows that the incidence rate of many solid tumors exceeds 20% (Beauchesne, 2010; Carasu et al., 2021; Nguyen et al., 2021), indicating that the number of patients diagnosed with LMD is far lower than the actual incidence. LMD is more common in middle-aged and elderly people, with no significant gender differences. The median time from the primary tumor to the diagnosis of LMD is approximately 1–2 years, and the median overall survival (OS) is only 3–4 months, with a high mortality rate (Glantz et al., 1999a; Glantz et al., 1999b; Le Rhun et al., 2019). Hematological metastasis, lymphatic system metastasis, and CSF dissemination are its transmission pathways. The diagnosis of LMD requires the combination of the patient's physical signs and symptoms with auxiliary examinations. In addition, the clinical manifestations are diverse and non-specific, with persistent headache as the main symptom of treatment (Wang et al., 2018). The overarching goals of treating LMD are twofold: first, to extend the survival of patients while ensuring a tolerable quality of life; second, to forestall or at least defer the progression of neurological deterioration. The LMD treatment plan encompasses supportive therapy, radiotherapy, chemotherapy, surgery, targeted therapy, immunotherapy, and intrathecal (IT) therapy (Pellerino et al., 2018). The specific treatment methods hinge on the histological characteristics, molecular expression, systemic disease progression, neurological function, prognosis, and other factors of the malignant tumor (Le Rhun et al., 2019; Thakkar et al., 2020).



The central nervous system is frequently regarded as a “pharmacological sanctuary” since the majority of anti-cancer drugs are incapable of effectively penetrating the blood–brain barrier (BBB). IT therapy is a key treatment strategy for patients with LMD, offering the advantage of directly bypassing the blood–cerebrospinal fluid barrier to effectively target meningeal lesions. Additionally, given the significantly smaller volume of the CSF than that of plasma, low-dose administration achieves higher drug concentrations in the CSF, resulting in enhanced anti-tumor efficacy with reduced systemic toxicity. The European Society for Medical Oncology (ESMO) (Le Rhun et al., 2023) expert consensus recommends that IT therapy is suitable for most CSF-positive nodular or linear nodules (IA/C type) and patients with LMD with a high tumor cell load in the CSF. IT therapy can be directly administered into the lateral ventricle via subcutaneous fluid storage sacs and ventricular catheters (using an implanted Ommaya reservoir) or into the lumbar dural sac by lumbar puncture (Figure 1). Researchers suggest that drugs should be directly injected into the lateral ventricle through subcutaneous fluid storage sacs and ventricular catheters for IT therapy. Pharmacokinetic studies have shown that drugs can be evenly distributed across various parts of the CNS through CSF circulation, and the concentration can reach up to 10 times that of the same dose administered through lumbar puncture (Roguski et al., 2015; Le Rhun et al., 2017), avoiding the risk of failing to inject drugs into the CSF cavity (approximately 10%) (Shapiro et al., 1975; Glantz et al., 2010). The use of Ommaya intraventricular therapy resulted in significantly longer OS than that of lumbar puncture (9.2 vs. 4 months, $p = 0.0006$) (Montes de Oca Delgado et al., 2018). In addition, in patients with solid tumor LMD, the incidence of complications in the intraventricular fluid reservoir is lower (Wang et al., 2016). However, the Ommaya sac needs to be surgically inserted, and common complications include intracranial hemorrhage and catheter translocation and blockage. Overall, IT therapy with Ommaya is recommended as a priority for patients with LMD.

The most commonly used IT chemotherapy drugs for solid tumor LMD are methotrexate, thiopeta, and cytarabine (Le Rhun et al., 2023). However, no randomized clinical trials comparing any of these drugs with the optimal treatment for systemic diseases and the best supportive care have provided unequivocal evidence of a survival benefit in the treatment of solid tumor LMD (Boogerd et al., 2004). Nevertheless, occasional anecdotal responders and long-term survivors have been observed with IT chemotherapy. With continuous research and new explorations of diverse IT therapies for LMD, apart from chemotherapy drugs, targeted drugs and immunotherapy drugs also represent potential and effective pharmaceutical options. An increasing number of anti-tumor drugs are being recommended for the treatment of LMD. This article reviews the current research landscape and progress of IT anti-tumor drugs for solid tumors with LMD.

2 Standard IT therapeutic drugs

2.1 Cytotoxic drugs

2.1.1 Methotrexate

As a classic anti-metabolic anti-tumor drug, methotrexate can effectively combat breast cancer and hematological malignancies. As early as 1977, researchers (Bleyer and Dedrick, 1977) recommended

methotrexate to treat patients with LMD, extending the survival period from 4 to 9 months. This study also investigated the pharmacokinetics of IT methotrexate, measuring the concentration of methotrexate in the CSF of 76 patients. The concentration of methotrexate in the lumbar CSF decreased in a biphasic metabolic manner with half-lives of 4.5 and 8 h. It is not metabolized within the CSF. The drug is slowly absorbed through the choroid plexus, released into the systemic circulation, partially bound to serum albumin, and then excreted through the kidneys (Rubin et al., 1968).

Several small-scale prospective studies focused on IT methotrexate in patients with solid tumor LMD. These studies included participants ranging from several dozen to one hundred, and the primary tumors were predominantly lung and breast cancer, with CSF cytological remission rates reaching 20%–61%. These studies can validate the therapeutic efficacy of IT methotrexate (Grossman et al., 1993; Siegal et al., 1994; Glantz et al., 1998). IT methotrexate combined with radiotherapy emerges as a promising approach in clinical practice. Multiple studies included patients with solid tumor LMD who underwent IT methotrexate treatment combined with radiotherapy. Such a retrospective study (Wasserstrom et al., 1982) included 90 patients. Approximately half of the patients exhibited improvement in neurological symptoms, with a median OS of 5.8 months (1–29 months). In another retrospective study that enrolled 98 patients who underwent radiotherapy in combination with the standard-dose IT methotrexate, 41 patients were evaluated as having achieved a CR, while 12 patients attained a PR. The median OS ranged from 3 months among breast cancer patients to 8 months for lymphoma patients. Notably, the survival duration of patients who responded to methotrexate was significantly prolonged (Pfeffer et al., 1988). Similarly, a phase-II prospective single-arm clinical study included 59 patients (42 with lung cancer, 11 with breast cancer, and 6 with other) with adverse prognostic factors. The treatment consisted of IT methotrexate (12.5–15 mg) and dexamethasone (5 mg) administered once a week, combined with brain radiotherapy at a dose of 40Gy/20fr. The clinical effective rate was 86.4% (51/59), and the median OS was 6.5 months (0.4–36.7 months). This study is the first to preliminarily explore, through prospective clinical research, that the treatment regimen of IT methotrexate combined with regional radiotherapy is highly effective for solid tumor leptomeningeal carcinomatosis, which can improve the quality of life of patients, alleviate neurological symptoms, and prolong survival (Pan et al., 2016). The abovementioned studies indicate that the treatment mode of IT methotrexate and synchronous regional radiotherapy has significant benefits for patients with LMD.

A common dosage regimen for IT methotrexate therapy involves induction treatment with two injections per week for 4 weeks, followed by consolidation treatment with one injection per week for 4 weeks, and maintenance therapy with one injection per month until disease progression (Shapiro et al., 1975; Le Rhun et al., 2023). However, the dosages of IT methotrexate in the current literature are not consistent, with most doses varying between 10 and 15 mg. Hou et al. (2021) conducted a prospective single-arm clinical trial to investigate the clinical efficacy and safety of different doses of IT methotrexate in the treatment of cancerous meningitis. The study encompassed 53 patients, who were randomly

assigned to a 15-mg dose group or a 10-mg dose group. The results showed that the median OS of the 15-mg dose group was 15.7 weeks, while that of the 10-mg dose group was 27.1 weeks, with no statistically significant difference ($p = 0.116$). Increasing the dosage of IT methotrexate appropriately did not prolong OS, but it was more effective in alleviating clinical symptoms and did not increase the incidence of adverse events. Continuous exposure to low concentrations of methotrexate throughout the body can lead to severe bone marrow suppression. Patients with renal insufficiency, pleural effusion or ascites, and abnormal CSF flow are at an increased risk of severe bone marrow suppression. IT methotrexate treatment can cause various neurological complications, including chemical meningitis, delayed white matter encephalopathy, acute encephalopathy, and transverse myelopathy (Boogerd et al., 2004; Stemmler et al., 2008; Pan et al., 2016). In order to minimize the risk of systemic methotrexate toxicity, all patients should be given oral folic acid (10 mg once, twice a day, for 3 days) simultaneously as a routine to reduce the systemic toxic reactions caused by methotrexate leaving the CSF and entering the bloodstream. Methotrexate, one of the classic IT chemotherapy drugs, has high efficacy in patients with solid tumor LMD. IT or combined radiation therapy can improve the quality of life of patients, prolong survival, and have good safety.

2.1.2 Thiotepa

Thiotepa is an ethyleneimine alkylating agent that forms unstable ethylimine groups under physiological conditions and has strong cytotoxic effects. As a multifunctional alkylating agent, it inhibits nucleic acid synthesis, crosslinks with DNA, and is effective against ovarian, breast, bladder, and digestive tract cancers (Maanen et al., 2000). Thiotepa is a small-molecule, non-polar, highly lipid-soluble drug with good tissue infiltration. It has the shortest half-life among IT chemotherapy drugs. After IT therapy, it can quickly diffuse out of the CSF within 4 hours without being distributed to the CSF space or brain tissue, and it can be cleared through cerebral capillaries. A patient received IT 10 mg of thiotepa. The peak concentration of the CSF in the ventricles was $>100 \mu\text{g/mL}$, approximately $10 \mu\text{g/mL}$ after 2 hours, and approximately $1 \mu\text{g/mL}$ after 8 hours. The average clearance rate of the CSF was 1.8 mL/min , and the average systemic clearance rate was 518 mL/min (Strong et al., 1986).

The efficacy of IT thiotepa is not as clear as that of methotrexate. The typical dosage is 10 mg. The induction regimen entails two injections per week for a period of 4 weeks. Subsequently, the consolidation regimen involves one injection per week for 4 weeks. Thereafter, the maintenance regimen entails one injection per month until the disease progresses. If required, one injection per week can be implemented (Le Rhun et al., 2023). In most studies, thiotepa was administered at a dose of 10–15 mg per dose twice a week or continuously for 3 days per month (Chamberlain and Kormanik, 1997; Witham et al., 1999; Schrappe et al., 2000; Cho et al., 2015). As early as 1976, a phase I–II study on IT thiotepa was conducted in LMD patients with malignant tumors. The study involved 11 patients with meningeal leukemia, lymphoma, and ependymoma who received IT thiotepa at a dose of $1\text{--}10 \text{ mg/m}^2$. Three patients achieved CR of the CNS symptoms, while five patients achieved PR. No hematological toxicity related to thiotepa was observed in this study, and

neurotoxicity was limited to mild transient lower limb toxicity. This study preliminarily confirms the effectiveness and safety of IT thiotepa in patients with LMD (Gutin et al., 1976). Another multi-center randomized trial compared IT thiotepa and methotrexate in 59 patients with LMD, including breast cancer (48%), lung cancer (23%), lymphoma (19%), and other tumors (10%). It is important to mention that in this trial, the results showed that approximately one-third of patients in both groups achieved complete cytological clearance, and the median OS was similar between the groups (14 vs. 16 weeks) (Grossman et al., 1993). In order to further verify whether IT thiotepa has efficacy in breast cancer patients, a single-center retrospective study included 66 breast cancer LMD patients who were given IT thiotepa (10 mg) and methylprednisolone (40 mg) once every 2 weeks. This study reported comparable results; 16 out of the 36 evaluable patients had cytological remission, and the median OS was 4.5 months (Comte et al., 2013). This study confirmed that LMD patients with breast cancer had a good response to thiotepa. The selection of IT drugs following the failure of methotrexate treatment has always been a research focus. IT thiotepa appears to have good efficacy in LMD cases after methotrexate treatment failure. Cho et al. (2015) evaluated 398 patients with LMD of non-small-cell lung cancer (NSCLC) and breast cancer who were treated with IT therapy based on methotrexate. According to the standard treatment scheme, they received 10 mg of thiotepa twice a week for the rescue treatment of patients after the failure of methotrexate. Among the 30 patients who could be evaluated, 14 (47%) had cytological reactions to thiotepa in the CSF, and the median OS after the treatment with thiotepa was 19.4 weeks. This study confirmed that patients who failed methotrexate treatment could choose to use thiotepa for rescue treatment with good effectiveness.

IT administration of thiotepa is often well-tolerated and may cause systemic bone marrow suppression (Grossman et al., 1993). Neurotoxicity is limited to mild, transient lower-limb sensory abnormalities, and there have been occasional reports of spinal cord disease. As a classic IT chemotherapy drug such as methotrexate, thiotepa has good safety and effectiveness for LMD. At the same time, it is also a meaningful salvage treatment for patients with LMD who have progressed after methotrexate treatment.

2.1.3 Cytarabine and cytarabine liposome

The structure of cytarabine is very similar to that of deoxycytidine, one of the components of DNA synthesis, which can interfere with DNA replication and kill tumor cells (Pace and Fabi, 2006). Cytarabine is mainly used for induction remission and maintenance therapy of acute non-lymphocytic leukemia in adults and children, and it is only effective for a small number of solid tumor patients. For solid tumors, such as lung and breast cancer, if the tumor has invaded the CSF or meninges, cytarabine can be used for IT therapy. The results were reported by Esteva et al. (2000) in seven patients with LMD from breast cancer, who were administered IT cytarabine three times in a single dose of 100 mg at 48-h intervals. The authors reported a mean ($\pm$ SD) peak cytarabine concentration of 16.7 ± 6.3 mmol/L and a mean concentration of 0.77 ± 0.53 mmol/L after 6 h. The cytarabine concentration in the CSF decreased in a monophasic pattern with a

concentration half-life of 1.45 ± 0.61 h. The accumulation of cytarabine in the CSF between the repeated injections was not observed, and cytarabine was not detectable in the plasma. The half-life of cytarabine in the CSF is less than 4 h, and it is completely cleared within 1–2 days (Fulton et al., 1982).

Cytarabine dosage is 25–100 mg, with an induction phase of two injections per week for 4 weeks, a consolidation phase of one injection per week for 4 weeks, and a maintenance phase of one injection per month until disease progression (Le Rhun et al., 2023). The researchers tested IT cytarabine in 32 patients with neoplastic meningitis from malignant gliomas and solid tumors (Fulton et al., 1982). The researchers administered cytarabine in two doses within just 1 week to the first group, which consisted of 20 patients, and 14 of these patients also received concurrent chemotherapy. All four patients exhibited clinical improvement. Among them, two patients remained alive for 8 weeks and 16 weeks, respectively, after the commencement of weekly cytarabine treatment. In another case, five patients demonstrated clinical improvement, and four of them were still alive without tumor recurrence at 26 weeks after the initiation of therapy. Although no distinct difference was observed between the two groups, it should be noted that these groups were not comparable in terms of prognostically significant clinical characteristics during diagnosis without recurrence. In the second group, three out of twelve patients were administered three consecutive doses. Shapiro et al. (2006) reported a series of 67 patients suffering from neoplastic meningitis caused by solid tumors, leukemia, and lymphomas. These patients received methotrexate or cytarabine alone or a combination of methotrexate and cytarabine through an intraventricular reservoir with or without CNS irradiation. Among these patients, 11 individuals underwent combination therapy with both drugs. Clinical improvement was observed in 58% of the patients with solid-tumor-related neoplastic meningitis. The median survival time for patients with breast cancer was 5 months. The above-mentioned research indicates that acute toxic effects associated with IT cytarabine included meningismus, nausea, vomiting, and myelosuppression.

Cytarabine liposome is a multi-vesicular system sustained-release liposome composition of cytarabine prepared using the sustained-release liposome platform. This nanostructure can encapsulate a large amount of drug and prolong the drug release time. It has been proven that the use of cytarabine liposome can delay the release of cytarabine, with a duration ranging from several days to several weeks and a longer half-life of over 80 h. Cytarabine liposome (50 mg) is administered intravenously every 2 weeks for LMD, with the number of IT does being only a quarter of that in conventional treatment (Jaeckle et al., 2001; Cole et al., 2003; Fusco et al., 2013). An open-label study (Phuphanich et al., 2007) investigated IT (50 mg) cytarabine liposome twice with a 14-day interval, analyzing the levels of cytarabine and metabolites in CSF and plasma samples. The results showed that the concentration range of free and encapsulated cytarabine in ventricular and lumbar CSF was 0.01–1,500 μ g/mL, and it could be detected within 14 days. Occasionally, free cytarabine was detected in the plasma, and the concentrations of cytarabine metabolites were low in all samples.

To compare the efficacy of cytarabine liposome and methotrexate in patients with LMD, a randomized controlled trial was conducted, enrolling 61 patients with histologically

confirmed cancer and positive CSF cytology. They were randomly divided into an IT cytarabine liposome group (31 patients) or a methotrexate group (30 patients). Patients received up to six doses of 50 mg cytarabine liposome or up to 16 doses of 10 mg methotrexate within 3 months. The trial results showed that in patients with LMD, cytarabine liposome was as effective as methotrexate and significantly increased the time of no progression in the nervous system while reducing the number of doses administered, providing convenience for patients (Glantz et al., 1999a). Another multicenter, randomized, controlled study also compared the benefits and safety of IT cytarabine liposome, methotrexate, and cytarabine in patients with solid tumor LMD ($n = 103$). The results showed that the benefits of cytarabine liposome and methotrexate in treating solid tumor LMD were comparable (Shapiro et al., 2006). These clinical trials encompassed a variety of cancer types, and the majority of the patients were non-primary cases. DEPOSEIN (NCT01645839) (Le Rhun et al., 2020) is a randomized open-label phase-III study that discusses the effectiveness and safety of IT cytarabine liposome combined with systemic therapy ($n = 36$) versus systemic therapy ($n = 37$) for patients with LMD from breast cancer at initial diagnosis. The study results indicate that the cytarabine liposome group significantly improved median PFS (3.8 vs. 2.2 months) and median OS (7.3 vs. 4 months) in patients with LMD. This study confirmed the role of IT cytarabine liposome in LMD patients with breast cancer. Cytarabine liposome, as a sustained-release liposome composition with a polycyclic vesicle system, can encapsulate more drugs in its nanostructure, thereby reducing the administration time and increasing patient convenience. Clinical trials have also confirmed that for patients with solid tumor LMD, cytarabine liposome is as effective as methotrexate, which may be a good choice. Multiple studies have shown that the main adverse events of IT cytarabine liposome are headache and arachnoiditis. To prevent chemical meningitis caused by IT chemotherapy, each patient needs to be pretreated with dexamethasone.

2.1.4 Etoposide

Etoposide acts on DNA topoisomerase II, forming a stable and reversible drug–enzyme–DNA complex that impedes DNA repair. Etoposide is mainly used for treating small-cell lung cancer (SCLC), malignant lymphoma, and malignant germ cell tumors (Issell and Croke, 1979). The systemic administration of etoposide is effective in treating metastatic, recurrent, or refractory brain tumors, but its permeability to CSF is extremely low, almost not entering the CSF (<3%). Even after high-dose intravenous injection of etoposide, the CSF concentration does not exceed 0.1–0.5 $\mu\text{g/mL}$ (Postmus et al., 1984; Kiya et al., 1992). The *in vitro* experimental results show that etoposide at concentrations ranging from 0.1 to 10 $\mu\text{g/mL}$ exhibits cytotoxicity (Henwood and Brogden, 1990). IT etoposide can directly penetrate the BBB, and low-dose medication can lead to higher drug concentrations in the CSF. In a published case report (van der Gaast et al., 1992), IT (0.5 mg) etoposide was administered twice daily, with a 2-hour interval, for 5 days, followed by a second course of treatment 21 days later. Two hours after daily administration, the peak concentrations of the CSF in the ventricles were 3.6–5.2 $\mu\text{g/mL}$, and the trough concentrations of the CSF in the ventricles were 0.2–0.6 $\mu\text{g/mL}$. Another study reported that the peak concentration of etoposide in the CSF

after IT administration exceeded that of intravenous infusion by more than 100 times. The steady-state distribution volume and total clearance rate showed significant individual differences, with a double exponential decrease. Etoposide was not detected in the plasma (Fleischhack et al., 2001).

Slavc et al. (2003) evaluated the long-term feasibility of IT etoposide in 11 children with disseminated non-hematological metastatic brain tumors. Two patients received IT mafosfamide before switching to intraventricular etoposide treatment, and nine patients received alternating IT etoposide and mafosfamide treatment. The dose of etoposide was 0.5 mg $\times$ 5 days, once every 3–6 weeks, for a total of 122 courses (1–29 courses/patient) until disease progression. The median time of etoposide treatment was 12 months (1–31 months), and the disease remission rate among the 11 patients was 54.5% (6/11). No long-term toxicity that could be attributed to IT therapy was observed. This study confirms that IT etoposide is both safe and effective for patients over 2 years old with LMD. In order to further validate the efficacy of IT etoposide in LMD of adult patients, another phase-II clinical trial (Chamberlain et al., 2006) was carried out, which included 27 adult patients (the median age was 55 years old); IT etoposide 0.5 mg per day was administered once every 5 days for 8 weeks to induce treatment. At the end of induction treatment, patients were evaluated through CSF cytology and neurological examination. Patients who had responded continued to receive etoposide treatment (every 4 weeks for 5 days) and undergo monthly evaluations. Among the 27 patients receiving etoposide treatment, 7 (26%) had stable or improved neurological status and 12 (44%) had no response to treatment. Among the patients who responded to treatment, the median PFS was 20 weeks (ranging from 8 to 20 weeks), and the 6-month PFS rate for neurological disease was 11%. For newly diagnosed LMD patients, the median OS is 10 weeks (4–52 weeks), and the survival probabilities at 3 months, 6 months, and 12 months are 30% (95% CI, 12%–47%), 11% (95% CI, 1%–21%), and 4% (95% CI, 0%–11%), respectively. During the treatment, five patients experienced transient chemical arachnoiditis, and no hematological toxicity related to etoposide was observed. The abovementioned report evaluated the efficacy of IT etoposide in the treatment of LMD, and patients demonstrated good tolerance to this treatment (Fleischhack et al., 2001; Slavc et al., 2003; Chamberlain et al., 2006). Based on the results of previous clinical trials on the efficacy of IT etoposide, Park (2015) first used IT etoposide to rescue a patient with NSCLC who failed first-line methotrexate treatment. The patient received 1 mg of etoposide intravenously once a week, and after 2 weeks of treatment, the brain lesion and clinical symptoms improved. However, the patient ultimately died due to liver metastasis. Unlike other studies, this study designed a weekly regimen of etoposide administration to rescue patients who failed methotrexate treatment, providing preliminary evidence for the feasibility of etoposide rescue therapy. In recent years, there has been relatively little research on IT etoposide. The safety of IT etoposide in children and young adults with refractory or recurrent malignant brain tumors was investigated (Pajtler et al., 2016). The research results showed that IT etoposide was well-tolerated, and the potential treatment-related adverse reactions included transient headache, epileptic seizures, nausea, and neuropsychological symptoms. No hematological side effects were observed.

2.1.5 Topotecan

Topotecan is a topoisomerase I inhibitor with good therapeutic effects on various solid tumors in adults and children. The hydrolysis of topotecan lactone is pH-dependent, and the open carboxylate form as topoisomerase-I inhibitor has no activity. When topotecan is administered intravenously, approximately 30% reaches the CNS. After IT topotecan (0.4 mg) administration, the peak concentration of cerebroventricular CSF was $28 \pm 11 \mu\text{mol/L}$, and it was not detected in the plasma. The clearance of topotecan is mainly achieved through its conversion to inactive forms and CSF circulation, with only approximately 10% cleared through microvasculature (Stewart et al., 1994; Blaney et al., 2003; Glaberman et al., 2005).

A phase-I clinical trial (Blaney et al., 2003) enrolled 23 patients aged 3 years and above with tumor-associated LMD, who received IT topotecan to determine the optimal dose and dose-limiting toxicity. The study determined a maximum tolerable dose of 0.4 mg through a dose ramp-up test, and chemical subarachnoid inflammation was the dose-limiting toxicity that occurred within 24 h of administration, which was characterized by fever, nausea, vomiting, headache, and back pain. Among 23 assessable patients, six patients had stable disease, providing the optimal dosage for phase-II clinical trials. Another phase-II, open-label, non-randomized single-arm trial (Groves et al., 2008) included 62 patients with malignant LMD who received IT topotecan (0.4 mg) twice a week for 6 weeks. Primary cancers included breast cancer ($n = 19$), lung cancer ($n = 13$), CNS cancer ($n = 14$), and other cancers ($n = 16$). The estimated PFS rates at weeks 13 and 26 were 30% (20%–45%) and 19% (11%–34%), respectively. The median OS is 15 weeks (13–24 weeks). The most common side effect was chemical meningitis, which occurred in 32% of the patients (5% grade 3). IT topotecan was well-tolerated and had no obvious side effects. However, topotecan does not have significant advantages over other IT chemotherapy options. It can be combined with radiotherapy or systemic treatment to maximize the benefits for patients. Researchers treated two patients with recurrent or progressive CNS cancer with LMD using IT topotecan (0.2 mg/day) for 7 days in order to increase the exposure of topotecan in the CSF as much as possible (Tran et al., 2014). After administration, neither patient showed hematological toxicity or arachnoiditis that was grade 3 or above. Although this study is a case report, it offers a novel approach to individualized treatment options in clinical practice. In the latest study by Yamada et al. (2023), high-dose chemotherapy combined with stem cell rescue, followed by IT topotecan maintenance therapy, was used to treat six children with atypical rhabdomyoma. This treatment model successfully avoided whole-brain radiation therapy and prolonged their survival. IT topotecan maintenance therapy can improve CNS symptoms. The sample size of this study is small, and further large-scale research is needed to confirm this treatment model. IT topotecan is safe, with the most common adverse reactions being nausea, vomiting, headache, anorexia, and fever. Arachnoiditis is dose-limited toxicity, and no hematological toxicity was observed. Although topotecan does not have significant advantages over other IT chemotherapies, it can also be used as one of the drug choices for solid tumor LMD patients.

2.1.6 Pemetrexed

Pemetrexed, as an anti-metabolic antitumor drug, is a first-line chemotherapy drug for NSCLC and exhibits good cytotoxicity. The incidence of NSCLC-LMD reaches as high as 5.0%–15.8% (Kwon et al., 2020). The pharmacokinetics of IT pemetrexed was investigated (Li et al., 2023). After IT 30 mg of pemetrexed was administered, a series of CSF samples were collected from eight patients, with 62.5% (5/8) reaching peak concentrations within 2–4 h, and the effective concentration level of pemetrexed in the CSF remained at an average of $93.44 \mu\text{g/mL}$ after 24 h of IT pemetrexed administration. The research results showed that pemetrexed exhibited a biphasic elimination mode in the CSF, while its concentration in the blood was extremely low.

To determine the efficacy of IT pemetrexed in lung adenocarcinoma patients with LMD who had undergone at least two previous treatments, a prospective phase-I study enrolled 23 lung adenocarcinoma LMD patients with failed multi-line treatment. According to the dose ramp-up test, IT pemetrexed at a dose of 30 mg was considered the optimal dose, with a median PFS of 6.3 months (ranging from 0.8 months to data not available (NA)) and a median OS of 9.5 months (ranging from 2.9 months to NA), respectively. In 2019, a study was reported on the use of IT pemetrexed for the salvage treatment of NSCLC-LMD, which was the world's first phase-I clinical trial. The research results showed that the clinical effective rate of IT 10 mg pemetrexed was 31% (4/13), the disease control rate was 54% (7/13), and it had good tolerability. Subsequently, Pan et al. (2020) conducted a phase-I/II clinical study of IT pemetrexed and concurrent radiotherapy for solid tumor LMD. Based on the combination of affected field radiotherapy, the clinical effective rate of 10 mg pemetrexed was 68% (23/34). The median OS is 5.5 months (0.3–16.6 months). The research results suggest that the combination of IT pemetrexed and radiotherapy is effective for the treatment of LMD. In another prospective, single-center phase-II study conducted in the latest report (Fan et al., 2021), 30 patients with an epithelial growth factor receptor (EGFR) mutation and NSCLC-LMD were included and evaluated with IT pemetrexed and dexamethasone. The results showed a clinical efficacy rate of 84.6% and a median OS of 9 months, confirming that IT 50 mg pemetrexed has a high response rate and few adverse reactions in the treatment. These data are encouraging and offer new effective treatment methods for NSCLC-LMD patients. Multiple studies have demonstrated that pemetrexed exhibits good anti-tumor activity in the CSF without significant accumulation in the body. Bone marrow suppression and hepatotoxicity are the most prevalent adverse reactions (Pan et al., 2016; Geng et al., 2022). To reduce adverse reactions to pemetrexed, pre-treatment with oral folate, intramuscular injection of vitamin B12, and IT dexamethasone are equally important. Most studies use the classic dose escalation model to observe the tolerated dose and the relevant dose-limiting toxicity of IT pemetrexed. However, the sample size in these studies is relatively small. The dosages of pemetrexed vary significantly among different studies, and further larger sample studies are needed to further verify safety and effectiveness. Perhaps, after more clinical trials validating the safety and efficacy of IT pemetrexed in lung adenocarcinoma LMD patients, pemetrexed can replace methotrexate as a first-line treatment.

2.2 Targeted drugs

2.2.1 Trastuzumab

Previous studies have shown that trastuzumab, whether as a single drug or in combination with chemotherapy, can delay the disease progression and improve the OS of breast cancer patients with human epidermal growth factor receptor 2 (HER-2) positivity (Kak et al., 2015; Malani et al., 2020). However, HER-2-positive breast cancer patients receiving systemic trastuzumab treatment often develop CNS metastasis, with approximately 20% of these cases occurring in LMD (Bendell et al., 2003). One of the reasons for stable or relieved CNS recurrence of systemic diseases may be the high molecular weight (145 kDa) of trastuzumab, which leads to its low CNS penetration rate. Research reported that when trastuzumab was administered intravenously on a weekly basis, there was a 300-fold difference in drug concentration between the patient's serum and CSF (Pestalozzi and Brignoli, 2000). IT trastuzumab is a new treatment approach for HER-2-positive cancer, especially for patients with LMD from breast cancer. Multiple small-scale studies have shown that trastuzumab 25–150 mg has good stability when dissolved in physiological saline without preservatives (Laufman and Forsthoefel, 2001; Platini et al., 2006; Stemmler et al., 2006; Stemmler et al., 2008; Oliveira et al., 2011; Zagouri et al., 2013). The latest investigation shows that after IT (80 mg) trastuzumab administration, the distribution volume is 73 ± 48 mL, the CSF clearance rate is 14 ± 5 mL/h, and the apparent CSF half-life is 4.1 ± 3.0 h. The CSF half-life is much shorter than the 18–30 days half-life of trastuzumab intravenous administration. After repeated administration, the CSF concentration stabilizes, indicating that trastuzumab will not accumulate to toxic concentrations in the CSF (Kumthekar et al., 2023).

Trastuzumab was initially injected intrathecally for HER-2-overexpressing breast cancer patients. In these reports, patients who had received systemic treatment, IT chemotherapy, whole-brain radiotherapy, neurosurgery, and other treatment methods still did not experience relief. IT trastuzumab was used for rescue treatment. The literature has reported that the dose of IT trastuzumab ranges from 5–160 mg, either twice a week or once every 3 weeks. However, in most studies, the dose range is from 20 to 30 mg per week. Patients' headaches, mental state, hemiplegia, ataxia, and other disorders were significantly improved. The duration of disease control with IT trastuzumab ranged from 39 days to over 72 months, and researchers observed no serious adverse reactions associated with trastuzumab (Laufman and Forsthoefel, 2001; Platini et al., 2006; Stemmler et al., 2006; Mir et al., 2008; Stemmler et al., 2008; Colozza et al., 2009; Ferrario et al., 2009; Oliveira et al., 2011; Smith et al., 2022). Some researchers believe that the effectiveness of IT trastuzumab seems to be dose-dependent. In the case that had been reported (Ferrario et al., 2009), patients who received IT trastuzumab 30 mg had better clinical benefits than those who received 20 mg. In another report (Mir et al., 2008), the IT dose of trastuzumab was increased to 50 mg in combination with 12 mg of thiotepa, which showed better efficacy than 20–30 mg. In a phase-I clinical trial (Bonneau et al., 2018), based on IT trastuzumab with a CSF concentration close to the conventional treatment plasma concentration (30 mg/L), a dose ramp-up test was conducted, and the maximum tolerable dose was determined to be 150 mg per week. In a large-scale phase-I/II multi-

center study (Kumthekar et al., 2023), HER-2-positive histological cancer patients were included in the phase-I study, and the maximum tolerable dose for IT trastuzumab was determined to be 80 mg, twice a week, while ensuring both treatment effectiveness and safety. These studies indicate that the maximum tolerable overall dose per week for IT trastuzumab does not exceed 160 mg, rather than the traditional administration of 20–30 mg.

For patients with HER-2 overexpression in LMD associated with breast cancer, IT trastuzumab provides good benefits. Zagouri et al. (2013) retrospectively summarized and analyzed the effectiveness and safety of IT trastuzumab. A total of 13 articles were included, involving 17 patients with an average age of 48.2 years (38–66 years), and the average total dose was 399.8 mg (35–1,110 mg). With trastuzumab alone or in combination treatment, 88.2% of patients did not report serious adverse events, 68.8% of patients observed significant improvement in clinical symptoms, 31.2% of patients were stable or progressive, and the median OS was 13.5 months. The CNS-PFS is 7.5 months, and the longer CNS-PFS seems to be related to the improvement of clinical symptoms and CSF response. This retrospective study demonstrates the effectiveness and safety of IT trastuzumab. Based on a phase-I dose escalation test (Oberkamp et al., 2023), the study established the recommended dose of IT trastuzumab as 150 mg per week for the phase-II study. A total of 19 patients with LMD and HER-2-overexpressing breast cancer were included. All patients received at least one systemic anti-HER-2 treatment. After 8 weeks of IT trastuzumab administration, 74% (14/19) of patients had no neurological progress, the median LMD-related PFS was 5.9 months, and the median OS was 7.9 months. No adverse reactions of grade 3 or above were observed in all patients. Furthermore, a large-scale phase-I/II multi-center study (Kumthekar et al., 2023) included 23 HER-2-positive cancer patients who received IT trastuzumab 80 mg (twice a week for 4 weeks, then once a week for 4 weeks, followed by maintenance therapy every 1–2 weeks) for treatment. The partial response rate, disease stability rate, and disease progression rate were 19%, 50%, and 30%, respectively. At a median follow-up of 10.5 months, the median PFS and OS were 2.8 months and 10.5 months, respectively, indicating longer survival than that of the recognized historical control (median OS of approximately 3–4 months). In patients receiving treatment with IT trastuzumab, the most common side effects are headache, nausea, vomiting, and meningeal spasms, with only one case of grade-4 toxic reaction (arachnoiditis) found. However, in another meta-analysis (Lazaratos et al., 2023), the total survival period and PFS of IT trastuzumab (92 patients) and oral or intravenous HER-2 targeted therapy alternative route of administration (183 patients) in HER-2-positive breast cancer patients with LMD were compared. In univariate and multivariate analysis, the research results showed that there was no significant difference in OS and CNS-specific PFS between IT trastuzumab and oral or intravenous HER-2 targeted therapy. The study suggests that the reason for this result can be explained as follows: intravenous trastuzumab may reach the subarachnoid space at a sufficient concentration, effectively treating LMD in patients with trastuzumab-sensitive diseases. The patient had already received an intravenous injection of trastuzumab before IT therapy, which may have led to drug resistance. Even for patients receiving whole-brain radiotherapy, the concentration of trastuzumab in CSF is still one order of magnitude lower than the serum concentration

(Stemmler et al., 2007; Steeg, 2021). Although this meta-analysis suggests that IT trastuzumab and HER-2 targeted therapy do not offer obvious advantages as an alternative route of administration, IT therapy can quickly alleviate patients' neurological symptoms, and many clinical trials have shown that it has considerable efficacy and good tolerability for HER-2-positive breast cancer LMD patients. However, most of the enrolled clinical trials involve breast cancer with HER-2 positivity, which may not be applicable to the overall LMD population. Follow-up studies can include more HER-2-overexpressing solid tumor LMD patients. The most common adverse reactions of IT trastuzumab are headache, nausea, vomiting, and meningeal spasms.

2.3 Checkpoint inhibitors

2.3.1 Nivolumab

Nivolumab is a programmed cell death protein 1 (PD-1) inhibitor approved for the treatment of NSCLC, head and neck squamous cell carcinoma, and gastric cancer, among others. Nivolumab specifically binds to PD-1 receptors on T cells, preventing signals from reaching T cells from malignant tumor cells and allowing T cells to function normally, thereby exerting anti-tumor effects. In multiple prospective clinical trials, it has been demonstrated to bring long-lasting benefits to patients with metastatic melanoma (Kluger et al., 2019; Tawbi et al., 2021; Wolchok et al., 2022). The intracranial objective response rate (ORR) of nivolumab was 20% in asymptomatic encephalomyelitis patients, 55% in the checkmate-204 trial ($n = 101$) (Long et al., 2018), and 51% in the ABC trial ($n = 35$) (Tawbi et al., 2021). In all these studies, the 3-year survival rate of responsive patients exceeded 90% (Tawbi et al., 2021). Therefore, nivolumab may have a significant benefit for patients with melanoma LMD.

For symptomatic patients with melanoma LMD, rapid relief of neurological symptoms is necessary. Compared with interleukin-2, anti PD-1 therapy has improved efficacy and safety. Therefore, experts hypothesized that IT anti PD-1 therapy for melanoma is safe and feasible, and a phase-I clinical study was conducted (Glitz Oliva et al., 2023). First, the toxicity of IT administration of anti-PD-1 was evaluated in an immunocompetent mouse model. Then, a first-ever human dose exploration study was designed using both IT and intravenous administration of anti-PD-1. A total of 25 melanoma LMD patients were included and treated with a dual-pronged approach, injecting nivolumab into two sites: IT 50 mg every 2 weeks to treat meningeal metastases and intravenous 240 mg to treat extra brain lesions. The median OS of these patients was 4.9 months, with 44% and 26% of patients surviving over 26 and 52 weeks, respectively. Notably, four patients survived for 74 weeks, 115 weeks, 136 weeks, and 143 weeks after receiving the first IT nivolumab dose, which far exceeded the expected survival period for patients with LMD after multiple treatments. The most common grade-1 or grade-2 adverse events in the study were nausea (36%), diarrhea (24%), decreased lymphocyte count (24%), elevated aspartate aminotransferase and/or alanine aminotransferase (24%), and rashes (24%). Patients who used IT nivolumab alone did not show grade 3 or higher toxicity. This is the first clinical study that has utilized two different routes of administration for nivolumab therapy in patients

with LMD secondary to melanoma. This study provides a detailed explanation of the dose escalation, recommended IT dosage, safety profile, and preliminary efficacy of synchronous IT and intravenous administration of nivolumab in melanoma LMD patients, supporting the feasibility of prospective clinical trials in LMD melanoma patients. At the same time, this trial is the largest prospective clinical trial of IT immunotherapy among all types of cancers, and it is also the first systematic evaluation of the application of IT anti-PD-1 drugs. Although this study is focused on melanoma patients, it also holds reference significance for other types of tumors. This study represents an important step forward and a new treatment method for patients with LMD. Perhaps, in the future, IT therapy will no longer be limited to chemotherapy drugs such as methotrexate but could also be combined with PD-1 inhibitors.

3 Potential IT drugs

3.1 Targeted drugs

3.1.1 Nimotuzumab

EGFR is a transmembrane glycoprotein with a molecular weight of 170 kD, and its intracellular region has special tyrosine kinase activity. *In vivo* and *in vitro* studies have shown that nimotuzumab can block the binding of EGFR and its ligand, and it has anti-angiogenic, anti-proliferative, and pro-apoptotic effects on tumors overexpressing EGFR (Crombet-Ramos et al., 2002). Nimotuzumab is suitable for combined treatment with radiotherapy for stage-III/IV nasopharyngeal carcinoma with positive EGFR expression. The efficacy of IT nimotuzumab in the treatment of solid tumor LMD may be related to the antibody-dependent cell-mediated cytotoxicity (ADCC) induced by nimotuzumab, and the effect of ADCC is usually observed 1–3 months after IT treatment. There are relatively few studies on IT nimotuzumab, with only two relevant reports. One of the reports (Ju et al., 2016a) included 20 patients with NSCLC-LMD who received IT nimotuzumab 50 mg per week. The average number of IT therapy sessions was 8.9 (range: 1–35), and the median OS after treatment was 5 months (2.4–7.6 months). The average CSF open pressure decreased from 270 mmH₂O (202–338 mmH₂O) before treatment to 140 mmH₂O (127–153 mmH₂O) ($p < 0.001$). All patients experienced LMD-related symptoms, especially headaches, in the nimotuzumab group. After IT nimotuzumab, all patients were relieved, and their quality of life improved. In the same year, another case report was published by Ju et al. (2016b), in which two patients were diagnosed with NSCLC-LMD. The first patient received IT nimotuzumab weekly at the dose of 25 mg, with an OS of 35 months after the diagnosis of LMD. The second patient received 50 mg, with an OS of 12.5 months. Both patients received systemic treatment before IT therapy. After 2–3 sessions of IT treatment, the neurological symptoms of both patients were significantly improved, and the opening pressure of CSF decreased. Neither of these reports mentioned the adverse reactions of IT nimotuzumab. There are relatively few studies on IT nimotuzumab, but it can also provide preliminary evidence of its effectiveness and safety. In the future, more research and larger sample sizes are needed for further verification.

3.1.2 Bevacizumab

Bevacizumab is the first anti-tumor angiogenesis-targeting drug approved for marketing. It plays an anti-tumor role by specifically targeting vascular endothelial growth factors (VEGF), and it has definite efficacy in a variety of malignant tumors. At present, it has been approved for a variety of indications, including metastatic colorectal cancer and advanced/metastatic or recurrent NSCLC (Cao, 2008). Angiogenesis in solid tumor brain metastases occurs through multiple pathways, among which the VEGF-mediated pathway is the most important and extensively studied one. Research has confirmed that brain metastasis relies on VEGF, and the high expression of VEGF in brain metastases is associated with poor prognosis (Hanibuchi et al., 2014; Ebben and Ming, 2016). Basic studies have shown that bevacizumab can reach high concentrations in brain metastases, exerting its anti-angiogenic effect and significantly inhibiting the proliferation of brain metastases (Ilhan-Mutlu et al., 2016). Numerous studies have confirmed that bevacizumab can effectively inhibit the development of LMD in lung cancer patients without increasing the risk of cerebral hemorrhage (Besse et al., 2015; Gubens et al., 2018). The meta-analysis showed that in NSCLC patients with LMD, the ORR and disease control rate (DCR) of the anti-tumor treatment regimen with bevacizumab were significantly better than those of the current therapy. Patients treated with bevacizumab have better intracranial DCR and PFS than those treated extracranially (Liang et al., 2020). Bevacizumab can reduce the risk of new-onset LMD by 70%, indicating its potential to prevent brain metastasis, and the risk of intracranial hemorrhage caused by bevacizumab treatment is lower. The abovementioned research provides a theoretical basis for IT bevacizumab in the treatment of solid tumor LMD patients. There is relatively scant research on IT bevacizumab. In the experimental group, four New Zealand white rabbits were randomly selected to receive IT bevacizumab at a dose of 1.5 mg per week, while four rabbits in the control group received IT physiological saline for 4 weeks, aiming to explore the safety of IT bevacizumab. The experimental results indicate that all rabbits in the experimental group can tolerate repeated IT bevacizumab, and there is no alteration in the baseline neurological function state of the control group. In addition, no adverse reactions were observed. IT bevacizumab has no clinical or pathological neurotoxicity, and this neurotoxicity study provides safety data for IT bevacizumab (Brastianos et al., 2012). The first use of IT bevacizumab in humans was for the treatment of LMD in a glioblastoma patient (Holdaway et al., 2023). The 19-year-old female patient had a recurrence of glioblastoma in the thalamus and received two arterial infusions of bevacizumab. The disease remained relatively stable until it progressed in 2022. Magnetic resonance imaging then showed glioblastoma with LMD. Subsequently, five doses of IT bevacizumab at 25 mg, 37.5 mg, 50 mg, 50 mg, and 37.8 mg were administered, with a two-week interval between the first and fourth doses. As the patient had good tolerance and requested discharge, the interval between the fifth and fourth doses was only 1 day, thus reducing the dosage of the fifth dose. Throughout the treatment period, the patient had good tolerance and did not experience any serious adverse reactions or dose-limiting toxicity. After the first IT bevacizumab, the patient survived for 110 days. Due to the rapid progression of glioblastoma with LMD, the patient's PFS could not be

determined. Although the survival benefits of the patient could not be evaluated, the research team preliminarily verified the effectiveness and safety of IT bevacizumab through dose escalation experiments. IT bevacizumab may have good application prospects in solid tumor LMD.

3.2 Checkpoint inhibitors

3.2.1 Pembrolizumab

Pembrolizumab, a humanized macromolecular monoclonal antibody, can selectively bind to PD-1 immune checkpoints on the surfaces of immune cells. It can accurately block the interaction between PD-1 and programmed death ligand 1 (PD-L1), relieve immune suppression mediated by the PD-1 pathway, promote T-cell proliferation and cytokine generation, and exert anti-tumor effects. It is approved for use in various solid tumors such as melanoma, NSCLC, esophageal cancer, and head and neck squamous cell carcinoma. Multiple studies have shown that systemic administration of pembrolizumab has good efficacy and safety for solid tumor LMD (Hendriks et al., 2019; Brastianos et al., 2020; Naidoo et al., 2021; Zheng et al., 2021). A phase-II clinical study included 13 patients with solid tumor LMD who received intravenous 200 mg pembrolizumab once every 3 weeks (Naidoo et al., 2021). The results showed that at 12 weeks, 38% of patients exhibited a CNS response (95% CI: 13.9%–68.4%). The median CNS-PFS and OS were 2.9 months (95% CI: 1.3-NA) and 4.9 months (95% CI: 3.7-NA), respectively. Only 15% of patients experienced treatment-related adverse events of grade 3 or higher.

Pembrolizumab has a limited amount of drug reaching the CSF through the BBB during systemic administration. A case report described a patient with triple-negative breast cancer who developed LMD following systemic administration of pembrolizumab and subsequently received IT pembrolizumab (Ensign et al., 2021). The patient was diagnosed with triple-negative breast cancer. After 2 years of systemic treatment, brain metastases occurred. Pembrolizumab was administered intravenously and combined with local radiotherapy for the brain. One year later, the patient developed LMD accompanied by cerebral edema, and two cycles of high-dose systemic methotrexate were given to control the neurological symptoms. However, imaging examinations indicated the progression of the CNS disease. Subsequently, 5 mg of pembrolizumab was injected intrathecally once every 3 weeks. During the first IT therapy, the patient did not have any adverse reactions. However, after the second administration, the patient experienced pain, weakness, nausea, and diplopia. Eventually, the patient died 9 weeks after receiving IT therapy. Coincidentally, the latest published study also reported the effects of IT pembrolizumab on a melanoma LMD patient (Dan et al., 2024). The patient was diagnosed with cutaneous melanoma, and genetic testing showed BRAF V600E mutation, PD-L1-positive expression, and a combined positive score of 10. After being diagnosed with LMD, the patient received whole-brain radiation therapy combined with targeted therapy and immunotherapy for 2 months, and the patient experienced disease progression. In May of the same year, the patient developed paraplegia, spinal hyperalgesia, nausea, vomiting, and headache, but the CSF cell pathology results were negative. The research group adjusted the anti-tumor regimen to

25 mg of pembrolizumab via IT injection and 175 mg via intravenous injection and continued with targeted therapy. No adverse events were reported after the first IT administration. The patient's symptoms of nausea, vomiting, and headache were relieved, and muscle strength in both lower limbs improved. The patient then received the second IT pembrolizumab, but the symptoms did not improve significantly, and intermittent breathing and cardiac arrest occurred. Ultimately, the patient died of respiratory arrest 2 weeks after receiving IT pembrolizumab. The abovementioned reports suggest that it is not possible to draw a conclusion from case studies that IT pembrolizumab benefits patients with LMD, but it may help temporarily alleviate neurological symptoms. The findings of the abovementioned studies provide a new exploration direction for the administration of pembrolizumab; further studies using IT pembrolizumab are required before any therapeutic treatment decisions can be made concerning its efficacy and safety in the treatment of LMD.

3.3 Other immunotherapeutic drugs

3.3.1 Chimeric antigen receptor natural killer

Numerous studies have shown that depletion of the natural killer (NK) cell population before tumor transplantation induces a more aggressive phenotype in metastatic tumors. The presence of a large number of tumor-infiltrating NK cells is associated with a good prognosis. NK cells play an important role in brain metastases and meningiomas, which have become the basis for the development of NK cell-based brain tumor therapies (Avril et al., 2012; Poli et al., 2013; Blaylock, 2015). Chimeric antigen receptor natural killer (CAR-NK) cell therapy is an innovative adoptive cell therapy that, through genetic engineering modification, connects antibodies or receptors that recognize target cell surface antigens with signaling molecules required to activate immune cells, thereby activating NK cells and allowing them to specifically attack tumor cells (Pan et al., 2022). As early as 2004, clinical trials were conducted using NK cells to treat recurrent malignant gliomas, and four out of nine patients who received treatment experienced tumor regression (Ishikawa et al., 2004). At present, there are no large-scale clinical trials reporting the effectiveness of NK cells in treating brain tumors or solid tumor LMD. Researchers first injected CAR-NK cells derived from umbilical cord blood intrathecally into T-cell acute lymphoblastic leukemia patients who experienced symptoms of CNS after receiving stem cell transplantation (Yuan et al., 2023). After a short period of infusion, the patient developed neurological symptoms including high fever, headache, nausea, vomiting, and spinal cord transection incontinence. However, the symptoms of the patient, including upper eyelid ptosis and blurred vision, were completely improved. After 9 months of IT CAR-NK cells, the patient's bone marrow was completely chimeric with the donor. Although IT CAR-NK cells have certain neurotoxicity, adverse reactions can be reduced by increasing the dose of CAR-NK cells administered, increasing the number of IT, and improving the preparation process of CAR-NK cells. A phase-I clinical trial based on CAR-NK (NCT03383978) is currently underway, using IT NK-92/5.28.z CAR-NK to treat leptomeningeal spread caused by recurrent HER-2-positive glioblastoma. The abovementioned

evidence suggests that IT CAR-NK cells may be a potentially feasible and effective option for treating tumor LMD in the future. For patients with solid tumor LMD, the BBB is a challenge faced by CAR-NK, including all macromolecular drugs. At present, changing the administration method, such as local delivery of NK cell therapeutic drugs through intraventricular and/or IT pathways, can increase drug concentration in the ventricular CSF, adjust NK cell distribution, and further kill tumor cells. However, further research and clinical trials are needed to verify the efficacy of IT NK cells.

4 Conclusions and future perspectives

IT anti-tumor drugs are a common method for treating LMD. IT chemotherapy drugs and targeted and immunotherapy drugs are summarized in Tables 1, 2, respectively. Based on current clinical data of antineoplastic drugs in patients with solid tumor LMD, methotrexate, thiotepa, cytarabine, and cytarabine liposomes are recommended by the ESMO (Le Rhun et al., 2023), while methotrexate, thiotepa, cytarabine, etoposide, topotecan, pemetrexed, trastuzumab, and nivolumab are endorsed by the National Comprehensive Cancer Network for the treatment of solid tumors with LMD (Central nervous system cancers V, 2024). Meanwhile, nimotuzumab, bevacizumab, and pembrolizumab have also been used by researchers for IT therapy. They may be potential IT drugs for patients with LMD from solid tumors. Although some efficacy has been achieved, the role of IT in the treatment of LMD patients with solid tumors is still controversial. Due to the short OS of patients with LMD, evaluating therapeutic efficacy is challenging. In addition, most immunotherapy and targeted drugs are reported as individual cases, lacking large-scale clinical trials and prospective evidence, and are less commonly used in clinical practice. Some studies have examined the effectiveness of combination IT therapy in solid cancers, such as IT administration of two anti-tumor drugs, IT therapy combined with radiotherapy, and IT therapy combined with systemic therapy (Martens et al., 2013; Pan et al., 2020), but most of the IT combination therapies we identified in the present review were case reports. Although these case reports suggested suitable efficacy and safety, these drug combinations should be validated in large, randomized, placebo-controlled clinical trials to enable clinicians to make accurate judgments concerning combination IT therapies for LMD.

Some studies have proposed that when there are circulating tumor cells in the CSF, IT therapy should be considered first (Montes de Oca Delgado et al., 2018). The European Association of Neuro-Oncology and the ESMO recommend IT therapy when there is an absence of blood-cerebrospinal fluid barrier dysfunction (Le Rhun et al., 2023). In addition, the NCCN guidelines recommend that patients with LMD who have a Karnofsky Performance Status score greater than or equal to 60, no severe neurological deficits, a small systemic tumor burden, and can tolerate systemic therapy should receive IT therapy (Horbinski et al., 2023). Meanwhile, different types of IT anti-tumor drugs, such as chemotherapeutic, immunotherapeutic, and targeted drugs, have distinct advantages. IT chemotherapy drugs can directly deliver high concentrations of chemotherapeutic agents to the CSF,

TABLE 1 IT cytotoxic drugs for solid tumors with LMD.

Drug name	Conventional intravenous dosage	Intrathecal administration dosage	Intrathecal administration pre-treatment	Pharmacokinetic characteristics of intrathecal chemotherapy	Adverse reactions of intrathecal chemotherapy	Study type	Recommended guidelines for intrathecal administration	Reference
Methotrexate	40–60 mg/m ² , once weekly	Induction therapy: 10–15 mg, twice weekly for 4 weeks Consolidation therapy: 10–15 mg, once a week, for 4 weeks Maintenance therapy: 15 mg, once a month	Folic acid (10 mg, twice a day, for 3 days) and IT or oral dexamethasone	The concentration of methotrexate in the lumbar CSF decreases in a biphasic metabolic manner, with half-lives of 4.5 and 8 h. The drug is slowly absorbed through the choroid plexus, released into the systemic circulation, partially bound to serum albumin, and excreted through the kidneys.	Chemical meningitis, delayed leukoencephalopathy, acute encephalopathy, and transverse myelopathy	Phase-II study, retrospective study	ESMO and NCCN	Rubin et al. (1968) , Beauchesne (2010) Pan et al. (2016) Le Rhun et al. (2023)
Thiotepa	10 mg (0.2 mg/kg) per day for 5 days; adjusted to three times per week, with a total cumulative dose of 300 mg	Induction therapy: 10 mg, twice weekly for 4 weeks Consolidation therapy: 10 mg, once a week for 4 weeks Maintenance therapy: 10 mg, once a month	IT or oral dexamethasone	Thiotepa exhibits good tissue infiltration. It has the shortest half-life among IT chemotherapy drugs; it rapidly diffuses into the CSF within 4 h after IT, without distributing the CSF space or the brain parenchyma, and is cleared through cerebral capillaries.	Systemic bone marrow suppression, neurotoxicity limited to mild transient lower-limb sensory abnormalities, and spinal cord disease	Phase-I/II study, retrospective study	ESMO and NCCN	Gutin et al. (1976) , Gutin et al. (1976) , Strong et al. (1986) , Beauchesne (2010) , Le Rhun et al. (2023)
Cytarabine	100–200 mg/m ² , administered once daily	Induction therapy: 25–100 mg, twice weekly for 4 weeks Consolidation therapy: 25–100 mg, once a week for 4 weeks Maintenance therapy: 25–100 mg, once a month	IT or oral dexamethasone	Accumulation of cytarabine in the CSF between the repeated injections was not observed, and cytarabine was not detectable in the plasma. The half-life of cytarabine in the CSF is less than 4 h, and it is completely cleared within 1–2 days.	Meningismus, nausea, vomiting, and myelosuppression	Phase-I/II study	ESMO and NCCN	Fulton et al. (1982) , Le Rhun et al. (2023)
Cytarabine liposome	NA	50 mg, twice weekly for 8 weeks, followed by once monthly for a total duration of 24 weeks	IT or oral dexamethasone	The half-life is relatively long, exceeding 80 h IT 50 mg cytarabine liposome twice every 14 days; the concentration range of free and encapsulated cytarabine in the CSF of the ventricles and lumbar vertebrae is 0.01–1,500 µg/mL, which can be detected within 14 days after administration. Occasionally, free cytarabine is detected in the plasma, and the concentration of metabolites of cytarabine in the CSF and plasma is relatively low.	Headache and arachnoiditis	Phase-III/IV study	ESMO	Glantz et al. (1999a) , Glantz et al. (1999b) , Jaekle et al. (2001) , Shapiro et al. (2006) , Phuphanich et al. (2007) Le Rhun et al. (2019)

(Continued on following page)

TABLE 1 (Continued) IT cytotoxic drugs for solid tumors with LMD.

Drug name	Conventional intravenous dosage	Intrathecal administration dosage	Intrathecal administration pre-treatment	Pharmacokinetic characteristics of intrathecal chemotherapy	Adverse reactions of intrathecal chemotherapy	Study type	Recommended guidelines for intrathecal administration	Reference
Etoposide	60–100 mg/m ² for 3–5 days, either once every 3 weeks or once every 4 weeks	Induction therapy: 0.5 mg/day for 5 days, every other week for 8 weeks Consolidation therapy: 0.5 mg/day for 5 days, every other week for 4 weeks Maintenance therapy: 0.5 mg/day for 5 days, once a month	IT or oral dexamethasone	The peak concentration of etoposide in the CSF in the ventricles of the brain exceeds that of intravenous infusion by more than 100 times. The steady-state distribution volume and total clearance rate show significant individual differences, showing a double exponential decrease. No etoposide was detected in the plasma.	Transient headache, epileptic seizures, Ommaya sac infection, nausea, and neuropsychological symptoms	Phase-II study	NCCN	Fleischhack et al. (2001), Slavic et al. (2003), Chamberlain et al. (2006)
Topotecan	1.25 mg/m ² , once daily for 5 days, repeated every 3 weeks	Induction therapy: 0.4 mg, twice weekly for 6 weeks Consolidation therapy: 0.4 mg, once weekly for 6 weeks Maintenance therapy: 0.4 mg, twice monthly for 4 months, followed by monthly administration thereafter	IT or oral dexamethasone	IT 0.4 mg topotecan, the peak concentration in the ventricles was 28 ± 11 μ mol/L, and it was not detected in the plasma. The clearance was mainly achieved through its conversion into inactive forms and CSF circulation, with only approximately 10% clearance through microvasculature.	Nausea/vomiting, headache, anorexia, fever, and arachnoiditis (which indicates dose-limiting toxicity)	Phase-I study, phase-II study, and case report	NCCN	Blaney et al. (2003), Glaberman et al. (2005)
Pemetrexed	500 mg/m ² , once every 3 weeks	Standard dosing: 50 mg, once every 3 weeks Induction therapy: 10 mg, twice weekly for 2 weeks Consolidation therapy: 10 mg, once weekly, with a total of no more than 8 doses	Take 400 μ g of folic acid before IT therapy continuously for 5 days. Stop taking folic acid during and 1 month after discontinuing the therapy Inject 1,000 μ g of vitamin B12 intramuscularly once a week before the first administration, and then administer it every 3 cycles thereafter IT or oral dexamethasone	IT 30 mg pemetrexed, the peak concentration reached 2–4 h. After 24 h of IT pemetrexed administration, the effective concentration level remained in the CSF, with an average value of 93.44 μ g/mL. It showed a biphasic elimination pattern in the CSF and extremely low concentration in the blood.	Bone marrow suppression and hepatotoxicity	Phase-I study and phase-II study	NCCN	Pan et al. (2020), Fan et al. (2021), Li et al. (2023)

Abbreviations: IT, intrathecal; CSF, cerebrospinal fluid; ESMO, European Society for Medical Oncology; NCCN, National Comprehensive Cancer Network; NA, not available.

TABLE 2 IT targeted drugs and immunotherapy drugs for solid tumors with LMD.

Drug name	Conventional intravenous dosage	Intrathecal administration dosage	Adverse reactions of intrathecal targeted and immunotherapy drugs	Study type	Recommended guidelines for intrathecal administration	Reference
Trastuzumab	Two-week dosing regimen: an initial loading dose of 4 mg/kg, followed by a maintenance dose of 2 mg/kg Three-week dosing regimen: an initial loading dose of 8 mg/kg, followed by a maintenance dose of 6 mg/kg	80 mg–150 mg, twice weekly for 4 weeks, then once weekly for 4 weeks, followed by maintenance therapy every 1–2 weeks	Headache, nausea, vomiting, and meningeal spasms	Systematic review, phase-I study, phase-II study, phase-I/II study, and case report	NCCN	Zagouri et al. (2013), Kumthekar et al. (2023), Oberkamp et al. (2023), Lazaratos et al. (2023)
Nimotuzumab	100–400 mg, once a week, for a total of 8 weeks	50 mg, once a week	NA	Case report	—	Ju et al. (2016a), Ju et al. (2016b)
Bevacizumab	5 mg/kg, once every 3 weeks 7.5–15 mg/kg, once every 3 weeks	25–50 mg, once every 2 weeks	NA	Case report	—	Holdaway et al. (2023)
Nivolumab	3 mg/kg or 240 mg every 2 weeks or 480 mg every 4 weeks	50 mg, once every 2 weeks	Nausea, diarrhea, decreased lymphocyte count, elevated aspartate aminotransferase and/or alanine aminotransferase, and papules/rashes	Phase-I study	NCCN	Glitza Oliva et al. (2023)
Pembrolizumab	200 mg, once every 3 weeks, or 400 mg, once every 6 weeks	5 mg or 25 mg, once every 3 weeks	Pain, weakness, nausea, diplopia, intermittent breathing, and cardiac arrest	Case report	—	Dan et al. (2024)

Abbreviations: NCCN, National Comprehensive Cancer Network; NA, not available.
Notes: CAR-NK was not included in Table 2 due to the lack of recommended dosage and description of adverse reactions in the literature.

effectively killing tumor cells that have metastasized to the meninges. They have a good effect on controlling local tumors in the meninges and help alleviate symptoms such as headache and dizziness caused by meningeal metastasis. Therefore, IT chemotherapy drugs are effective for most solid tumors with LMD (Pfeffer et al., 1988). Targeted drugs can target specific targets of tumor cells with minimal damage to normal tissues. For example, for HER-2-positive breast cancer and glioblastoma with LMD, targeted drugs can more accurately inhibit tumor growth signaling pathways (Ferrario et al., 2009; Holdaway et al., 2023). Compared with traditional chemotherapy, due to its clear targeting, side effects are relatively easier to manage, and patients may have better tolerance to treatment, which can help improve their quality of life. Immunotherapy drugs can activate the patient’s own immune system, allowing immune cells to recognize and attack tumor cells that have metastasized to the meninges. In some patients who have melanoma and renal cell carcinoma with LMD, IT immunotherapy drugs can enhance the body’s anti-tumor immune response. After the immune system is activated, it may generate immune memory, inhibit tumor cell growth for a long time, potentially prolong the patient’s survival, and control tumor progression (Glitza Oliva et al., 2023).

However, the occurrence of adverse reactions has been repeatedly reported, and the safety of IT anti-tumor drugs has

gained widespread attention in the medical community. Due to the BBB, most drugs injected within the sheath gather in the ventricles of the brain and do not circulate throughout the body. Therefore, the systemic toxicity of IT is relatively low. The most common complications of IT anti-tumor drugs are mainly related to their toxic effects (Beauchesne, 2010). Potential causes of adverse reactions are as follows: first, high concentrations of anti-tumor drugs exposed to the CSF have direct toxic effects on the CNS and induce changes in CSF biochemical levels (Rolf et al., 2006). Furthermore, after IT therapy, the outflow of CSF and the injection of medication result in an imbalance between intracranial pressure and spinal subarachnoid pressure, and brain tissue often flows into the lower pressure area. Therefore, patients often experience headaches and vomiting without fever, but anti-tumor drugs can damage white blood cells, leading to the release of endogenous pyrogens, which can also induce an increase in body temperature. In addition, the dura mater at the puncture site has a needle hole, and the CSF continuously flows out, causing a decrease in intracranial pressure and dilation of intracranial blood vessels, leading to headaches and vomiting. IT is usually performed simultaneously with systemic chemotherapy, which exacerbates the symptoms of adverse reactions. Finally, injecting impure drugs or introducing iodine from the skin into the CSF during puncture can trigger irritation due to puncture failure, bleeding, and

other reasons. Increased secretion of the CSF can lead to elevated intracranial pressure, causing headaches, vomiting, fever, and more adverse effects. To reduce and prevent adverse reactions caused by IT therapy of anti-tumor therapy, attention should be paid to the following points: first, the operation of IT therapy needs to be standardized. It is necessary to strictly control the medication time interval, injection speed, and drug dosage. Second, attention should be paid to the potential risk of drug allergies, and it should be inquired whether the patient has experienced adverse reactions to IT anti-tumor drugs before choosing anti-tumor drugs with minimal toxic side effects, high safety, and sufficient evidence-based medicine as much as possible.

Although IT therapy for LMD has achieved some promising outcomes, it still faces many challenges. First, the optimal drug combinations and treatment regimens for IT therapy remain undetermined. Different drug combinations and treatment regimens may influence treatment outcomes and side effect profiles in various ways, necessitating further research and exploration. In addition, the long-term safety of IT therapy remains controversial. Although IT therapy can reduce the side effects caused by systemic chemotherapy, long-term use may cause damage to the nervous system and affect the quality of life. Moreover, clinical research on IT therapy for LMD also faces challenges. Addressing ethical issues related to patients, such as the risks of invasive procedures, complex risk explanations, therapeutic misconceptions, and strict ethical review standards, is particularly difficult. There is considerable heterogeneity among study participants, with patients often presenting with varying tumor types, stages, and treatment histories. The range of anti-tumor drugs available for IT therapy is limited, and their selection is frequently guided by empirical evidence or preliminary study findings, lacking precise screening mechanisms. Additional challenges include inaccurate evaluation criteria, insufficient safety monitoring, and difficulties in follow-up. In conclusion, although IT therapy for LMD has made some progress, it is still in the exploratory stage. In the future, it is necessary to further strengthen basic research and clinical research, explore more effective treatment regimens and drug combinations, improve treatment efficacy and safety, and ultimately provide greater hope for patients with LMD.

With the continuous progress of medical technology, the prospect of IT therapy for LMD is full of hope. On the one hand, an increasing number of new drugs are under development, and these drugs may have higher efficacy and fewer side effects. For example, some targeted and immunotherapy drugs have achieved remarkable results in other tumor fields and are expected to be applied to IT therapy for LMD in the future. On the other hand, the development of precision medicine and artificial intelligence can provide assistance for the individualized treatment of patients (Adam et al., 2020; Miao et al., 2020). Perhaps, in the near future, these two technologies can be utilized to assist in the design of IT administration regimens and drug screening. First, artificial intelligence is used to integrate data such as patient's gene sequencing, pathology, and physical condition to accurately classify patients and screen out those suitable for IT treatment. Subsequently, it analyzes the multi-target characteristics of tumor cells, correlates them with the drug database, and selects matching multi-target drugs while also predicting their therapeutic

efficacy and potential adverse reactions. During the treatment, the CSF indexes and symptom changes of the patients are continuously monitored. Artificial intelligence provides real-time feedback to dynamically fine-tune the drug dosage and injection frequency. The synergy between artificial intelligence and multi-target drugs can overcome the obstacles of individual differences and build a precise and flexible personalized treatment framework for different patients. The new generation of precision oncology utilizes computational models to personalize the prediction of patient responses to IT injections of anti-tumor drugs, enabling the determination of the optimal treatment plan. This approach holds great promise for improving the chances of successful recovery in patients.

LMD, characterized by a poor prognosis and complex treatment regimens, is one of the most severe complications of malignant tumors. It is thus recommended to integrate multidisciplinary treatment, which encompasses active systemic and local treatment approaches. IT treatment emerged as an important therapeutic option for patients with LMD. Although optimal IT therapy has not yet been established through clinical trials, it has demonstrated initial advantages in enhancing the efficacy and quality of life for solid tumor LMD patients. This involves not only classic IT drugs such as methotrexate and others but also attempts to administer other medications such as immunotherapeutic and targeted drugs. Future research is required to further evaluate the efficacy and safety of various IT drugs and determine the most appropriate approach and treatment combination.

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XW: writing—original draft, resources, and visualization. CY: investigation and writing—original draft. LQ: writing—review and editing and investigation. JZ: conceptualization, supervision, and writing—review and editing.

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A pharmacoinformatic approach for studying *Atractylodes Lancea* DC's anticancer potential and control ROS-mediated apoptosis against prostate cancer cells

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Introduction: Prostate cancer (PCa) is a malignancy characterized by abnormal cell proliferation in the prostate gland, a critical component of the male reproductive system. *Atractylodes lancea* DC. (ALD), a medicinal herb commonly used in traditional Asian medicine, is highly regarded for its antioxidant, antidiabetic, and anticancer properties. Virtual docking studies have identified Atractylenolide II and III as active components of ALD, demonstrating strong binding potential to inhibit androgen receptor (AR) activity, with docking scores of -8.9 and -9.3, respectively. These findings suggest that ALD may exert a synergistic effect comparable to or greater than that of enzalutamide (ENZ) in inhibiting AR. However, its specific anticancer and anti-metastatic mechanisms in prostate cancer remain unclear.

Methods: The cytotoxic effects of ALD were evaluated on PC3 and DU145 prostate cancer cells, as well as on the normal prostate cell line BPH-1. Cell viability was assessed using the EZ-Cytotoxic kit, while colony formation assays and TUNEL staining were used to measure proliferation and apoptosis, respectively. Apoptosis was further analyzed through annexin V-FITC/PI staining and quantified by flow cytometry (FACS). Western blotting was performed to elucidate the underlying molecular mechanisms. Additionally, mitochondrial membrane potential ($\Delta\Psi_m$) and intracellular calcium levels were measured to evaluate mitochondrial function, while reactive oxygen species (ROS) generation was assessed with and without pretreatment with N-acetylcysteine (NAC).

Results: ALD selectively reduced the viability of PC3 and DU145 prostate cancer cells while sparing BPH-1 normal prostate cells, demonstrating cancer-selective cytotoxicity. ALD disrupted mitochondrial function by reducing $\Delta\Psi_m$ and increasing intracellular calcium levels. A concentration-dependent increase in ROS generation was observed in PC3 and DU145 cells, which was completely inhibited by NAC pretreatment, confirming a ROS-mediated mechanism. Colony formation assays revealed a significant reduction in proliferation, while TUNEL and annexin V-FITC/PI staining indicated enhanced apoptosis. Western blot analysis showed that ALD modulates critical survival pathways, leading to apoptotic cell death.

Discussion: These findings demonstrate that ALD exerts potent anticancer effects against metastatic prostate cancer cells through ROS-mediated apoptosis and mitochondrial dysfunction, while exhibiting minimal cytotoxicity toward normal prostate cells. The presence of active compounds such as Atractylenolide II and III suggests a synergistic interaction that enhances AR inhibition and promotes apoptosis. ALD's ability to engage multiple pathways highlights its therapeutic potential as a selective and multifaceted treatment for aggressive prostate cancer.

KEYWORDS

Atractylodes lancea DC., prostate cancer, reactive oxygen species, apoptosis, mitochondrial membrane potential

1 Introduction

Prostate cancer (PCa), a malignancy originating in the prostate gland, a small organ responsible for producing seminal fluid to support and transport sperm, is among the most prevalent cancers affecting men worldwide (1). Prostate cancer is more prevalent in industrialized nations, with GLOBOCAN estimating 1,276,106 new cases and 358,989 deaths worldwide in 2018. Each year, approximately 80,000 individuals globally succumb to prostate cancer, with an average of 190,000 new cases diagnosed annually (2). In Korea, recent data ranks prostate cancer as the third most commonly diagnosed cancer in men, highlighting a pressing need for effective prevention and treatment strategies (3, 4). The Korean Society of Medical Oncology (KSMO) established comprehensive guidelines in 2017 for managing metastatic prostate cancer, underscoring the increasing national and global concern regarding its impact (5). Understanding incidence trends and prognosis is essential, as outcomes are influenced by factors such as cancer stage, individual health, and response to therapy (6). While some prostate cancer cases progress slowly, allowing patients to live for many years with minimal health effects, the continuous advancement of therapies aims to improve survival and quality of life (7). Therefore, identifying novel therapies with minimal adverse effects is crucial for enhancing patient outcomes. Traditional Chinese Medicine (TCM) is an invaluable source for discovering new cancer treatments, with many herbs showing significant pharmacological properties (8). Among these, *Atractylodes lancea* DC. (ALD) stands out due to its wide-ranging effects, including antioxidant, antidiabetic, and anticancer activities (9). However, the precise mechanisms by which ALD exerts

anticancer and anti-metastatic effects, particularly against PCa, remain underexplored. PCa progression is strongly linked to the androgen receptor (AR) signaling pathway, which promotes the growth and survival of cancerous cells (10). Advanced PCa treatment often involves androgen deprivation therapy (ADT) to reduce androgen levels or inhibit AR function (11). The AR plays a critical role in prostate gland development and in driving PCa progression by facilitating cell growth, survival, and migration (12). Virtual docking studies have revealed that components of TCM, including Atractylenolide II and III, possess the potential to bind and inhibit AR ligand activity, indicating a possible therapeutic pathway (13). Furthermore, *in vitro* and *in vivo* studies have demonstrated the therapeutic efficacy of ALD and its bioactive compounds, including atractylodin and β -eudesmol, in treating cholangiocarcinoma. To further assess the immunomodulatory effects of ALD in humans, a randomized, double-blind, placebo-controlled phase I clinical trial was conducted, providing additional insights into its clinical potential (14). Recent studies underscore Atractylenolide II as a powerful inducer of apoptosis in prostate cancer cells. By effectively modulating the androgen receptor (AR) and JAK2/STAT3 signaling pathways, it emerges as a compelling targeted therapeutic strategy, making it a promising candidate for treating metastatic prostate cancer (15). These insights underscore the need for further research on the therapeutic potential of ALD against PCa and inspired our investigation into its ability to trigger apoptosis in prostate cancer cells by disrupting AR signaling and other critical survival pathways. Mechanisms that selectively induce cancer cell death hold great promise for targeted therapies (16). Apoptosis, a programmed cell death process, is central to developing effective

anticancer drugs (17, 18). Disruption of mitochondrial function vital for cellular energy production and apoptosis regulation represents a powerful approach to inducing cancer cell death (19). Research increasingly shows that mitochondrial ROS production is a key trigger of apoptosis (20). Elevated ROS levels disturb cellular homeostasis, activating signaling pathways that lead to cell death (21). Thus, agents that promote mitochondrial ROS generation are considered promising therapeutic candidates. In this study, we explore the hypothesis that ALD exerts anticancer effects by inducing ROS-mediated apoptosis in prostate cancer cells. Using PC3 and DU145 cell lines, established models for PCa research, we examined ALD's effects on cell viability, mitochondrial activity, ROS production, and apoptotic protein expression. Through well-established *in vitro* assays, we evaluated ALD's impact on cell survival, colony formation, and key apoptotic markers, as well as its influence on mitochondrial function and ROS generation. This research aims to advance the development of novel therapies for metastatic prostate cancer by elucidating the mechanisms underlying ALD's anticancer effects.

2 Materials and methods

2.1 Preparation of *Atractylodes Lancea* DC. extract

In Gangwon Province, Korea, *Atractylodes lancea* DC. (ALD) was collected (200 g), and a voucher specimen (Registration number BK059) was preserved in the herbarium of the Cancer Molecular Targeted Herbal Research Center at Kyung Hee University. Following established protocols (22), dried ALD was extracted in 100% ethanol, concentrating the solution to 100 mL using an evaporator at 98°C for 24 hours. After lyophilization, the extraction yield was 18%, resulting in a powdered form. ALD was prepared as a stock solution at a concentration of 200 mg/mL in 100% dimethyl sulfoxide and stored at -20°C for future use.

2.2 Culturing of cell lines

PC3 (a prostate cancer cell line with high metastatic potential) and DU145 cells were obtained from the Korean Cell Line Bank (KCLB) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10,000 units/mL of penicillin and streptomycin (Gibco, Grand Island, NY, USA). The culture medium was refreshed every two to three days. The BPH-1 cell line (ATCC, PCS-440-030; Human Benign Prostatic Hyperplasia epithelial cells) was also cultured in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, and 10,000 units/mL of penicillin and streptomycin.

2.3 Cytotoxicity evaluation assay

Cell viability was assessed using a cytotoxicity assay. PC3, DU145 and BPH-1 cells were plated at a density of 1×10^4 cells per well in 96-well plates and treated with ALD at varying concentrations (12.5, 25,

50, 100, or 200 µg/mL) for 24, and 48 hours. At each time point, cell viability was evaluated using a cell viability assay kit according to the manufacturer's instructions (Daeil Lab Service, Seoul, Korea). Absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA) to determine cellular viability.

2.4 Colony formation assay

The colony formation assay was conducted to evaluate cell proliferation and the effects of ALD treatment on prostate cancer cells. PC3 and DU145 cells were seeded in triplicate into 6-well plates at a density of 1000 cells per well and treated with 50 and 100 µg/mL of ALD for 24 hours. After treatment, the cells were cultured in fresh growth medium for 9 days, with the medium replaced every two days, and maintained at 37°C in a 5% CO₂ incubator. Colony formation was assessed by staining: cells were fixed in 70% ethanol for 10 minutes and stained with 0.5% crystal violet to visualize colonies. Colonies consisting of at least 50 cells were counted as individual colonies using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.5 Reactive oxygen species detection assay

To assess ROS production, PC3 and DU145 cells were first incubated with 20 µM 2',7'-Dichlorofluorescein diacetate (DCFDA) for 45 minutes. Following this, cells were treated with ALD at concentrations of 50 and 100 µg/mL for 4 hours. Fluorescence was measured in a 96-well plate format using an ELISA reader (Bio-Rad, Hercules, CA, USA) at excitation/emission wavelengths of 490/595 nm to confirm ROS generation. For further validation, cells were treated with ALD for 24 hours and then incubated with 5 mM N-acetylcysteine (NAC) as a ROS inhibitor, followed by 30 minutes of DCFDA staining (20 µM). Fluorescence was then analyzed using a flow cytometry analysis (FACS) Calibur flow cytometer (Becton Dickinson, Bergen County, NJ, USA) to measure ROS levels and evaluate NAC's inhibitory effects on ALD-induced ROS production. This dual approach, using both ELISA and FACS, provided comprehensive confirmation of ROS activity in response to ALD treatment.

2.6 Analysis of mitochondrial membrane potential

PC3 and DU145 cells were incubated with JC-1 dye (20 µM) for 45 minutes to assess mitochondrial membrane potential (MMP), followed by treatment with ALD at concentrations of 50 and 100 µg/mL for 4 hours. The JC-1 dye, which differentiates between healthy and depolarized mitochondria based on fluorescence, was used to monitor mitochondrial health (Mitochondria Function Assay Kit, Thermo-Fisher Scientific, USA). MMP was evaluated by measuring fluorescence from JC-1 aggregates (intact mitochondria, $\lambda_{ex}/\lambda_{em} = 535/595$ nm) and JC-1 monomers (depolarized mitochondria, $\lambda_{ex}/\lambda_{em} = 475/535$ nm). This ratio provides insights into ALD's impact on

mitochondrial function in PC3 and DU145 cells. For validation, we included a positive control using 2 μ M FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), a known mitochondrial uncoupler, and analyzed the results with a FACS Calibur flow cytometer (Becton Dickinson, Bergen County, NJ, USA). Consistent gating for dye exclusion was applied across experiments to accurately reflect changes in mitochondrial health under different treatment conditions.

2.7 Cytosolic Ca^{2+} quantification assay

Cells were treated with ALD for 24 hours. After treatment, a chromogenic reagent and calcium buffer were added to each well and incubated for an additional 10 minutes. Absorbance was then measured at 575 nm using a microplate reader (Bio-Rad, Hercules, CA, USA) to quantify the response.

2.8 TUNEL apoptosis detection assay

PC3 and DU145 cells were seeded onto a 4-well culture slide (SPL, Pocheon, Republic of Korea) and treated with ALD for 24 hours. After treatment, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Bylabs, San Francisco, CA, USA), and permeabilized using 0.2% Triton X-100 solution (Promega, Madison, WI, USA), followed by additional PBS washes. Cell apoptosis was then analyzed using the DeadEndTM Fluorometric TUNEL System (Promega, Madison, WI, USA). After treatment with equilibrium buffer for 10 minutes, the Nucleotide Mix and rTdT enzyme were added, and samples were incubated for one hour. Following a 15-minute reaction, samples were washed with 2 $\times$ SSC solution and PBS, stained with 1 μ g/mL DAPI, mounted, and visualized using a Zeiss LSM 800 confocal microscope (Zeiss, Oberkochen, Germany). To further assess apoptosis, annexin V-FITC labeling was employed. PC3 and DU145 cells were plated in a six-well plate and incubated for 24 hours at 37°C. Cells were then treated with ALD for 24 hours, harvested, and incubated with 5 μ L annexin V-FITC on ice at 4°C for 15 minutes. After labeling, cells were centrifuged at 1000 g for 5 minutes. Apoptosis was quantified using a FACS Calibur flow cytometer (Becton Dickinson, Bergen County, NJ, USA), providing an accurate measurement of apoptotic cell populations.

2.9 Protein expression analysis by western blot analysis

After treating PC3 and DU145 cells with ALD at concentrations of 50 and 100 μ g/mL for 24 hours, the cells were lysed on ice for 30 minutes using a lysis buffer containing protease inhibitors (Translab, Daejeon, Korea). The cell lysates were then centrifuged at 13,000 rpm for 10 minutes to collect the supernatant containing the proteins. Protein concentrations were determined using the BSA assay to ensure equal protein loading. Equal amounts of protein were loaded onto 6–15% SDS-PAGE gels and separated by electrophoresis at 100 V for 100

minutes. Proteins were then transferred onto nitrocellulose membranes at 300 mA for 120 minutes. Following the transfer, membranes were rinsed three times for 10 minutes each with TBS containing 0.1% Tween 20 (TBST) and then blocked with 5% skim milk dissolved in TBST for 1 hour at room temperature. The membranes were incubated overnight at 4°C with specific primary antibodies diluted 1:10,000, including pro-PARP, cleaved PARP (c-PARP), Bcl-2, survivin, cytochrome c, ATF4, TGF- β , DNMT1, N-Cadherin, and E-Cadherin (Cell Signaling Technology, Beverly, MA, USA). After washing with TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies either anti-mouse (Abcam, Cambridge, UK) or anti-rabbit (Bioss Antibodies, Woburn, MA, USA) for 1 hour at room temperature. Membranes were washed again with TBST, and protein bands were visualized using chemiluminescence detection reagents (Davinchi-K, Seoul, Korea) and scanned for analysis.

2.10 Wound healing assay

The cell lines PC3 and DU145 were used for the wound healing assay. Cells were plated into 6-well plates at a density of 1×10^6 cells per well and cultured in RPMI complete medium for 24 hours to reach approximately 90% confluence. A scratch was made across the cell monolayer using a 200 μ L pipette tip to create a wound gap. Detached cells were removed by gently washing with PBS. The cells were then treated with ALD for a 24 hour intervention period. Following this treatment, the ALD-containing medium was replaced with fresh complete medium, and images of the wound closure were taken to assess the effects of ALD on cell migration.

2.11 Statistical analysis

Statistical analyses were conducted using one-way ANOVA followed by Tukey's *post-hoc* test for multiple group comparisons to determine statistically significant differences between experimental groups. Most experiments were performed in triplicate ($n=3$), and results are expressed as mean $\pm$ standard deviation (SD). For direct comparisons between control and treated groups, Student's t-test was applied where appropriate. Statistical analyses were conducted using SigmaPlot version 12 software (SysTest Software Inc., San Jose, CA, USA), and p-values were considered statically significant as follows $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered statically significant.

3 Results

3.1 The active components of ALD and their targeted pathways in prostate cancer

Active components and potential targets 8896 and 56 for Atractylenolide II and Atractylenolide III, respectively, were identified using the TCMSP, TCMIP, and BATMAN-TCM databases (23). To further understand the molecular interactions, KEGG pathway enrichment analysis and Gene Ontology (GO)

highest affinity. Notably, we highlight that the combined docking scores for Atractylenolide II and III suggest a potential interaction strength that may match or even surpass that of ENZ. This supports the concept that multiple bioactive constituents in herbal formulations like ALD can work synergistically to enhance biological effects, resulting in broader and more stable receptor binding. As illustrated in Figure 2, each ligand formed detailed interaction profiles with specific amino acid residues on the androgen receptor. Atractylenolide II engaged with multiple residues on Chain A, including LYS563, VAL564, LYS567, and ARG590, and with ARG568, GLN574, and TYR576 on Chain B, among others. Atractylenolide III showed a similar pattern, interacting with residues like TYR554, LYS563, and ASN593 on Chain A and with LYS588 and LYS592 on Chain B. ENZ, showing the highest binding affinity, formed interactions with critical residues such as VAL564, LYS567, and PHE589 in Chain A, as well as additional binding with ARG568, TYR576, and LEU577 in Chain B. These findings indicate strong and specific interactions between these ligands and the androgen receptor, with ENZ showing the most robust profile. However, the cumulative binding potential of Atractylenolide II and III may offer a multi-faceted interaction strategy, reinforcing the therapeutic value of ALD in targeting prostate cancer through enhanced receptor engagement.

3.3 ALD demonstrated potent cytotoxic activity against prostate cancer cells

The cytotoxic effects in prostate cancer cells refer to the ability of a compound or treatment to induce cell death or suppress the proliferation of these malignant cells. Understanding and refining these cytotoxic mechanisms is a critical focus in advancing potent cancer therapies, as it holds the potential to selectively target and eradicate cancer cells while preserving healthy tissue (25). The cytotoxic effects of ALD on prostate cancer and normal cells were

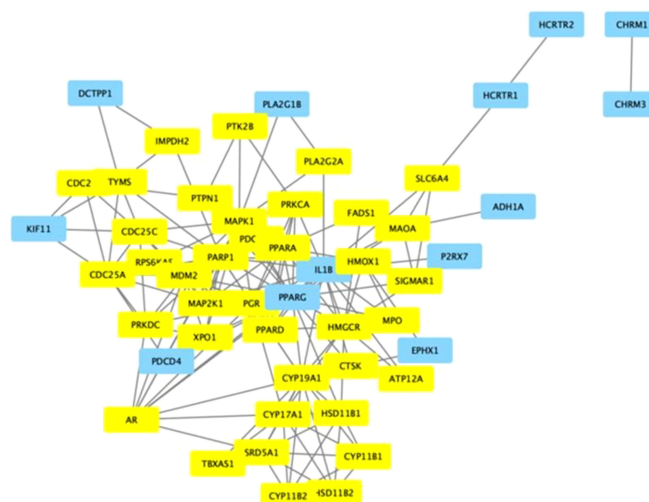


TABLE 1 Ligand interactions with the androgen receptor as a prostate cancer target marker (PDB ID: 1r4i).

Ligands	Docking Score	Amino Acid residue interaction
atractylenolideII	-8.9	Chain A: LYS563 VAL564 LYS567 ARG568 LYS588 PHE589 ARG590 ARG591 LYS592 ASN593 PRO595 Chain B: ARG568 GLN574 LYS575 TYR576 LEU577 LYS592 ASN593 PRO595
atractylenolideIII	-9.3	Chain A: TYR554 LYS563 VAL564 LYS567 ARG568 LYS588 PHE589 ARG591 LYS592 ASN593 Chain B: LYS588 LYS592 ASN593
Enzalutamide	-10.0	Chain A: VAL564 LYS567 ARG568 LYS588 PHE589 ARG590 ARG591 LYS592 ASN593 CYS594 PRO595 Chain B: PHE565 ARG568 ALA569 GLN574 LYS575 TYR576 LEU577 LYS592 ASN593 CYS594 PRO595

assessed at concentrations of 12.5, 25, 50, 100, and 200 µg/mL across 24 and 48 hours. **Figures 3A–C** demonstrate that ALD effectively inhibits the proliferation of PC3 and DU145 prostate cancer cells in a dose- and time-dependent manner, while exhibiting minimal impact on BPH1 normal prostate cells. This selective inhibition underscores ALD’s potential as a targeted therapeutic agent with specificity toward cancerous cells, sparing healthy prostate cells. These findings, illustrated in **Figures 3A–C** underscore ALD’s potential as a targeted therapeutic agent for prostate cancer. To further investigate its cytotoxic impact, cells were treated with ALD at 50 and 100 µg/mL, revealing pronounced cell death characteristics as early as 24 hours treatment. **Figures 3D, E** vividly capture these morphological changes, underscoring ALD’s potent and rapid cytotoxic effects on prostate cancer cells. Distinctive apoptotic features, including plasma membrane disintegration and the formation of apoptotic bodies, were prominently observed in ALD-treated cells. In contrast, untreated PC3 and DU145 cells remained viable up to 48 hours, displaying increased cell size, cell number, and

a progressively flatter morphology over time. ALD-treated PC3 and DU145 cells, however, exhibited consistent and striking apoptotic characteristics, with cell death morphology becoming more pronounced in a dose-dependent manner.

3.4 ALD suppressed colony formation in prostate cancer cells

The clonogenic, or colony-forming cell (CFC) assay, is widely regarded as the gold-standard quantitative method for assessing the proliferative capacity of progenitor cells *in vitro* by measuring the ability of a single cell to survive, proliferate, and form a colony (26). To investigate the antiproliferative effects of ALD on prostate cancer cells, a colony formation assay was performed with PC3 and DU145 cell lines cultured on 6-well plates, both in the presence and absence of ALD, over a 9-day period. ALD treatment at a concentration of 100 µg/ml significantly inhibited colony formation in both cell lines (**Figure 4**), underscoring its strong suppressive effect on the proliferation of PC3 and DU145 cells. These results highlight ALD’s potent antiproliferative action against prostate cancer cells, further validating its potential as an effective therapeutic agent.

3.5 ALD triggered apoptosis through Ca²⁺ uptake and mitochondrial membrane potential disruption in prostate cancer cells

Disruption of the mitochondrial membrane triggers a cascade of apoptotic events, including a reduction in mitochondrial membrane potential (MMP) and an increase in cytoplasmic Ca²⁺ concentration (27). Effective anticancer agents are known to induce apoptosis in cancer cells by elevating intracellular Ca²⁺ levels, a hallmark of mitochondrial dysfunction. In our study, we used the JC-1 assay to assess changes in MMP, which revealed a notable decrease in MMP (28) in ALD-treated PC3 and DU145 cells at 50 and 100 µg/ml

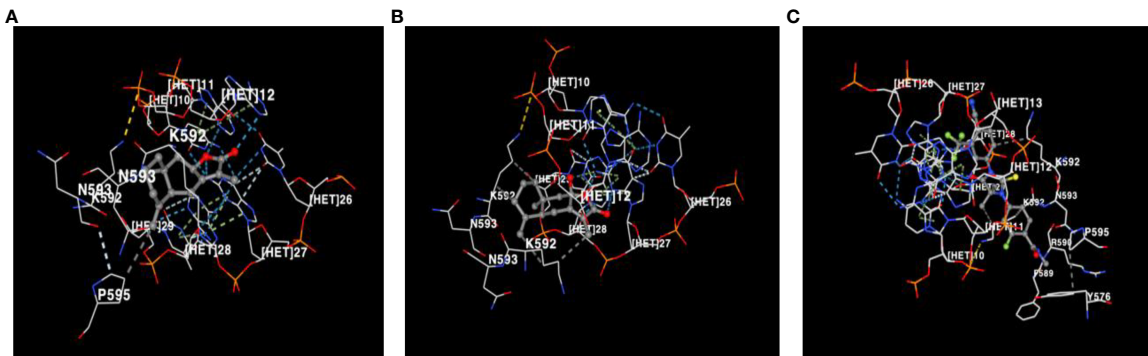


FIGURE 2 Visualization of amino acid residue interactions with (A) Atractylenolide II, (B) Atractylenolide III, and (C) Enzalutamide (ENZ). Interactions between amino acid residues of the androgen receptor and the ligands Atractylenolide II, Atractylenolide III, and ENZ are shown, highlighting the binding affinity and key contact points for each compound.

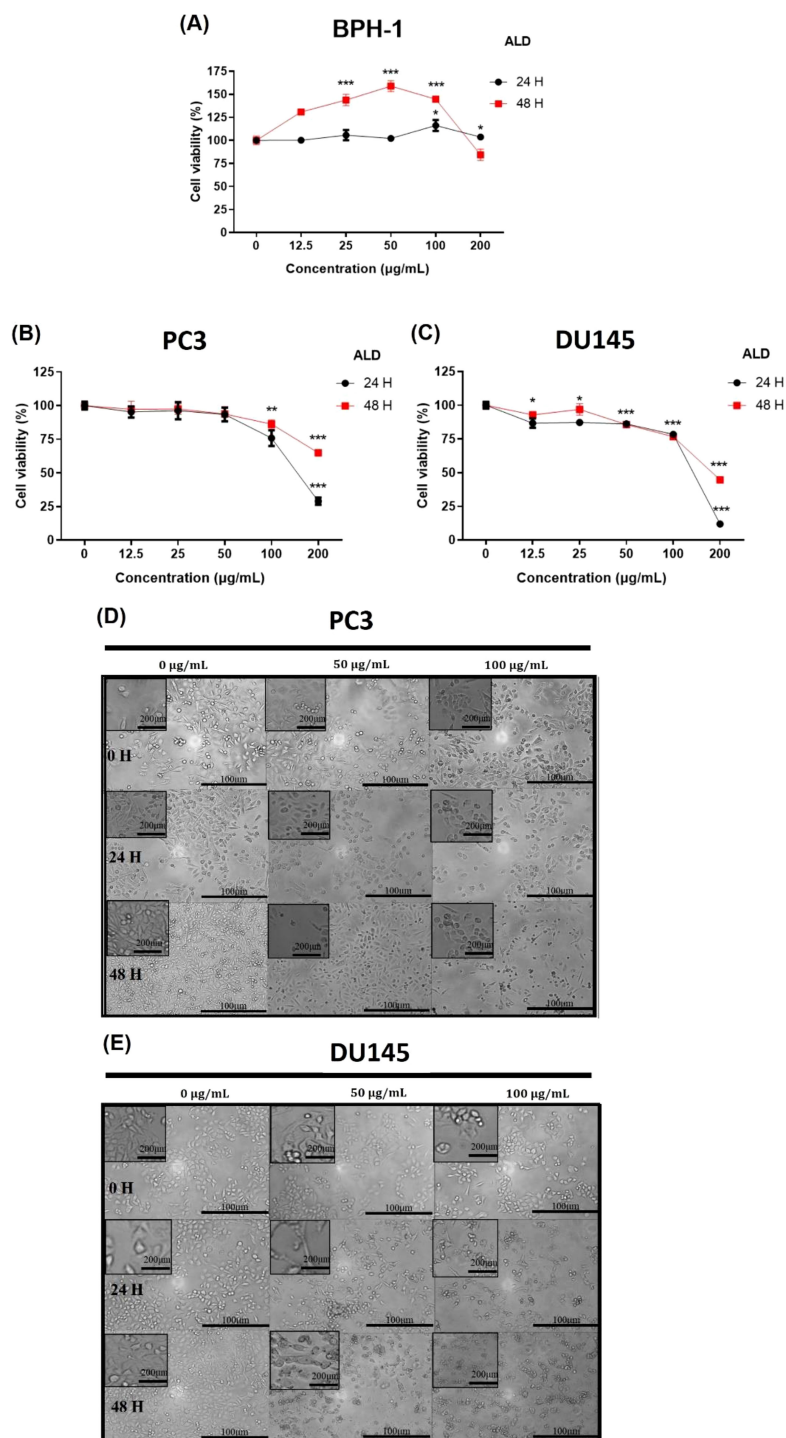


FIGURE 3

Cytotoxic effects of ALD on PC3 and DU145 cell lines. (A–C) BPH-1, PC3, and DU145 cells were treated with ALD at various concentrations (12.5, 25, 50, 100, and 200 μg/mL) for 24 and 48 hours in 96-well plates. Cell viability was assessed using EZ-Cytox, a cell viability assay reagent. Data represent the means of three independent experiments and are shown as Means ± SD; $p^* < 0.05$, $p^{**} < 0.01$, and $p^{***} < 0.001$ compared to control. (D, E) Effect of ALD on the morphology of prostate cancer cells. PC3 and DU145 cells (250,000 cells/well) were seeded into 6-well plates and treated with ALD at concentrations of 50 and 100 μg/mL for 24 and 48 hours. Images were captured using phase-contrast microscopy at 100× magnification. Scale bars = 100 and 200 μm.

concentrations. This was indicated by a reduced red-to-green fluorescence ratio in apoptotic cells (Figures 5A, C). Treatment with FCCP, a known disruptor of mitochondrial function, confirmed that the observed reduction in oxygen consumption

suggests irreversible mitochondrial impairment within certain treatment ranges (29). To further validate the JC-1 assay results, flow cytometry (FACS) analysis was conducted using FCCP as a positive control. The FACS results supported the JC-1 findings,

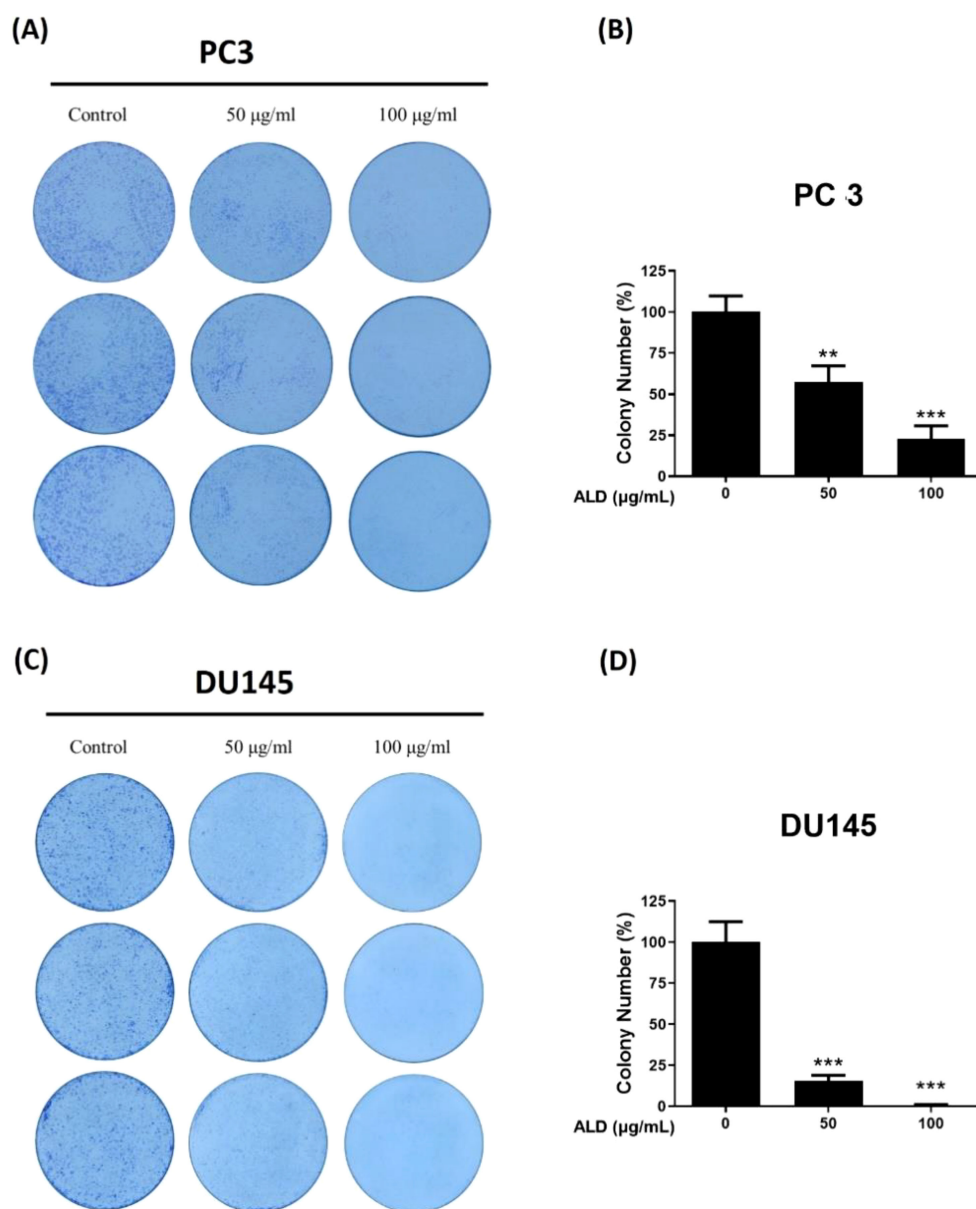


FIGURE 4

ALD inhibits cell proliferation in PC3 and DU145 cells. (A, B) PC3 and (C, D) DU145 cells were seeded at a density of 1000 cells/well in 6-well plates and allowed to adhere for 24 hours. Cells were then treated with 50 and 100 $\mu\text{g/mL}$ of ALD and cultured in fresh medium for 9 days, followed by fixation and staining with crystal violet to visualize colonies. Representative images of colony formation in PC3 cells (left) and quantitative analysis shown as a bar graph (right). Representative images of colony formation in DU145 cells (left) with corresponding quantitative analysis (right). Data are expressed as means $\pm$ SD; $p^{**} < 0.01$, and $p^{***} < 0.001$ compared to control.

showing that ALD treatment significantly reduced the red-to-green fluorescence ratio, indicating disrupted mitochondrial membrane potential. Interestingly, this reduction in the ALD-treated group was comparable to the effect observed with FCCP, the positive control, suggesting that ALD's effect on mitochondrial function is similar to that of FCCP in both PC3 and DU145 cells. This reinforces the potent effect of ALD in altering mitochondrial dynamics, as illustrated in Figures 5B, D. Given that changes in MMP can impact intracellular ion homeostasis, we also measured Ca^{2+} levels in ALD-

treated PC3 and DU145 cells, which revealed a concentration-dependent increase in Ca^{2+} levels in both cell lines (Figures 5C, D). This rise in Ca^{2+} supports the hypothesis that ALD induces apoptosis through mitochondrial destabilization. These findings align with previous studies suggesting that decreased MMP and elevated intracellular Ca^{2+} are essential mechanisms in apoptosis induced by anticancer agents. Future research should further investigate the specific molecular pathways through which ALD exerts these effects and explore its therapeutic potential in cancer treatment.

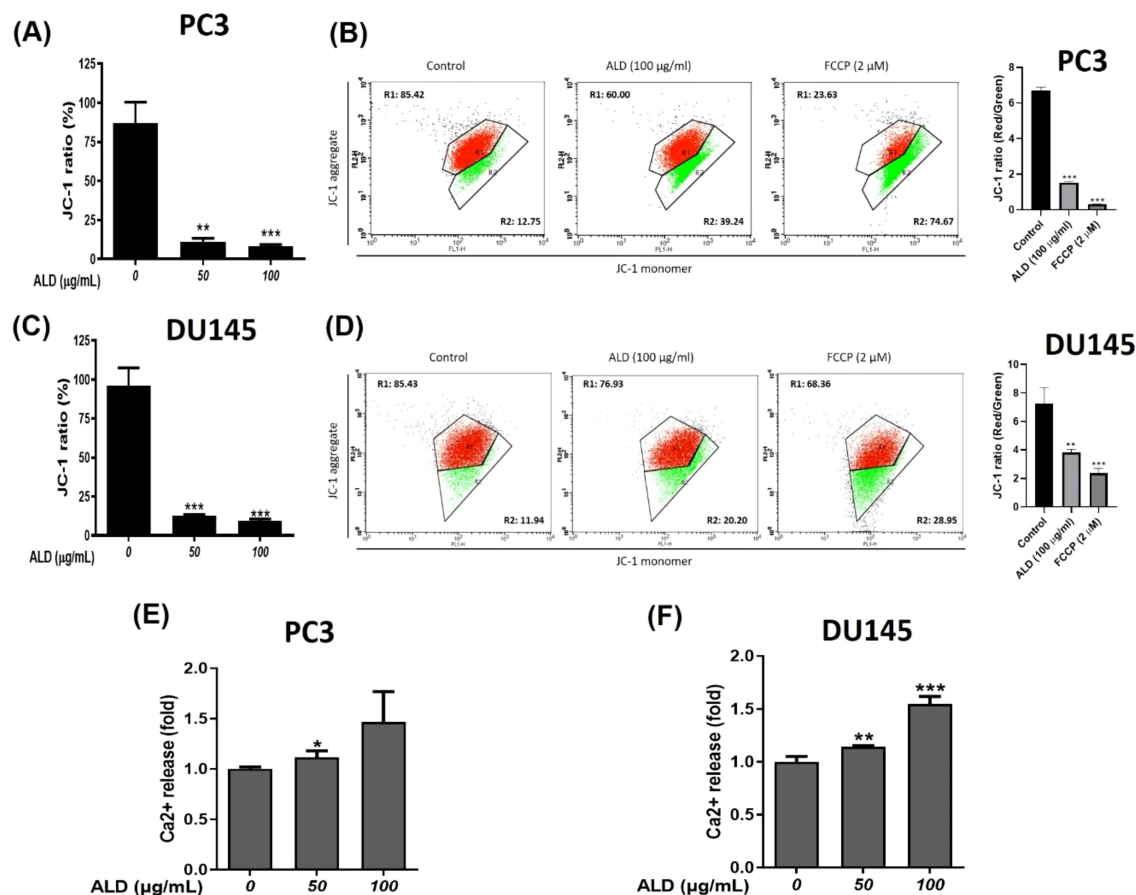


FIGURE 5

ALD-Induced Mitochondrial Dysfunction in PC3 and DU145 Cells. (A–C) Mitochondrial membrane potential in PC3 and DU145 cells was assessed after 4 hours of treatment with ALD at concentrations of 50 and 100 µg/ml. Cells were stained with JC-1 dye, and the fluorescence ratio of JC-1 aggregates (indicative of healthy mitochondria) to monomers (indicative of depolarized mitochondria) was analyzed. Data are presented as mean $\pm$ SD, with $p^{**} < 0.01$ and $p^{***} < 0.001$ compared to the untreated group. (B–D) PC3 and DU145 cells were treated with ALD at a concentration of 100 µg/ml for 24 hours, with FCCP (2 µM) used as a positive control for mitochondrial depolarization. Mitochondrial membrane potential was assessed using JC-1 staining followed by FACS. The red-to-green fluorescence ratio was measured to evaluate changes in membrane potential, with a decrease indicating mitochondrial depolarization. Results are shown as mean $\pm$ SD, with $p^{**} < 0.01$, and $p^{***} < 0.001$ versus the untreated group. (E, F) Cytosolic Ca^{2+} concentration in PC3 and DU145 cells was evaluated following 24 hours of ALD exposure at the same concentrations. Chemogenic reagent labeling was used, and fluorescence was measured with an ELISA reader. Results are shown as mean $\pm$ SD, with $p^{*} < 0.05$, $p^{**} < 0.01$, and $p^{***} < 0.001$ versus the untreated group.

3.6 ALD enhanced reactive oxygen species production and induced apoptosis in prostate cancer cells

Reactive oxygen species (ROS) are known to drive cancer progression by damaging DNA through oxidation, promoting lipid peroxidation, and causing oxidative modifications in proteins, which can lead to structural DNA changes (30). To investigate whether ROS generation and DNA damage are central to ALD's anticancer effects, we conducted DCFDA analysis and TUNEL assays on PC3 and DU145 cells treated with ALD at 50 and 100 µg/mL. ALD treatment led to a significant, dose-dependent increase in ROS production in both cell lines, suggesting that ROS is a critical mediator of ALD-induced apoptosis (Figures 6A, C). Higher ALD doses correlated with greater ROS generation, highlighting the role of oxidative stress (31) in ALD's cytotoxic mechanism against prostate cancer cells. To further explore the role

of specific ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\text{HO}^\bullet$), we used flow cytometry (FACS) to assess the impact of N-acetylcysteine (NAC), a ROS inhibitor, on ROS levels. Cells were treated with H_2O_2 to induce oxidative stress, with a subset also receiving NAC. FACS analysis, which used fluorescent probes to quantify ROS levels based on fluorescence intensity, showed that H_2O_2 treatment significantly elevated ROS levels compared to controls. Notably, ALD treatment induced ROS generation to a similar extent as H_2O_2 , suggesting that ROS plays a critical role in ALD's apoptotic effects. Simultaneous NAC treatment mitigated the ROS increase, demonstrating NAC's protective effect against oxidative stress and reinforcing ROS as a key mediator in ALD-induced apoptosis (Figures 6B, D). The TUNEL assay further supported these findings by revealing DNA fragmentation, a hallmark of apoptosis, in cells treated with ALD. TUNEL staining showed blue fluorescence marking nuclei and green fluorescence indicating fragmented DNA (Figures 6E, G).

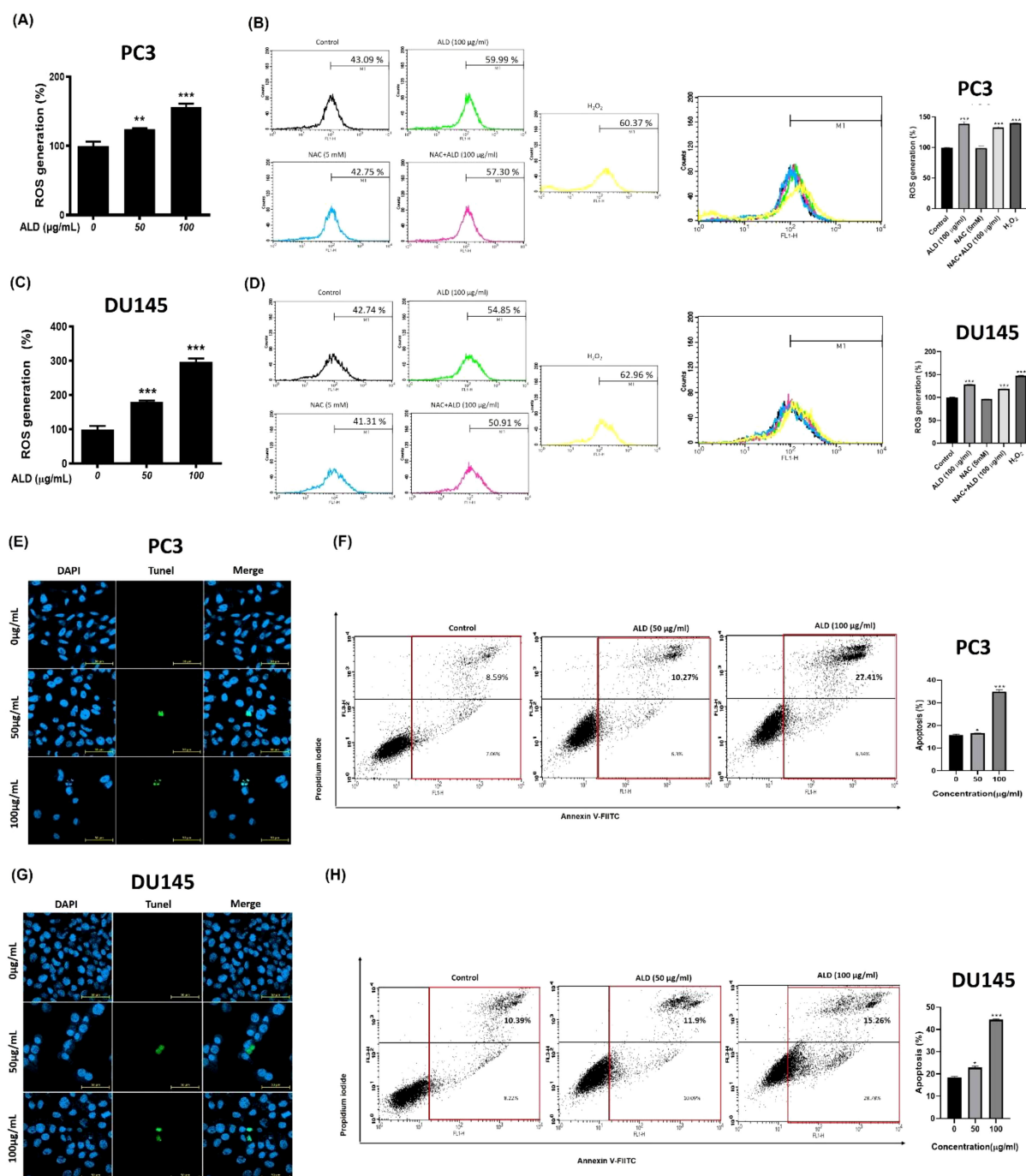


FIGURE 6

ALD increased ROS-mediated apoptosis in prostate cancer cells. (A–C) The cells were treated with ALD (50 and 100 μg/ml) for 4 hours in PC3 and DU145 cells. The reactive oxygen species generation results were analyzed using an oxidation-sensitive fluorescent dye. A 96-well microplate reader was employed to measure the fluorescence. Values represent the means of three experiments. Means ± SD; $p < 0.05$, $p < 0.01$ and $p < 0.001$ between two groups. (B–D) Flow cytometry analysis was conducted to measure ROS levels in PC3 and DU145 cells treated with H_2O_2 and NAC. Cells were exposed to H_2O_2 to induce oxidative stress, while NAC was used to evaluate its protective effects. ROS levels were quantified and compared between treated and control groups. (Right) The quantitative analysis of FACS results is illustrated in the bar graph. Data are presented as mean ± SD, with a significant difference indicated by $p < 0.001$ when compared to the untreated control group. The presence of cell death. (E–G) PC3 and DU145 cells was confirmed using a confocal microscope. The nucleus emits blue fluorescence with a wavelength of 465 nm, while dead cells display fluorescent green images with a wavelength of 520 nm. Scar bar = 50 μm. (F–H) Apoptotic rate of prostate cancer cells was assessed using Annexin V-FITC staining after a 24-hour treatment with 100 μg/mL of ALD. The results indicate the proportion of cells undergoing apoptosis in response to the treatment. (Right) The quantitative analysis of FACS results is illustrated in the bar graph. Data are presented as mean ± SD, with a significant difference indicated by $p < 0.05$, and $p < 0.001$ when compared to the untreated control group.

There was a positive correlation between ALD concentration and DNA fragmentation, confirming ALD's role in inducing apoptosis through ROS-mediated DNA damage. Additionally, Annexin V/FITC assays demonstrated that ALD-induced apoptosis involves DNA damage, providing further evidence that ALD effectively triggers apoptosis in prostate cancer cells (Figures 6F, H).

3.7 ALD promoted mitochondrial-driven apoptosis in prostate cancer cells

Cytochrome c, a mitochondrial protein, plays a crucial role in initiating apoptosis by activating caspases once released into the cytosol, thereby triggering a cascade that promotes programmed cell death (32). Elevated cytosolic cytochrome c levels indicate mitochondrial outer membrane permeabilization (MOMP), a pivotal early event in the apoptotic pathway (33). In addition, ATF4, a transcription factor responsive to cellular stress (34), especially the unfolded protein response (UPR) in the endoplasmic reticulum—signals increased stress levels and regulates genes associated with apoptosis (35). The cleavage of pro-PARP into c-PARP is another hallmark of apoptosis, as reduced levels of intact PARP confirm active apoptotic processes (36). Anti-apoptotic proteins like Bcl-2 prevent cytochrome c release by stabilizing the mitochondrial membrane (37), while survivin, an inhibitor of apoptosis protein (IAP), further supports cell survival by counteracting apoptotic signals (38). To confirm ALD's role in promoting apoptosis and inhibiting cell growth, we treated PC3 and DU145 prostate cancer cells with increasing concentrations of

ALD. Results demonstrated that ALD (50 and 100 $\mu\text{g/mL}$) significantly increased the levels of c-PARP, cytochrome c, and ATF4, while concurrently reducing the expression of pro-PARP, Bcl-2, and survivin in a dose-dependent manner, as shown in Figures 7A, B. This dose-dependent modulation of apoptosis-related proteins underscores ALD's mechanism of action, enhancing pro-apoptotic signals while suppressing anti-apoptotic factors. Together, these findings suggest that ALD effectively induces apoptosis in prostate cancer cells by shifting the cellular balance toward cell death through an increase in pro-apoptotic proteins and a decrease in survival-promoting factors.

3.8 ALD suppressed EMT via modulation of TGF- β signaling in prostate cancer cells

Androgens play a crucial role in promoting prostate cancer growth, with transforming growth factor-beta (TGF- β) known to synergize with androgens to facilitate the epithelial-to-mesenchymal transition (EMT) in prostate cancer cells (39). Furthermore, recent studies have highlighted DNMT1 as a key enhancer of tumor progression by promoting EMT (40). This transition is marked by a shift in cadherin expression: a decrease in E-cadherin (associated with cell adhesion) and an increase in N-cadherin (associated with motility), which collectively drive tumor invasion and metastasis (41). In this study, we investigated the effects of ALD on EMT-related proteins and cell migration in prostate cancer cells using western blot analysis (Figures 8A, B). ALD treatment led to a significant downregulation of TGF- β , N-

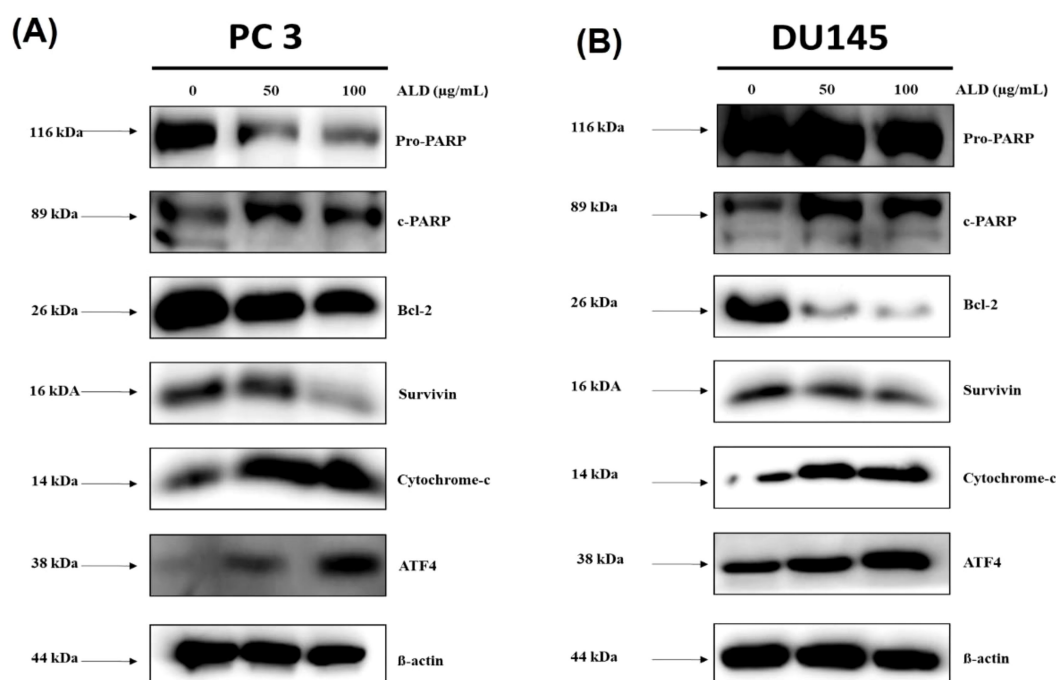


FIGURE 7

ALD enhances apoptosis pathway activation in PC3 and DU145 cells. (A, B) After a 24-hour treatment with specified doses of ALD, apoptosis-related protein expression was analyzed by western blotting in PC3 and DU145 cells. Probed proteins included pro-PARP, cleaved PARP (c-PARP), Bcl-2, survivin, cytochrome c, ATF4, and β -actin as a loading control, demonstrating ALD's effect on apoptotic signaling pathways.

cadherin, and DNMT1 levels, while upregulating E-cadherin expression in both PC3 and DU145 cells, suggesting a reversal of the EMT phenotype. Additionally, cell migration assays demonstrated that ALD treatment at concentrations of 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ significantly inhibited migration in PC3 and DU145

cells, respectively (Figures 8C, D). Western blotting results further confirmed this effect, showing a marked increase in E-cadherin alongside significant reductions in N-cadherin, Snail, and TGF- β levels in both cell lines (Figures 8A, B). These findings indicate that ALD effectively inhibits the EMT process, potentially reducing the

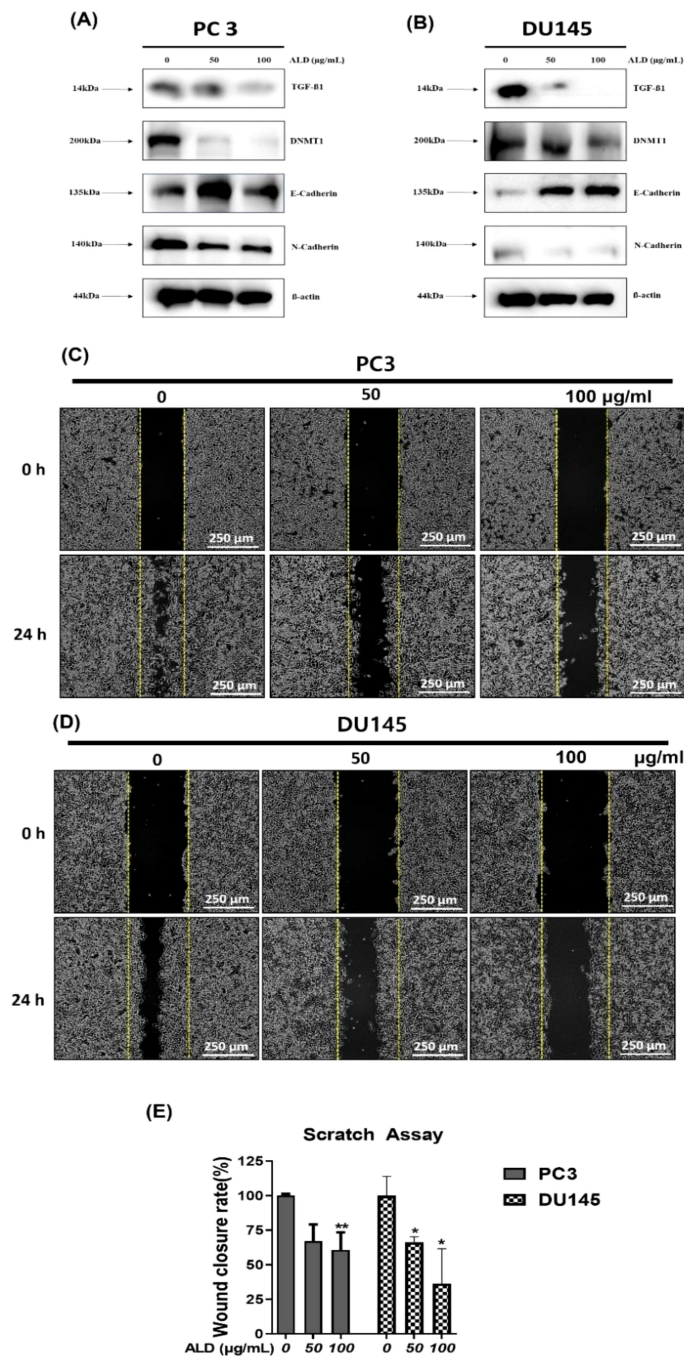


FIGURE 8
ALD inhibits EMT and cell migration in prostate cancer cells. (A, B) PC3 and DU145 cells were treated with ALD (50 and 100 $\mu\text{g/mL}$) or left untreated for 24 hours. Western blot analysis was performed to assess the expression of EMT-related proteins, including TGF- β , DNMT-1, E-cadherin, N-cadherin, with β -actin as the loading control. (C, D) Migration assay in PC3 and DU145 cells was conducted in 6-well plates to evaluate ALD's effects on cell migratory behavior. (E) Quantitative analysis of cell migration is presented in the bar graph. Data are expressed as mean $\pm$ SD; $p < 0.05$, and $p^{**} < 0.01$ compared to the untreated control.

metastatic capacity of prostate cancer cells. By modulating key EMT markers and impairing cell migration, ALD demonstrates significant potential as a therapeutic agent against metastatic prostate cancer.

4 Discussion

This study provides robust evidence that ALD, a widely used herb in traditional Chinese medicine, has significant therapeutic potential for prostate cancer. Through pharmacoinformatics, we examined ALD's anticancer effects in PC3 and DU145 cells and found that ALD disrupts mitochondrial function, promotes ROS generation, and induces apoptosis. These actions lead to the inhibition of cell migration and colony formation, underscoring ALD's multifaceted cytotoxic impact, including apoptosis induction, growth inhibition, and mitochondrial dysfunction. The bioactive components of ALD, such as Atractylenolide II and III (42), demonstrate strong molecular interactions with the androgen receptor (AR) as shown by molecular docking studies (43). Since AR signaling plays a crucial role in prostate cancer progression, these interactions suggest that ALD may effectively disrupt AR-driven pathways, thereby inhibiting PC3 and DU145 cell proliferation and survival (44). By simultaneously triggering ROS-mediated apoptosis and impairing cellular processes essential for metastasis (45, 46), ALD emerges as a potential multi-targeted therapeutic candidate for prostate cancer treatment. Our findings also indicate that the combined binding strength of Atractylenolide II and III may approximate or even surpass that of ENZ, highlighting the therapeutic advantage of herbal formulations like ALD, where multiple active compounds work synergistically to achieve a more stable and comprehensive interaction with target receptors. This multi-component synergy, typical of herbal medicines, may provide broader efficacy compared to single-agent therapies (Table 1, Figure 2). The examination revealed that ALD exerts a significant influence on the survival of prostate cancer (PCa) cells, particularly within the PC3 and DU145 cell lines (47), as evidenced by the results obtained from the EZ-Cytotoxic kit assays. Notably, these assays demonstrated that the cytotoxic effects of ALD are both time- and dose-dependent, indicating that prolonged exposure and higher concentrations of ALD correlate with increased inhibition of cancer cell proliferation. This observed cytotoxicity underscores ALD's capacity to directly impede the growth of cancer cells while sparing normal cells, thereby suggesting a cancer-specific effect of ALD. Further validation of the decrease in cell viability was achieved through colony formation assays, which demonstrated a marked reduction in the ability of PCa cells to form colonies following ALD treatment. This reduction in colony formation not only indicates the elimination of cancer cells but also highlights ALD's ability to hinder their capacity for proliferation and the establishment of new tumor colonies. These findings underscore the potential of ALD as a promising anti-cancer agent. Overall, the results are compelling, suggesting that ALD may not only eradicate existing cancer cells but also play a crucial role in preventing the formation of new tumors, thereby positioning it as a

valuable candidate for further investigation in cancer therapeutics (Figures 3, 4).

Mechanism of apoptosis and ROS generation

One of the significant discoveries in this study is ALD's ability to induce apoptosis in PCa cells through mitochondrial dysfunction and ROS generation. ALD's impact on mitochondrial membrane potential (MMP) suggests an intrinsic pathway of apoptosis, where mitochondrial instability leads to the release of pro-apoptotic factors like cytochrome C (48). This finding aligns with current research suggesting that MMP disruption and elevated Ca^{2+} levels are critical factors in apoptosis induction by anticancer agents (49). The JC-1 assay results confirmed ALD-induced MMP depolarization, and the increase in intracellular Ca^{2+} further supports its role in apoptosis initiation (Figure 5). Additionally, ALD-induced ROS elevation in PCa cells reinforces its pro-apoptotic activity. ROS, highly reactive molecules that cause oxidative stress, have been linked to cell death in various cancer studies. The observed increase in ROS production suggests that oxidative stress is a key mechanism underlying ALD's cytotoxicity (Figure 6). The involvement of ROS in facilitating apoptosis has been extensively studied, and the elevation of ROS production by ALD provides additional evidence for its pro-apoptotic properties (50). Our study further demonstrated that ALD treatment results in the release of cytochrome C, a critical step in mitochondrial-mediated apoptosis, which triggers downstream apoptotic pathways. The increased ATF4 levels in ALD-treated cells suggest endoplasmic reticulum stress and support the involvement of mitochondrial pathways in apoptosis (Figure 7).

Suppression of EMT and potential anti-metastatic effects

The epithelial-to-mesenchymal transition (EMT) is crucial in cancer metastasis, where epithelial cells transform, gaining motility and invasive potential (51, 52). Our findings indicate that ALD suppresses EMT in PC3 and DU145 cells by modulating the TGF- β signaling pathway (Figure 8). ALD treatment significantly downregulated TGF- β and N-cadherin while upregulating E-cadherin, signaling a reversal of EMT (53) and suggesting reduced metastatic potential. TGF- β inhibition by ALD indicates that it may hinder metastasis, as TGF- β is a known regulator of EMT (54). Future studies are warranted to clarify ALD's interactions with TGF- β pathway components and the molecular basis of its anti-EMT effects.

Therapeutic implications and multi-targeted approach

The results of this study indicate that ALD has the potential to act as a multi-targeted treatment for prostate cancer by inducing programmed

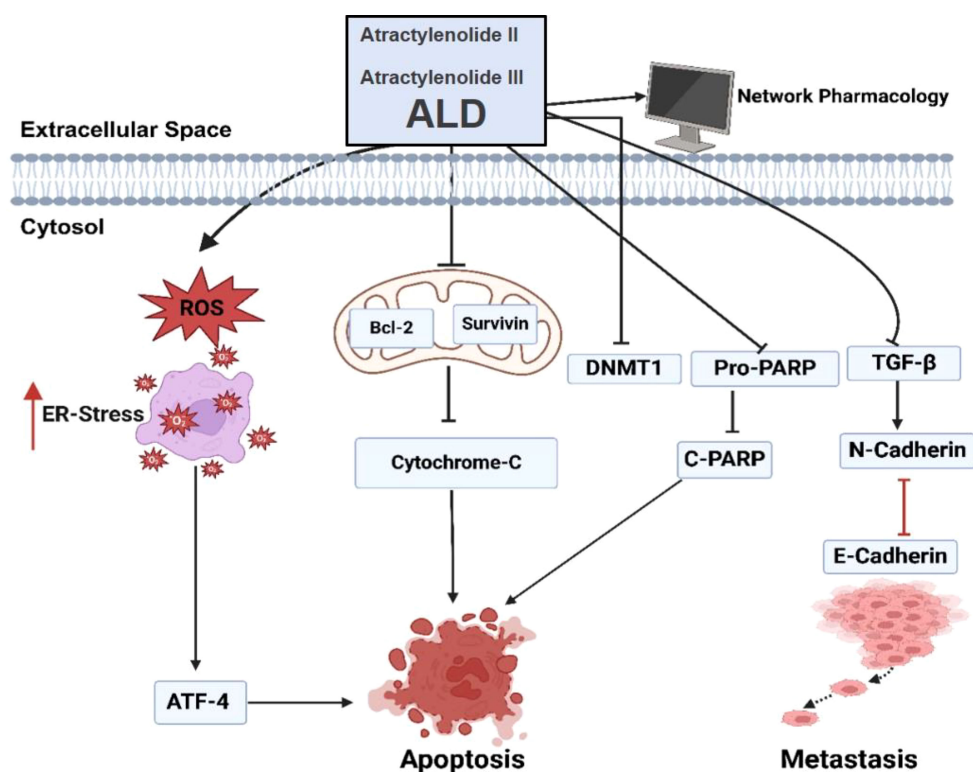


FIGURE 9

ALD-induced apoptosis and inhibition of EMT in PC3 and DU145 cells. Treatment with ALD (containing Atractylenolide II and Atractylenolide III) reduces mitochondrial membrane potential, leading to cytochrome c release and activation of apoptosis markers, including cleaved PARP, ATF4, and E-cadherin. Concurrently, ALD suppresses levels of pro-survival proteins (pro-PARP, survivin, and Bcl-2) and EMT-related proteins (DNMT1 and N-cadherin). These molecular changes suggest that ALD inhibits EMT progression, thereby promoting apoptosis in prostate cancer cells.

cell death, increasing ROS, altering mitochondrial function, and inhibiting EMT. Such a multi-targeted approach is highly advantageous in cancer therapy as it reduces the likelihood of cancer cells developing resistance. Moreover, being a natural compound, ALD may offer lower toxicity and fewer side effects than conventional chemotherapies, potentially providing a safer alternative.

Limitations and future directions

While this study provides strong evidence of ALD's anticancer potential, further research, including *in vivo* xenograft experiments and testing on primary human prostate cancer cells, is essential to strengthen the evidence of ALD's efficacy in cancer treatment. Such models would help validate these findings in a more physiological context and are crucial for translating these results into clinical applications. Future studies should investigate the detailed molecular pathways through which ALD affects prostate cancer cells, focusing on the complex mechanisms of mitochondrial dysfunction and ROS generation. Additionally, conducting clinical trials alongside animal studies, particularly xenograft models, would provide a more comprehensive understanding of

ALD's efficacy and safety. Exploring the potential synergy of ALD with other anticancer agents could also lead to innovative combination therapies. Further research should assess the long-term effects and potential side effects of ALD treatment, ensuring its safety and effectiveness for therapeutic use. Collectively, this study highlights ALD's promising role as a multi-targeted therapeutic agent against prostate cancer, laying a solid foundation for future research into its clinical applications.

Conclusion

In summary, this study highlights the significant anticancer properties of *Atractylodes lancea* DC. (ALD) in targeting prostate cancer. ALD exhibits cytotoxic effects, induces apoptosis through ROS generation and mitochondrial disruption, and inhibits epithelial-to-mesenchymal transition (EMT) by modulating TGF- β signaling pathways (Figure 9). These findings suggest that ALD could be a promising therapeutic agent, offering a multifaceted approach to combat prostate cancer. However, further research, including clinical trials, is essential to validate and expand upon these therapeutic effects in clinical settings.

Data availability statement

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

Author contributions

KA: Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. JM: Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing. MAR: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. MF: Data curation, Visualization, Writing – review & editing. MC: Data curation, Visualization, Writing – review & editing. MR: Visualization, Writing – review & editing. JC: Formal analysis, Visualization, Writing – review & editing. KK: Visualization, Writing – review & editing. SM: Visualization, Writing – review & editing. MD: Visualization, Writing – review & editing. RS: Visualization, Writing – review & editing. MP: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. S-WS: Visualization, Writing – review & editing. AH: Visualization, Writing – review & editing. BK: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Validation, Visualization, Writing – review & editing. H-SY: Project administration, Supervision, Validation, Visualization, Writing – review & editing. CA: Writing – review & editing.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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A comprehensive review of Schisandrin B's preclinical antitumor activity and mechanistic insights from network pharmacology

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As an active constituent in the extract of dried fruits of *Schisandra chinensis*, Schisandrin B exhibits diverse pharmacological effects, including liver protection, anti-inflammatory and anti-oxidant. Numerous studies have demonstrated that Schisandrin B exhibits significant antitumor activity against various malignant tumors in preclinical studies, which is achieved by inhibiting cell proliferation and metastasis and promoting apoptosis. As a potential antitumor agent, Schisandrin B holds broad application prospects. This review systematically elaborates on the antitumor effect of Schisandrin B and the related molecular mechanism, and preliminarily predicts its antitumor targets by network pharmacology, thereby pave the way for further research, development, and clinical application.

KEYWORDS

Schisandra chinensis, Schisandrin B, antitumor, molecular mechanisms, network pharmacology

1 Introduction

In recent years, the incidence and mortality of malignant tumors on a global scale continue to ascend without interruption. In 2022, there were approximately 20 million newly diagnosed cancer cases worldwide. According to demographic projections, by 2050, the number of new cancer cases will attain 35 million (Bray et al., 2024). Consequently, it is of utmost importance to identify effective treatment plans and discover new therapeutic targets for malignant tumors. Natural drugs boast abundant sources, diverse structures, and extensive activities. On this basis, the development of new antitumor drugs and prodrugs with superior efficacy has become a hot field for numerous tumor scholars at present (Ma L. et al., 2021). Among them, small molecule drugs originating from the active ingredients of natural drugs, which are diverse in variety and possess the characteristics of multiple targets, multiple signaling pathways, and extensive pharmacological activities, have obtained remarkable achievements in both antitumor drug screening and mechanism research, thereby providing new thoughts for discovering new tumor treatment strategies (Basmadjian et al., 2014; Chopra and Dhingra, 2021).

Schisandra chinensis (called bei-wuweizi in Chinese, *S. chinensis*) is a plant of the *Magnoliaceae* family. Its dried fruits are traditional Chinese medicines (Yang K. et al., 2022). Schisandrin B (Sch B) is the most notable dibenzocyclooctene-type lignan in *S. chinensis* (Nasser et al., 2020). At present, most of the research on Sch B focuses on its effects in protecting the liver (Hong et al., 2017; Słuzalec et al., 2024), treating cardiovascular and cerebrovascular diseases (Chen et al., 2013; Duan et al., 2021), anti-inflammation (Ran et al., 2018; Ma Z. et al., 2021), anti-oxidation (Kopustinskiene and Bernatoniene, 2021). In addition, certain preclinical studies have indicated that Sch B possesses antitumor potential in liver cancer (Song et al., 2023; Yang and Wu, 2023), colorectal cancer (Pu et al., 2021; Co et al., 2024), breast cancer (Fei et al., 2020; Li W. et al., 2024), gastric cancer (He et al., 2022) and other cancer types through promoting tumor cell apoptosis, inhibiting tumor cell proliferation, invasion and migration. In this review, we introduce the physicochemical properties and pharmacokinetics of Sch B, systematically summarize its antitumor effects and mechanisms in different tumors through preclinical studies, analyze and predict the main targets and signaling pathways of Sch B in antitumor through network pharmacology, with the objective of providing references for its further research, development and clinical application.

2 Methods

In August 2024, the author carried out an extensive search in the PubMed database for relevant literature published from January 2001 to August 2024. By using a collection of keywords such as “*Schisandra chinensis*”, “Schisandrin B”, “Sch B”, “antitumor”, “cancer”, “molecular mechanisms” and “network pharmacology”, 806 articles were retrieved in total. When it came to the literature screening stage, strict inclusion criteria were carefully established. The literature to be incorporated was required to meet multiple conditions. In the section of “Sch B Physicochemical and Pharmacokinetic Properties”, the literature should show a high relevance to Schisandrin B and have a high citation rate. For other literature, a strong link to the antitumor effect of Schisandrin B was necessary. Among comparable literature, those with a relatively high impact factor and a high standing in the journal ranking were preferred. Additionally, recently published works were given priority. After this rigorous selection procedure, 74 articles were finally cited in this paper.

3 Schisandrin B: physicochemical and pharmacokinetic properties

Sch B ($C_{23}H_{28}O_6$) manifests as white flaky crystals. It has a molecular weight of 400.46 and its melting point ranges from 120°C to 121°C. Sch B is insoluble in water but soluble in organic solvents such as ethanol and dimethyl sulfoxide (DMSO). Studies have indicated that following oral administration in rats, Sch B may possess enterohepatic circulation, predominantly accumulate in the liver, and it is mainly excreted through the kidneys in the form of metabolites. The pharmacokinetics of Sch

B is related to gender, with the absolute oral bioavailability of female mice being significantly higher than that of male mice. Sch B, which is widely distributed in the ovary and adipose tissue, shows linear pharmacokinetic characteristics within a certain concentration range (10 mg/kg, 20 mg/kg and 40 mg/kg) (Zhu et al., 2013; Wang et al., 2018). Shao et al. demonstrated in a myocardial infarction mouse model study that the utilization of matrix metalloproteinase-sensitive peptide-modified PEGylated lipid nanoparticles for carrying Sch B can enhance its solubility and bioavailability (Shao et al., 2017). A study has confirmed that Sch B exerts protective effects on various normal cells including liver parenchymal cells, cardiomyocytes, neurons, renal cells, and umbilical vein endothelial cells. Different concentrations of Sch B (1.67 µg/mL, 5 µg/mL, 15 µg/mL) show no cytotoxicity to nerve cells M146L. Moreover, Sch B can significantly increase the viability of human proximal tubular epithelial cells (HK-2) at 2.5 µM, 5 µM and 10 µM (Zhu et al., 2012). In animal experiments, low, medium and high concentrations of Sch B (20 mg/kg, 40 mg/kg, 80 mg/kg) were respectively administered intragastrically to rat models of myocardial ischemia-reperfusion injury. The apoptosis of myocardial cells was inhibited and myocardial ischemia-reperfusion injury in rats was improved (Zhang et al., 2017). These findings are sufficient to indicate that Sch B is characterized by low toxicity and high safety.

4 Role of Schisandrin B in different tumors

In the investigations on the antitumor efficacy of Sch B, researchers have determined that Sch B predominantly exerts its tumor-suppressing function via mechanisms including inhibiting the proliferation, invasion, and migration of tumor cell lines, promoting the apoptosis of tumor cells, and suppressing tumor angiogenesis (Table 1).

4.1 Schisandrin B in liver cancer

Malignant liver tumors are among the most prevalent malignant tumors globally. The oncogenesis is typically accompanied by a dismal prognosis and a low overall survival rate. Among them, hepatocellular carcinoma (HCC) constitutes 80%–90% of primary liver cancer. It is the fourth leading cause of cancer-related deaths worldwide (Li X. et al., 2021). Multiple studies have demonstrated that Sch B can inhibit the progression of liver cancer cell lines in various ways. Inducing apoptosis of tumor cells is an important approach in tumor treatment. Researchers have found that the RhoA/ROCK1 pathway is involved in regulating the proliferation and migration of HCC cells (Lin et al., 2018; Zhang et al., 2020). Sch B promotes the apoptosis of Huh-7 cells and restrains the proliferation, migration and invasion capabilities of Huh-7 cells via the RhoA/ROCK1 pathway (Yang and Wu, 2023). Additionally, Sch B can downregulate heat shock protein 70 (hsp70) and upregulate the expression of Caspase-3 to induce apoptosis of human hepatoma cells (SMMC-7721) (Wu et al., 2004). Pyroptosis refers to inflammatory programmed cell death

TABLE 1 Antitumoral effects and molecular targets of Sch B in various cancers.

Tumor type	Cell line	Target	Effect	References
Liver cancer	Huh-7 SMMC-7721 HepG2 Hepa1-6	RhoA, ROCK1, hsp70↓ Caspase-3, ROS, Th1/Th2↑	Proliferation Migration Apoptosis Pyroptosis Autophagy	Wu et al. (2004), Tan et al. (2022), Song et al. (2023), Yang and Wu (2023)
Colorectal cancer	HCT116 HT29 SW620 Caco2	Bcl-2, SIRT1↓ CHOP, Bax/Bcl-2, Caspase-3, SMURF2, p-FAK↑	Apoptosis Proliferation Cell Cycle Migration	Li et al. (2019), Pu et al. (2021), Sana-Eldine et al. (2023), Co et al. (2024), Li et al. (2024b)
Breast cancer	MS MDA-MB-435S 4T1, MCF-10A MDA-MB-231 BT-549 MDA-MB-468	P-glycoprotein, VEGF MMP-9, VIM, NOX4, TGF-β1, NLRP3, IL-1β, p-STAT3↓ E-cad↑	Multidrug resistance Migration Invasion Apoptosis Proliferation	Liu et al. (2012), Zhang et al. (2013), Dai et al. (2018), Fei et al. (2020), Jing et al. (2020), Chang et al. (2024), Li et al. (2024b)
Gastric cancer	SGC-7901 BGC-823	p-STAT3, VEGF, E-Cad HIF-1a, PI3K, MMP-2, FAK↓	Proliferation Migration Invasion Autophagy Apoptosis	Li et al. (2021a), He et al. (2022)
Glioma	U251, U87 SHG-44	p-Akt, p-mTOR, MMP-9 HOTAIR, Bcl-2, CDK4↓ miR-125a-5p, Caspase-3↑	Migration Invasion Apoptosis	Jiang et al. (2015), Li et al. (2015), Qi et al. (2016), Jiang et al. (2017)
Gallbladder cancer	GBC-SD NOZ	Bcl-2, NF-κB Cyclin D1, CDK-4↓	Apoptosis Proliferation Cell Cycle	Xiang et al. (2014)
Non-small cell lung cancer	A549	VIM, VEGF, Cyclin D1, CDK-4↓ E-cad, p53↑	Migration Invasion Cell cycle	Lv et al. (2015), Cai et al. (2021)
Ovarian cancer	SK-OV-3	MMP-2, MMP-9↓	Angiogenesis VM Invasion Metastasis	Zhang et al. (2021)
Osteosarcoma	143B MG63 SaOS2 U2OS	Wnt/β-catenin, PI3K/Akt↓ miRgg708-5p↑	Proliferation Metastasis	Wang et al. (2020), Wang et al. (2022)
Prostate cancer	DU145 LNCaP	p-PI3K/AKT p-STA3/JAK2↓	Cell cycle Apoptosis	Nasser et al. (2019)
Melanoma	B16F10	Wnt/β-catenin↓	Cell cycle Migration Invasion	Chen et al. (2023)

(Cookson and Brennan, 2001). The apoptosis of HepG2 cells induced by Sch B transforms to pyroptosis in the presence of NK cells, which also indicates that Sch B has the potential to be a combined partner for immunotherapy against HCC (Song et al., 2023). Autophagy is a regulatory mechanism that eliminates unnecessary or dysfunctional cellular components and recycles metabolic waste (Xia et al., 2021). At the early stage of tumors, autophagy is capable of inhibiting tumorigenesis. The research conducted by Siran Tan et al. indicated that Sch B led to an excessive accumulation of reactive oxygen species (ROS), thereby inducing autophagy and reducing the activity of Hepa1-6 (Tan et al., 2022).

4.2 Schisandrin B in colorectal cancer

Colorectal cancer, ranking as the fourth most prevalent cancer globally, was seldom diagnosed several decades ago. According to statistics, nearly 900,000 people succumb to colorectal cancer annually (Zhou et al., 2021). In the early stage of colorectal cancer, patients typically exhibit no symptoms. When patients present with symptoms such as anemia, stomach discomfort, or rectal bleeding, it often indicates that colorectal cancer has advanced to an advanced stage and transformed into an aggressive, malignant, and metastatic tumor. The advancements in the pathophysiology of colorectal cancer have provided a variety of treatment options for it,

including targeted therapy (Kasprzak, 2023). Vanessa Anna Co et al. discovered that Sch B had induced apoptosis both *in vivo* and *in vitro* by interacting with C/EBP homologous protein (CHOP) and up-regulating its expression level. Additionally, it was verified that Sch B inhibits cell proliferation and tumor growth (Co et al., 2024). Sch B also indirectly regulates Smad ubiquitination regulatory factor 2 (SMURF2) by suppressing the protein expression of Sirtuin 1 (SIRT1) and restrains the growth and metastasis of colon cancer cells (Pu et al., 2021). Preclinical studies have indicated that colitis-associated colorectal cancer (CAC) is closely associated with ulcerative colitis (UC) (Zhang et al., 2023). Sch B can activate focal adhesion kinase (FAK) to enhance the protective effect of the intestinal epithelial barrier. Moreover, Sch B can affect the intestinal flora, which can effectively prevent UC and thereby achieve the purpose of preventing CAC (Li et al., 2019). In addition to having the potential to become a targeted therapeutic drug for colorectal cancer, Sch B is also promising in combination therapy. It is well known that the overexpression of epidermal growth factor (EGFR) and its related pathways are the key regulatory factors of colorectal cancer (Koustas et al., 2017). Panitumumab, a monoclonal antibody targeting EGFR, is utilized in the treatment of colorectal cancer. The combination of Sch B and panitumumab can induce apoptosis of colorectal cancer cell lines by activating caspase-3 and down-regulating B-cell lymphoma-2 (Bcl-2), and its cytotoxic effect is more remarkable than that of panitumumab when used alone (Sana-Eldine et al., 2023).

4.3 Schisandrin B in breast cancer

Breast cancer, a malignant disease originating in the cells of the breast, is currently one of the leading causes of cancer-related deaths among women worldwide (Akram et al., 2017). Chemotherapy is one of the most commonly employed methods for treating breast cancer. However, breast cancer stem cells exhibit multiple drug resistance to chemotherapy drugs. Weinan Li et al. fabricated pH-sensitive nanoparticles (Sch B/AP NPs) to reverse the multidrug resistance of breast cancer stem cells, presenting a novel approach for the treatment of breast cancer (Li W. et al., 2024). To address the problem of poor targeting efficacy of traditional chemotherapy drugs, Ming Jing et al. developed a PFV-modified epirubicin and Sch B liposome which showed a remarkable anti-breast cancer effect (Jing et al., 2020). There is also a type of F127-modified lipid-polymer hybrid nanoparticles carrying Sch B (Sch B-F-LPNs), which can significantly reduce the number of metastatic lung nodules and inhibit the lung metastasis of breast cancer to a certain extent (Fei et al., 2020). Furthermore, the mechanism through which Sch B inhibits the progression of breast cancer is of great concern. Research results indicated that Sch B significantly inhibited the lung and bone metastasis of 4T1 mouse breast cancer cells by inhibiting TGF- β -induced epithelial-mesenchymal transition (EMT), suggesting the targeted effect of Sch B on breast cancer metastasis (Liu et al., 2012; Zhang et al., 2013). Sch B hinders the progression of triple-negative breast cancer (TNBC) by suppressing the production of interleukin-1 β (IL-1 β) induced by nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) (Chang et al., 2024). Sch B can also restrain the phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3), induce cell cycle

arrest and apoptosis, thereby inhibiting the growth, migration and clone formation of TNBC cell cells and exerting its anti-breast cancer effect through preclinical studies (Dai et al., 2018).

4.4 Schisandrin B in gastric cancer

Gastric cancer (GC) is one of the most prevalent malignant tumors in the digestive tract system. Due to the characteristics of easy metastasis, high recurrence rate, and chemotherapy resistance of advanced GC, the survival rate of patients with advanced GC is low (He et al., 2020). Therefore, it is of utmost importance to investigate the pathogenesis of GC and identify effective treatment regimens. Previous research has demonstrated the role of traditional Chinese medicine in the treatment of GC (Hung et al., 2017; Liu et al., 2017). Peng Yang et al. analyzed the functional gene network in the occurrence of gastric cancer through bioinformatics, screened target genes, and thus discovered that four potential active components of traditional Chinese medicine, including Sch B, were expected to become therapeutic drugs for GC (Yang P. et al., 2022). There are conclusive research findings suggesting that Sch B is capable of suppressing the proliferation, migration and invasion of gastric cancer cells both *in vitro* and *in vivo*, inducing autophagy of gastric cancer cells by restraining the phosphorylation of STAT3; and it can enhance the therapeutic efficacy of the chemotherapy drug 5-fluorouracil (5-FU) (He et al., 2022). Furthermore, certain researchers have successfully fabricated R8 (RRRRRRRR)-modified vinpocetine and Sch B liposomes. This targeted liposome is capable of inducing apoptosis in BGC-823 cells, inhibiting the metastasis of tumor cells, and strengthening the targeting effect on tumor cells, demonstrating remarkable antitumor efficacy both *in vitro* and *in vivo* (Li X.-Y. et al., 2021).

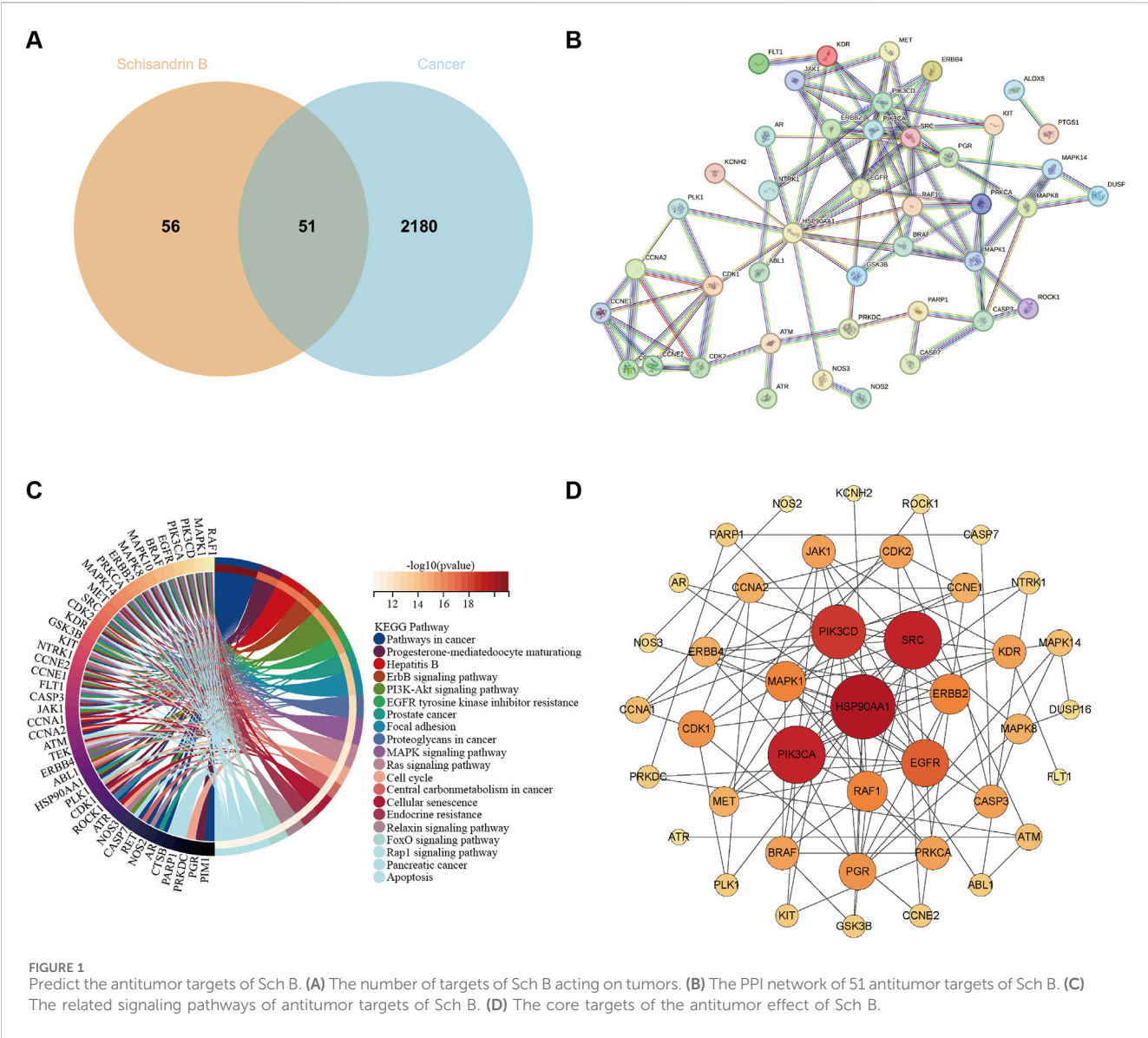
4.5 Schisandrin B in glioma

Glioma represents a group of heterogeneous brain tumors, accounting for 81% of malignant tumors within the central nervous system (Xu et al., 2020). Despite the development of numerous treatment approaches for glioma to date, the presence of the blood-brain barrier impedes the entry of most antitumor drugs into the brain, resulting in a scarcity of drugs currently used for the treatment of glioma (Ballabh et al., 2004; Oberoi et al., 2016). Research has shown that Sch B can inhibit the migration and invasion of glioma cells by suppressing the expression of p-Akt, p-mTOR, and MMP-9 in the PI3K/Akt-mTOR-MMP-9 signaling pathway (Jiang et al., 2015). Additionally, Sch B regulates the HOTAIR-miR-125a-mTOR pathway by reducing hox transcript antisense intergenic RNA (HOTAIR) expression and increasing miR-125a-5p expression to exert its function in suppressing the migration and invasion of glioma cell lines (Jiang et al., 2017). Moreover, Sch B induces cell cycle arrest of glioma cells in the G0/G1 phase, triggers apoptosis through the activation of caspase-3, caspase-9, poly (ADP-ribose) polymerase (PARP), and Bcl-2, and significantly inhibits the tumor growth of glioma cells *in vivo* (Li et al., 2015; Qi et al., 2016). Evidently, Sch B holds the potential for the treatment of glioma.

4.6 Schisandrin B in other tumors

In addition to the aforementioned cancer types, the antitumor effects and mechanisms of Sch B in other cancers have also been initially revealed in preclinical studies. Sch B has been demonstrated to inhibit the proliferation of human cholangiocarcinoma cells both *in vitro* and *in vivo* and to induce their apoptosis (Yang et al., 2016). Sch B can also trigger apoptosis of gallbladder cancer cells by modulating the expression of apoptosis-related proteins (up-regulating Bcl-2-associated X protein [Bax], cleaved caspase-9, cleaved caspase-3, cleaved PARP and down-regulating Bcl-2, NF-κB, cyclin D1 and cyclin-dependent kinase 4 [CDK-4]) (Xiang et al., 2014). These preclinical research results imply the potential application of Sch B in the treatment of biliary tract tumors. Over a decade ago, researchers discovered that Sch B was able to restrain the proliferation, migration and invasion of human lung adenocarcinoma A549 cells (Lv et al., 2015). Fu-Yi Cai et al. fabricated co-delivery liposomes featuring PFV-modified doxorubicin plus Sch B. The experimental results

demonstrated that this targeted liposome had excellent targeting characteristics and was capable of suppressing the migration and invasion of non-small cell lung cancer cells, while simultaneously enhancing the cytotoxicity of doxorubicin and reducing its side effects, thereby providing a novel concept for the therapeutic approach of non-small cell lung cancer chemotherapy (Cai et al., 2021). RPV-modified paclitaxel and Sch B liposomes exhibit an anti-ovarian cancer effect by disrupting angiogenesis channels, inhibiting angiogenesis, and restraining the proliferation and migration of ovarian cancer cells (Zhang et al., 2021). Sch B remarkably induces apoptosis and autophagy in human head and neck squamous cell carcinoma cells. Notably, the inhibition of autophagy augments the apoptotic effect of Sch B (Li M. et al., 2024). In the early stage, osteosarcoma is often accompanied by lung metastasis. Sch B exerts its effect on the PI3K/Akt signaling pathway by modulating the circ_0009112/miR-708-5p axis, promoting the apoptosis of osteosarcoma cells and significantly suppressing the growth and lung metastasis of osteosarcoma in

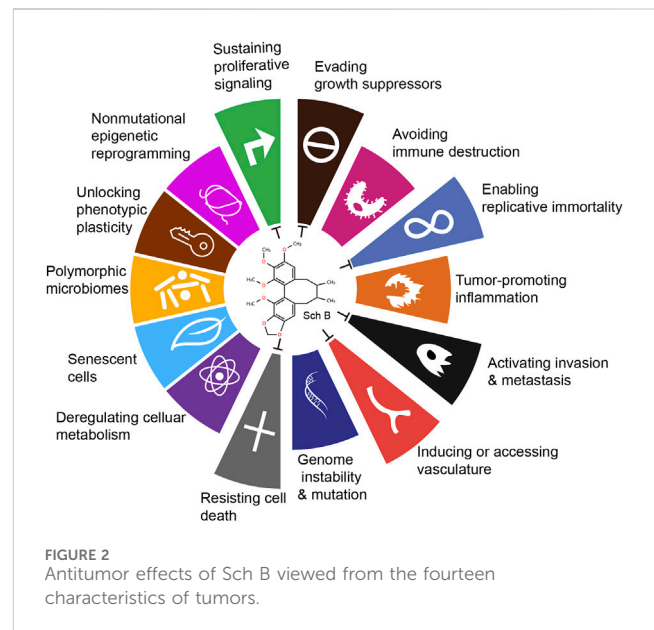


animal models (Wang et al., 2020; Wang et al., 2022). Researches have demonstrated that Sch B induces the apoptosis of prostate cancer cell lines by generating oxidative stress, suppressing androgen receptors, as well as the phosphorylation of PI3K/AKT and JAK2/STAT3 (Nasser et al., 2019). Furthermore, Sch B significantly reduces the cellular activity and malignant progression of melanoma cells by inhibiting the Wnt/ β -catenin signaling pathway and exhibited favorable antitumor effects in both *in vitro* and *in vivo* experiments (Chen et al., 2023).

5 Prediction of potential targets of Schisandrin B in tumors

Most drugs exert their effects by means of the binding of drug molecules to biological macromolecules such as proteins in the body. At present, the action targets of many bioactive molecules are still unknown. Predicting the targets of drug molecules is of great significance for the development of new drugs and the exploration of new uses for old drugs (Gfeller et al., 2013). Researchers mainly employ two approaches, namely experimentation and computation, for target prediction. The experimental method has relatively high accuracy but is rather time-consuming and laborious. In the case of conducting a large number of screening tasks, the cost is extremely high. Conversely, computational prediction consumes relatively less time. However, the reliability of current algorithms is inferior to that of the experimental method. Generally, the results obtained through theoretical calculations need further verification by experiments (Zheng et al., 2004; Forouzesht et al., 2019). Existing research has shown that Sch B is a specific inhibitor of ataxia telangiectasia and rad3 - related (ATR) protein kinase (Nishida et al., 2009). The gomisin N (GN), which is the diastereoisomer of Sch B, inhibits DNA damage checkpoint signal transduction by interacting stereospecifically with ATR (Tatewaki et al., 2013). Pan et al. discovered that Sch B could reverse the drug resistance of multiple multidrug resistance cell lines overexpressing p-glycoprotein. Furthermore, the interaction between Sch B and p-glycoprotein could fully restore the intracellular drug accumulation, thereby proving that Sch B was a novel p-glycoprotein inhibitor (Qiangrong et al., 2005).

We employed a series of network pharmacology analysis methods to explore more potential targets of Sch B in tumor therapy. Initially, the targets of Sch B were retrieved from the SwissTargetPrediction database. We selected only those targets labeled “*homo sapiens*” for research (<http://www.swisstargetprediction.ch/>). Subsequently, on the Genecards database (<https://www.genecards.org/>), a search was conducted with “cancer and tumor” as the keyword and a relevance score greater than 10 was set to obtain tumor targets. By intersecting the outcomes of these two components, 51 targets of Sch B acting on tumors were ultimately obtained (Figure 1A). The 51 targets were used to establish a protein-protein interaction (PPI) network on the String database (<http://string-db.org/>) to observe the interactions among different targets and performing KEGG analysis. The disconnected nodes were concealed, and shared proteins with a minimum interaction score of 0.9 were selected. The results indicated that Sch B might exert its antitumor efficacy by influencing signal pathways related to cell cycle, apoptosis, cell migration and invasion, which is in accordance with the existing research findings (Nasser et al., 2020) (Figures 1B, C). To determine the core targets that play a crucial role in the entire target network, these



targets were analyzed in depth on Cytoscape. According to the important parameter of degree, core targets such as heat shock protein 90 alpha family class A member 1 (HSP90AA1), phosphatidylinositol - 4,5 - bisphosphate 3 - kinase catalytic subunit alpha (PIK3CA), and proto - oncogene tyrosine - protein kinase Src (SRC) can be obtained. And ATR, which has been proven to be a target of Sch B, is also among them (Figure 1D). Although we have predicted the targets of Sch B in antitumor via network pharmacology, the limitation of this method is that the prediction results are all obtained based on the calculation of protein structural similarity. Whether it truly exerts its effect through these target remains to be confirmed by more experiments.

6 Conclusion

Currently, surgery, radiotherapy and chemotherapy remain the main approaches for cancer treatment. Drawing on the current research findings on the antitumor mechanism of Sch B, it is evident that Sch B exerts its antitumor role through various means and pathways in preclinical studies. Douglas Hanahan and Robert A. Weinberg authoritatively released the fourteen major characteristics of tumors (Hanahan, 2022). Upon comparing the antitumor mechanism of Sch B, it is apparent that Sch B inhibits the progression of different tumors through six aspects, including: sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis, inducing or accessing vasculature, resisting cell death (Figure 2).

However, the current research on Sch B does reveal several potential limitations. Despite the identification of multiple antitumor targets of Sch B, as of now, there is a scarcity of relevant research reports on its clinical application. This deficiency significantly hampers the translation process from basic research to clinical implementation. Traditional treatment modalities, such as surgical intervention, often enable radical resection of early-stage localized tumors. During extensive long-term clinical practice, radiotherapy and chemotherapy

have accumulated substantial experience and demonstrated well-defined curative efficacy in a diverse range of tumors. In sharp contrast, the clinical evidence related to Sch B remains relatively limited. Current research results are insufficient to rule out the potential for long-term or high-dose administration of Sch B to induce toxicity in specific organs or systems. This may include adverse effects on the functions of crucial organs like the liver and kidneys, as well as potential impacts on the immune system. Additionally, the current research on the interactions between Sch B and existing clinical medications is still inadequate. This deficiency has the potential to undermine the safety and efficacy of combination therapies.

Undoubtedly, as researchers embark on a more systematic exploration of Sch B's toxicity characteristics with the aim of ensuring its safe clinical application, delving deeper into its formulation technology and developing a more efficient, stable, and targeted drug delivery system to enhance its bioavailability and therapeutic efficacy, they will also need to assess the efficacy and safety of Sch B across diverse tumor types and stages. Simultaneously, clarifying its optimal combination regimens and the appropriate patient populations is of crucial importance. On the other hand, whether Sch B can enhance the efficacy of radiotherapy and act as a radiation sensitizer is also a research direction worthy of in-depth exploration by researchers. Through these efforts, the application of Sch B in the antitumor field is expected to expand in both scope and depth. Consequently, Sch B is anticipated to emerge as a potential component of novel antitumor medications, thus offering new hope to cancer patients.

Author contributions

YF: Funding acquisition, Writing–review and editing. JP: Writing–original draft. PW: Data curation, Software, Writing–review and editing. RW: Conceptualization, Funding

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CXCR3 inhibitors for therapeutic interventions: current status and perspectives

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CXC chemokine receptor 3 (CXCR3) is a G protein-coupled chemokine receptor that plays a key role in regulating immune responses and is involved in various pathological processes, particularly in tumor development and inflammatory diseases, making it a novel target for clinical therapy. The expression of CXCR3 and its ligands—CXCL9, CXCL10, CXCL11, CXCL4, and CXCL4L1—is closely associated with the onset and progression of numerous diseases. With a deeper understanding of the mechanisms underlying CXCR3 function, significant progress has been made in the development of small molecule antagonists targeting CXCR3, some of which have entered clinical trials and demonstrated therapeutic potential. This review provides an overview of the structure and signaling pathways of CXCR3, its biological functions in cancer and inflammatory diseases, and highlights the innovative roles of CXCR3 in these diseases. Furthermore, it discusses recent advances in the development of small molecule antagonists, particularly those that have been tested in clinical settings, such as AMG 487 and ACT-777991. These studies provide a scientific foundation for the development of novel CXCR3 antagonists and may offer new directions for future clinical treatments.

KEYWORDS

CXCR3, tumor, inflammatory diseases,, CXCR3 inhibitors, molecule antagonists,, future clinical treatments

1 Introduction

CXCR3 is a G protein-coupled chemokine receptor, belonging to the CXCR subfamily (CXCR1–CXCR7) of the G protein-coupled receptor (GPCR) family. It is also referred to as G protein-coupled receptor 9 (GPR9) and is designated as CD183. Since its initial discovery, CXCR3 has been shown to play a crucial role in a wide range of physiological and pathological processes. CXCR3 antagonists were initially proposed for the treatment of inflammatory and autoimmune diseases; however, recent studies suggest that they may have therapeutic potential in a broader spectrum of diseases (Wijtmans et al., 2014; Wijtmans et al., 2011; Wijtmans et al., 2008).

Chemokines are a class of low molecular weight soluble cytokines, typically ranging from 8 to 15 kDa, that exert biological effects through their interaction with G protein-coupled receptors (GPCRs). GPCRs are one of the key targets in drug development. Chemokines play essential roles in various biological processes, including development, angiogenesis, inflammatory responses, immune diseases, and cancer (Andrews and Cox, 2016).

The CXC chemokine family is an important branch of the chemokine family, characterized by the presence of an “X” amino acid located between cysteine residues at the amino terminus. CXC chemokines are involved in various biological processes, including cell chemotaxis, regulation of cell growth, induction of apoptosis, and modulation of anti-angiogenic activity (Billottet et al., 2013). CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, CXCL4/PF-4, and CXCL4L1/PF4V1 are all members of the CXC chemokine family, and they exert biological effects by binding to the CXCR3 receptor.

The gene encoding CXCR3 (Gene ID: 2833) is located on the X chromosome (Q13). This receptor is expressed on activated T cells, NK cells, mast cells, and dendritic cells. Its expression is not limited to leukocytes but also includes fibroblasts, endothelial cells, and tumor cells.

CXCR3 has been recognized to play a critical role in several human diseases, with the expression of CXCR3 and its ligands associated with various inflammatory diseases (Qin et al., 1998), tumors (Ma et al., 2015), transplant rejection (Sørensen et al., 1999), and others. Three subtypes of CXCR3 have been identified (CXCR3-A, CXCR3-B, and CXCR3-alt), with the two major subtypes, CXCR3-A and CXCR3-B, believed to induce opposing physiological functions (Va et al., 2015). CXCR3-A, primarily found on hematopoietic cells, appears to mediate tumor progression signals by promoting cell proliferation, survival, chemotaxis, invasion, and metastasis. In contrast, CXCR3-B, mainly expressed on endothelial cells, seems to mediate tumor suppression signals by promoting growth inhibition, apoptosis, and vascular regression (Ma et al., 2015). Therefore, the dysregulated expression of CXCR3-A and CXCR3-B may influence tumor progression.

The development of drugs targeting chemokine receptors has always been a challenge. Although G protein-coupled receptors (GPCRs) are popular drug targets, very few chemokine receptor antagonists have reached the market, aside from HIV entry inhibitors. This may be due to the relatively late discovery of chemokine receptors and the high redundancy within the chemokine system, which complicates the prediction of the therapeutic efficacy of chemokine receptor antagonists. Nonetheless, the upregulated expression of CXCR3 and its ligands in a variety of diseases has been associated with several inflammatory conditions, including multiple sclerosis, rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease, inflammatory bowel disease, inflammatory skin disorders such as psoriasis, as well as hepatitis C infection in the liver, sarcoidosis, and SARS. Additionally, CXCR3 plays a key role in transplant rejection, metastasis of melanoma and colon cancer, and may act as a co-receptor for certain HIV strains. To explore the therapeutic potential of the CXCR3 receptor system, various preclinical approaches have been employed, including: 1) generating CXCR3 knockout mice; 2) using antibodies targeting CXCR3 or its endogenous ligands; 3) inhibiting CXCR3 with protein-based antagonists; and 4) targeting CXCR3 with small molecule drugs. These studies have provided valuable insights for the further development of CXCR3 antagonists.

Due to the critical role of CXCR3 and its ligands in various diseases, CXCR3 has emerged as a promising target for anti-tumor and anti-inflammatory therapies. CXCR3 antagonists can block the binding of CXCR3 to its ligands, thereby inhibiting the biological signaling axis, which may offer therapeutic benefits for conditions

such as tumors and autoimmune diseases. In recent years, many research institutions have focused on developing CXCR3 antagonists. Among them, Chemocentryx has been particularly prominent, identifying a series of dihydro-1,2,4-triazolo[3,4-b]quinolin-3-yl derivatives, including potent and effective CXCR3 antagonists (e.g., AMG487). In preclinical studies, AMG487 was shown to block immune cell migration and exhibited excellent potency, high selectivity, and good oral bioavailability. Unfortunately, this inhibitor failed to demonstrate any signs of efficacy in Phase II clinical trials, and the trial was terminated. In the latest research, ACT-777991, a novel CXCR3 antagonist, has undergone preliminary human studies in healthy adults to evaluate its pharmacokinetics, pharmacodynamics, and safety. The study demonstrated that ACT-777991 was well-tolerated at both single and multiple escalating doses and exhibited pharmacokinetic and pharmacodynamic properties suitable for further clinical development. Moreover, ACT-777991, in combination with an anti-CD3 antibody, showed synergistic effects in experimental models, increasing the sustained remission rate in a type 1 diabetes model.

Although CXCR3 antagonists have demonstrated therapeutic efficacy in animal models of disease, studies validating the effectiveness of these models in humans are still lacking. Furthermore, more in-depth research is needed on the pharmacokinetic properties, dose-dependency, signaling bias, and potential side effects of these drugs.

2 Structures, subtypes, and ligands of CXCR3

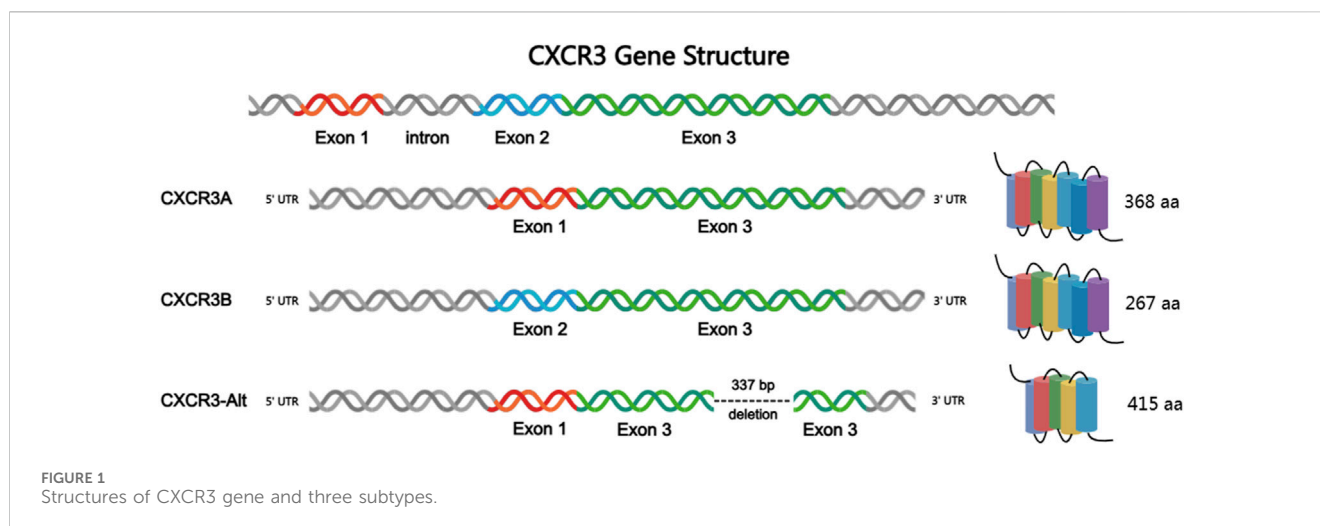
The precise structure of a protein is crucial for understanding its interactions with various molecules and may facilitate the discovery of new ligands with better affinity, selectivity, or safety profiles.

2.1 Structure of CXCR3

As shown in Figure 1, CXCR3 is a typical seven-transmembrane G protein-coupled receptor, featuring an external N-terminus, three extracellular loops (ECL1-3), three intracellular loops (ICL1-3), and an intracellular C-terminus. Recent structural biology studies have revealed that the second extracellular loop (ECL2) of CXCR3 plays a key role in receptor activation, while the N-terminus is involved in initial ligand recognition (Jiao et al., 2024; Xanthou et al., 2003). CXCL11 promotes receptor activation by interacting with the N-terminus and ECL2 of CXCR3 through its N-terminus (Jiao et al., 2024). Additionally, small-molecule agonists, such as VUF10661 and VUF11418, activate CXCR3 through distinct binding modes, offering new insights for drug design (Jiao et al., 2024; Saha et al., 2025).

2.2 Three subtypes of CXCR3

Based on the composition of the receptor's N-terminal, CXCR3 can be classified into three subtypes (Figure 1): CXCR3-A, CXCR3-B, and the truncated variant CXCR3-alt (Singh et al.,



2013). Among these, CXCR3-A and CXCR3-B are the most extensively studied isoforms. CXCR3-A is primarily expressed on activated T lymphocytes and natural killer (NK) cells, whereas CXCR3-B is predominantly found on endothelial cells. The research on CXCR3-alt is relatively limited, and it is currently believed to mainly exert its biological effects in conjunction with interferon-inducible T cell alpha chemoattractant (I-TAC) (Billottet et al., 2013). Both CXCR3-A and CXCR3-B encode seven transmembrane domains, and both proteins are coupled to heterotrimeric G proteins (HTGPs), mediating their downstream signaling. The number of CXCR3-alt transmembrane helices is predicted to be 4 to 5 (Ehlert et al., 2004). CXCR3-A consists of 368 amino acids and is encoded by two exons separated by an intron, coupling with a Gai protein. The coding sequence of CXCR3-A includes three amino acids from exon 1, with the remainder from exon 2. CXCR3-B, composed of 415 amino acids, results from selective splicing at the 5' end of the second exon. The coding sequence of CXCR3-B comes from exon 2 and a retained intronic sequence that is adjacent to the 5' end of exon 2. Furthermore, the inserted sequence contains a selective start codon, leading to the loss of the four N-terminal residues encoded by the first exon, which are replaced by a new 51 amino acid N-terminal tail. In terms of sequence, the only difference between CXCR3-A and CXCR3-B lies in their N-terminal regions. Specifically, CXCR3-B has an additional 48 amino acids at its N-terminus, which are absent in CXCR3-A. Despite their sequence similarities, the behavior of CXCR3-A and CXCR3-B is quite distinct. Activation of CXCR3-A has been shown to induce chemotaxis and proliferation in various cell types, increase intracellular calcium levels, and enhance cell survival upon overexpression. In contrast, activation of CXCR3-B inhibits migration and proliferation, and induces apoptosis. Overexpression of CXCR3-B significantly reduces DNA synthesis and upregulates the apoptosis-associated HMEC-1 death signaling pathway.

CXCR3 subtypes exhibit distinct organ-specific distribution patterns. Study (Boyé et al., 2017) showed that CXCR3-A is predominantly enriched in CD8⁺ tissue-resident memory T cells (Trm) and tumor-infiltrating NK cells, accounting for up to 68% of portal vein-associated lymphocytes in the liver. CXCR3-B is

specifically expressed in liver sinusoidal endothelial cells (with expression levels 3.2 times higher than CXCR3-A) and glomerular mesangial cells. Recent cryo-electron microscopy studies have revealed that the N-terminal extension of CXCR3-B forms β -sheets to interact with laminin in the hepatic extracellular matrix, which may explain its high expression in the liver. CXCR3-alt is expressed in choroid plexus epithelial cells at levels over five times higher than other subtypes, and its truncated structure may be involved in blood-brain barrier regulation.

2.3 Ligands of CXCR3

As shown in Table 1 the ligands of CXCR3 are mainly divided into two categories: IFN- γ -induced ligands CXCL9, CXCL10, and CXCL11, and platelet-derived ligands CXCL4 and CXCL4L1. CXCL9, CXCL10, and CXCL11 are regulated by different promoters, exhibit distinct expression patterns, and bind to CXCR3-A and CXCR3-B with different affinities (Karin, 2020). CXCR3-A has high affinity for CXCL9, CXCL10, and CXCL11, while CXCR3-B has a higher affinity for CXCL4 and CXCL10. CXCL4 does not activate CXCR3-A, but CXCL4L1 binds with moderate affinity to both CXCR3-A and CXCR3-B. Unlike the IFN- γ -induced ligands, CXCL4 and CXCL4L1 are not induced by IFN- γ , but are released by activated platelets. CXCL4L1 shares high sequence similarity with CXCL4, but subtle structural differences result in distinct functions, such as differences in their potential to inhibit angiogenesis (Zhou et al., 2019).

2.4 Oligomerization of CXCR3

CXCR3 exhibits dynamic oligomerization properties that critically regulate its signaling bias and cellular trafficking. As a GPCR, CXCR3 forms homo-oligomers (CXCR3-A/CXCR3-A, CXCR3-B/CXCR3-B) and hetero-oligomers with other chemokine receptors such as CXCR4 and CCR5 (Breitwieser, 2004; Watts et al., 2013). Structural studies reveal that CXCR3-A homo-oligomerization occurs via transmembrane domain (TM) interactions (TM1, TM4, TM5), stabilizing active conformations

TABLE 1 Detailed information on CXCR3 ligands.

	CXCL4	CXCL4L1	CXCL9	CXCL10	CXCL11
Gene(HGNC)	NM_002619	NM_002620	NM_002416	NM_001565	NM_005409
	4q13.3	4q13.3	4q21.1	4q21.1	4q21.1
Mature protein	70 AA	70 AA	103 AA	77 AA	73 AA
Producer cells	Platelets Monocytes T cells pDC	Platelets Smooth muscle cells	HMVEC Fibroblasts Macrophages PBMC	Fibroblasts Monocytes T cells Keratinocytes	Fibroblasts Astrocytes PBMC
Inducers	Thrombin (platelets) Phorbol ester (T cells)	Thrombin (platelets)	IFN- γ	TNF- α (fibroblasts) IFN- α ,IFN- β ,IFN- γ	TNF- α (fibroblasts) IFN- γ ,IFN- β
Processing					
-Protease	N.D.	N.D.	DPPIV/CD26	DPPIV/CD26	DPPIV/CD26
-Peptide product	CXCL4 ^{17–70}	N.D.	CXCL9 ^{3–103}	CXCL10 ^{3–77}	CXCL11 ^{3–73}
-Effect on					
Chemotaxis	N.D.		↓	↓	↓
Angiostasis	↑		=	=	=

essential for Gai coupling and cAMP/PKC pathway activation (Boyé et al., 2017). In contrast, CXCR3-B oligomerization is modulated by its extended N-terminal domain, which restricts surface expression while enhancing β -arrestin recruitment and ERK phosphorylation (D’Uonno et al., 2022). Hetero-oligomerization with CXCR4 induces negative binding cooperativity—CXCL12 binding to CXCR4 suppresses CXCL11-mediated CXCR3 activation, a mechanism implicated in tumor immune evasion (Watts et al., 2013). Notably, CXCR3-Alt acts as a dominant-negative variant by forming nonfunctional oligomers with CXCR3-A/B, effectively reducing their membrane localization (Nguyen et al., 2023). These oligomeric states directly influence ligand selectivity; for example, CXCR3-CXCR4 heterodimers redirect signaling toward ERK/MAPK pathways while suppressing calcium flux, highlighting their context-dependent roles in cancer metastasis and autoimmune responses (Saotome et al., 2025).

3 Signal pathways of CXCR3

Most chemokine receptors transduce cellular signals through heterotrimeric GTP-binding proteins (G proteins) composed of α , β , and γ subunits. Binding of a chemokine to its receptor results in the activation of the α subunit and the subsequent dissociation of the $\beta\gamma$ subunit complex (Berchiche and Sakmar, 2016).

Upon activation of CXCR3, the G protein regulates a wide range of signaling pathways, including adenylate cyclase, phospholipase C (PLC), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK), which influence various cellular responses such as calcium influx, proliferation, integrin activation, actin remodeling, and migration (Smith et al., 2017).

The two distinct receptor subtypes of CXCR3, CXCR3-A and CXCR3-B, can activate different downstream signaling pathways (Figure 2). Both receptors can activate phospholipase C β (PLC β),

which hydrolyzes phosphatidylinositol bisphosphate (PIP2) to release two products: inositol trisphosphate (IP3, a general calcium ion influx second messenger) and diacylglycerol (DAG, a PKC activator). IP3 induces the downstream flow of Ca²⁺ into the cell, thereby activating u-calpain to weaken cell–substrate adhesion and promote cell motility; activated PKC enhances the activation of extracellular signal-regulated kinase (ERK), and the phosphorylation and activation of m-calpain (Billottet et al., 2013). In summary, CXCR3 signaling promotes cell migration via PLC β activity. Additionally, CXCR3-B uniquely induces the accumulation of cAMP and activates protein kinase A (PKA, a cAMP-dependent kinase), which in turn inhibits the activation of m-calpain, blocking the release of signaling and suppressing cell migration.

Moreover, studies have shown that the different subtypes of CXCR3 may also activate distinct signaling pathways:

1. Gai Activation: CXCR3-A can activate Gai through CXCL10 and CXCL11, which is manifested as inhibition of forskolin-induced cAMP production. CXCR3-B only shows Gai activation at a concentration of 100 nM CXCL11. It is noteworthy that CXCR3-B does not exhibit activation of classical G proteins (Gai/o/q) under physiological ligand concentrations (1–10 nM CXCL11) (D’Uonno et al., 2022). Its N-terminal extended structure restricts conformational changes in the transmembrane domain, thereby hindering G protein coupling (Saha et al., 2025). However, CXCR3-B can still activate ERK signaling through a β -arrestin-dependent pathway and mediate chemokine internalization (Saha et al., 2025). This signaling bias may account for the unique role of CXCR3-B in tumor angiogenesis inhibition and provides a theoretical basis for the development of biased ligands (Zheng et al., 2022). CXCR3-Alt, however, fails to activate Gai, even in the presence of any tested chemokine.

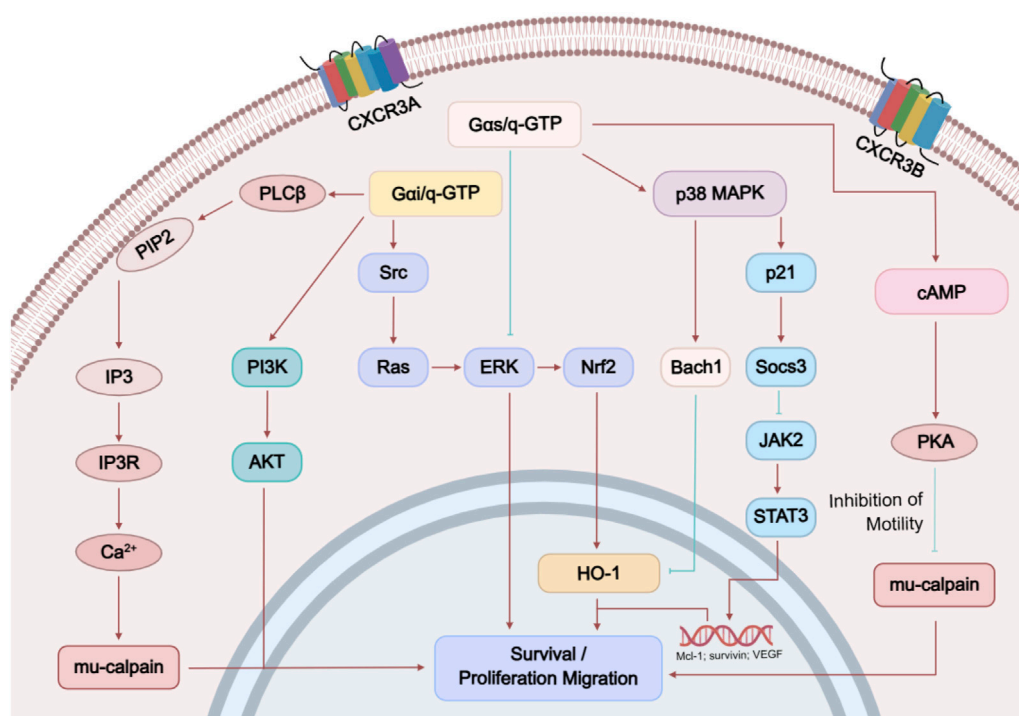


FIGURE 2
CXCR3 signal pathways.

2. β -Arrestin: Both CXCR3-A and CXCR3-B exhibit ligand-dependent and ligand-independent interactions with β -arrestin1 and β -arrestin2. In the absence of a ligand, CXCR3-A shows significant basal BRET (bioluminescence resonance energy transfer) signals with both β -arrestins, which are further increased after CXCL11 stimulation. CXCR3-B exhibits a preference for β -arrestin2, observed both in the presence and absence of ligand. CXCR3-Alt does not show any ligand-dependent or independent interactions with β -arrestin1 or β -arrestin2.
3. Receptor Internalization: All CXCR3 variants undergo internalization in response to CXCL11, CXCL10, CXCL9, and CXCL4. CXCL4, in particular, induces strong receptor internalization in all three variants.
4. ERK1/2 Phosphorylation: CXCR3-A strongly induces ERK1/2 phosphorylation through CXCL11, CXCL10, and CXCL9, while the response to CXCL4 is weaker. CXCR3-B induces weaker ERK1/2 phosphorylation in response to CXCL11 and CXCL9, while CXCL10 and CXCL4 fail to induce significant responses. CXCR3-Alt shows limited ERK1/2 phosphorylation upon stimulation with CXCL11, CXCL10, and CXCL9.
5. Biased Agonism: Different splice variants of CXCR3 activate distinct signaling pathways, indicating the complexity of CXCR3 signaling. For example, CXCL11 and CXCL10 are full agonists for CXCR3-A, capable of activating both Gai and ERK1/2 phosphorylation, while CXCL9 only shows a weaker response in ERK1/2 phosphorylation.
6. PTX Sensitivity: Studies also observed that β -arrestin recruitment to CXCR3-A and CXCR3-B is insensitive to PTX (pertussis toxin) treatment, suggesting that these responses may occur independently of Gai signaling.

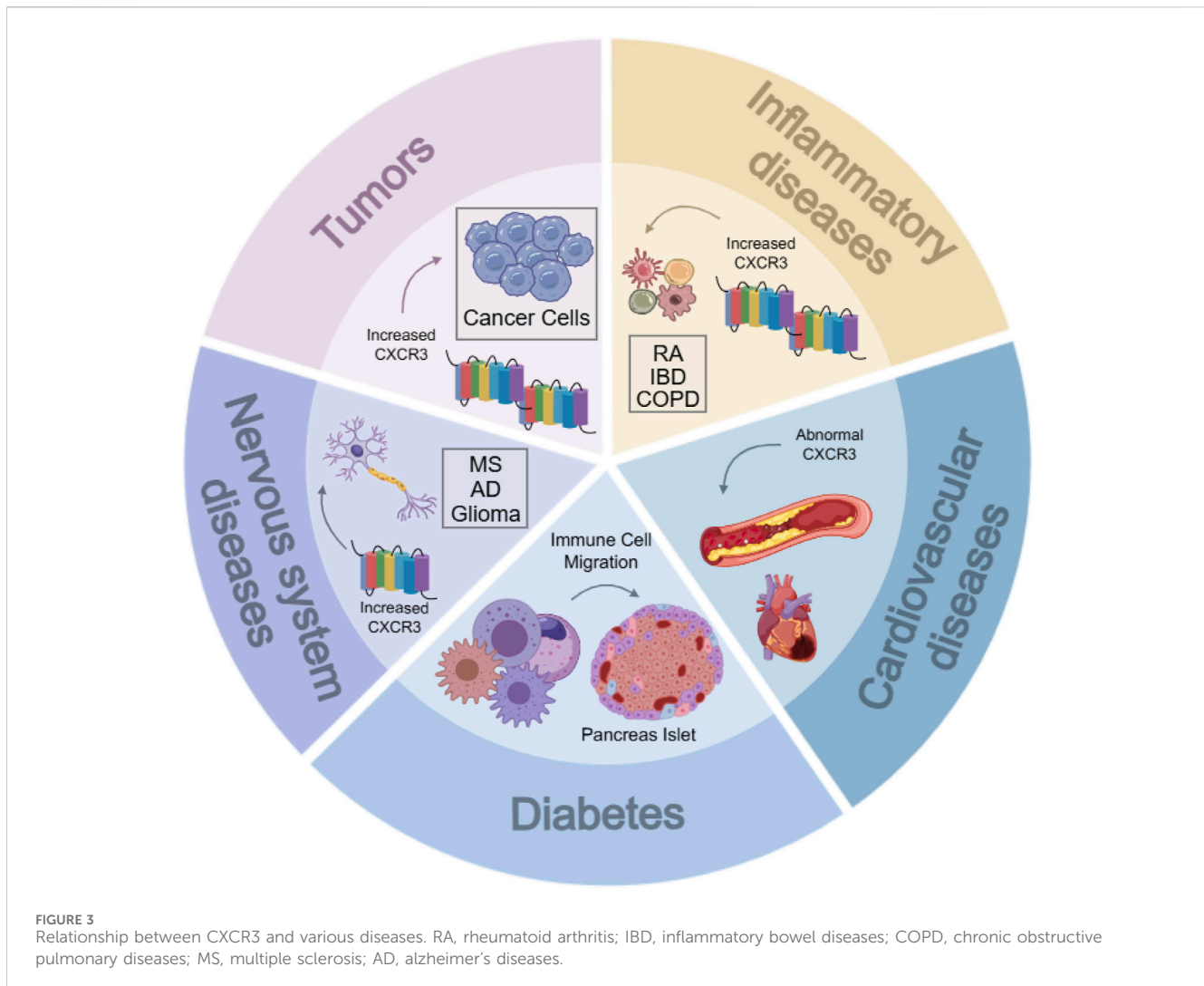
These findings highlight the distinct signaling properties of the various CXCR3 splice variants. They offer new insights into the role of CXCR3 in different biological processes and may aid in the development of targeted therapeutic strategies for specific CXCR3 variants.

4 The role of CXCR3 in the occurrence and development of diseases

CXCR3 is directly or indirectly involved in tumor progression by regulating tumor growth, migration, invasion, angiogenesis, and immunity. CXCR3 is upregulated in many primary and metastatic tumors, including prostate, breast, colorectal, lung, ovarian, kidney, melanoma, and multiple myeloma. CXCR3 and one or more of its ligands have been found to be overexpressed in a variety of inflammatory diseases, including allograft rejection, atherosclerosis, autoimmune diseases. Examples include rheumatoid arthritis (RA), inflammatory bowel disease (IBD), chronic obstructive pulmonary disease (COPD), multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (Figure 3).

4.1 Tumors

CXCR3 signaling is associated with tumor progression. CXCR3-A promotes mesenchymal cell migration, while CXCR3-B may inhibit movement. The expression of CXCR3-B regulates and is regulated by E-cadherin expression. Overexpression of CXCR3-A decreases E-cadherin, whereas overexpression of CXCR3-B leads to



its upregulation. Both E-cadherin and CXCR3-B play roles in tumor suppression, but during early tumor metastasis, E-cadherin contributes to cell dormancy and survival. CXCR3-B may be involved in the early adaptation phase of metastasis, although the exact mechanisms remain unclear. Studies have also shown that E-cadherin expression is regulated by various mechanisms, including DNA methylation, transcriptional repression, and signal-induced unstable internalization. CXCR3-B may maintain the stability of adhesion complexes by inhibiting vinculin phosphorylation, though this hypothesis requires further validation. Furthermore, these findings may not be applicable to all cancer types, as methylation of E-cadherin may be the primary mechanism for its downregulation in other cancers. CXCR3 is a key chemokine receptor for Treg cell accumulation and immune suppression in tumors. Targeting CXCR3 could enhance anti-tumor immune responses, offering a new strategy for cancer treatment (Moreno Ayala et al., 2023).

4.1.1 Hepatocellular carcinoma (HCC)

A study on tumor recurrence after liver transplantation found that the increase in circulating endothelial progenitor cells and CXCL10 was associated with a higher rate of tumor recurrence

in patients who received small-volume liver transplants (Ling et al., 2014). In contrast, the recurrence rate was significantly lower in CXCL10-and CXCR3 knockout (KO) animals in a liver transplantation-like model. Importantly, in an orthotopic nude mouse model (Ding et al., 2016), injecting CXCL10 into the portal vein increased the number of pulmonary metastatic tumors. The authors of this study suggested that CXCL10 administration enhanced angiogenesis within tumors, leading to increased metastatic spread. However, this finding contradicts the classical understanding of CXCR3 function, as tumors in CXCL10-treated mice exhibited more CD34-positive cells, but these regions appeared to be smaller compared to untreated mice. Moreover, mice injected with endothelial progenitor cells exhibited larger tumors and multiple lesions in the liver, but did not show an increase in lung metastasis. Due to these findings, the involvement of angiogenesis in metastasis observed in this study is questionable and may require further investigation.

Other studies (Lan et al., 2014) have highlighted the role of CXCR3 signaling in HCC cells and its impact on metastasis. CXCR3 signaling, through the AKT/PI3K pathway and ERK1/2 signaling, plays a crucial role in promoting the invasive and

migratory phenotypes of HCC cells. CXCR3 may influence HCC progression by promoting tumor cell proliferation and migration, as well as modulating the activity of immune cells within the tumor microenvironment. These findings suggest that CXCR3 could serve as a potential therapeutic target for HCC treatment (Ren et al., 2017).

4.1.2 Lung cancer

In lung cancer, CXCR3 promotes tumor progression by regulating the expression levels of receptors on inflammatory cells or the expression levels of ligands on tumor cells (Gemelli et al., 2022). Unlike other cancers, in clinical specimens of non-small cell lung cancer (NSCLC), tumor cells and blood vessels are predominantly negative for CXCR3, while infiltrating immune cells show strong CXCR3 staining.

In NSCLC, the expression of CXCR3 and its ligands is elevated within the tumor microenvironment (TME), influencing the activity of natural killer (NK) cells and T cells, which are critical for the surveillance and elimination of cancer cells (Butler et al., 2017). The increased CXCR3 signaling may lead to the suppression of NK cell function, thereby promoting a “nurturing” phenotype that supports tumor growth and immune evasion. Additionally, CXCR3-positive NK cells may develop resistance to immune checkpoint inhibitor (ICI) therapy.

4.1.3 Breast cancer

CXCR3 has been associated with poor survival outcomes in breast cancer patients. CXCR3-A signaling promotes breast cancer proliferation, while CXCR3-B may inhibit tumor growth. In the breast cancer cell lines MCF-7 and MDA-MB-231, the expression of CXCR3 splice variants A and B, as well as CTSB, is commonly observed. Upon binding to CXCR3, the chemokine ligands CXCL9 and CXCL10 can trigger the upregulation of CTSB, while the CXCR3-B specific ligand CXCL4 does not induce this response, suggesting that CXCR3-A is involved in the regulation of CTSB (Kundu et al., 2019). In breast cancer, the expression of CXCR3 is closely linked to tumor metastasis and prognosis. For example, high CXCR3 expression is associated with poor prognosis in breast cancer patients, and its expression level correlates positively with tumor size and the number of metastatic lymph nodes. Certain studies (Bronger et al., 2017) showed that CXCR3B expression is significantly upregulated in the breast cancer stem cell (CSC) subpopulation, and CXCL11 and CXCL10 can directly induce CSCs, indicating that CXCR3B may be involved in the characteristics of breast cancer stem cells and the metastatic spread of the tumor. Furthermore, CXCR3 expression in breast cancer is also linked to the tumor's immune microenvironment (Cannon et al., 2021). In CXCR3-deficient mouse models, breast cancer progression is accelerated, which is associated with increased M2 polarization of macrophages, suggesting that CXCR3 may play a suppressive role in regulating the tumor immune microenvironment. Therefore, CXCR3 not only participates in the initiation and progression of breast cancer but may also regulate tumor metastasis and prognosis by influencing the tumor immune microenvironment. Its expression levels and function may vary depending on the tumor microenvironment, providing new potential therapeutic targets for breast cancer treatment.

4.1.4 Prostate cancer (PC)

Prostate cancer (PC) is a global male urological disease known for its high incidence and mortality, particularly challenging to diagnose once bone metastasis occurs. The chemokine receptor CXCR3 plays a critical role in tumor angiogenesis and cell migration. Certain studies (Shen and Cao, 2015) used the PC-3 cell line model and gene silencing techniques to specifically downregulate two CXCR3 isoforms, CXCR3-A and CXCR3-B, confirming that downregulation of CXCR3-A led to an upregulation of CXCR3-B in PC-3 cells, further inhibiting cell proliferation and invasion. Furthermore, research by Wu et al. (2012) showed that CXCR3B is the major splice variant in normal human prostate tissues and epithelial cells, and that CXCL10 upregulates cAMP through CXCR3B, which in turn weakens m-calpain activity, thereby inhibiting the migration and invasion of RWPE-1 endothelial cells.

These findings provide a rationale for targeting CXCR3 as a potential therapeutic strategy to inhibit prostate cancer metastasis and progression. However, further experimental studies are needed to clarify the specific mechanistic role of CXCR3 in prostate cancer (Cannon et al., 2021).

4.1.5 Renal cancer

The ratio of CXCR3-A to CXCR3-B is higher in renal cell carcinoma (RCC) tissues compared to normal kidney tissues, and the total expression of CXCR3 and CXCR3-A is significantly higher in metastatic RCC tissues than in non-metastatic RCC tissues (Gudowska-Sawczuk et al., 2020). A study by Sun et al. (2005) demonstrated that the angiogenesis-inhibiting protein IP-10 could be released by various mammalian cells through transduction with retroviral vectors carrying the human IP-10 gene (RCR vectors). These cells, cultured *in vitro* for at least 3 months, indicated that gene therapy based on RCR vectors could provide a long-term supply of anti-angiogenesis proteins during tumor treatment, thus being more effective and convenient for inhibiting tumor growth. The expression of CXCR3 and its ligands on monocytes was found to be elevated in metastatic renal cancer patients treated with high-dose IL-2, exerting some anti-angiogenic effects (Reckamp et al., 2007). Datta et al. (2010) confirmed via flow cytometry that the high expression of CXCR3-B in the human renal cancer cell line Caki-1 promoted apoptosis. Using protein chip technology and small interfering RNA (siRNA), they discovered that high expression of CXCR3-B significantly downregulated the expression of heme oxygenase-1 (HO-1), an anti-apoptotic protein, and that inhibiting HO-1 expression enhanced renal cancer cell apoptosis. These findings help explain the possible mechanism by which CXCR3-B inhibits renal cancer cell growth.

4.1.6 Colorectal cancer

18%–34% of colorectal cancer (CRC) patient samples show strong CXCR3 staining, and most of these CXCR3-positive patients are also diagnosed with lymph node metastasis (Abrón et al., 2017). Patients expressing CXCR3 have a poorer prognosis compared to those who do not express CXCR3 or express CXCR4 or CCR7. Previous studies have demonstrated high expression of cytotoxic CXCR3 ligands on CD8⁺ T cells in colorectal cancer tissues. CXCR3 and its ligands exhibit differential expression at inflammatory sites and within tumors. Moreover, the expression of

CXCR3 and its ligands correlates with the presence of effector T cells in tumor tissues and with patients' disease-free survival (Jin et al., 2018).

One of the leading causes of mortality in CRC is invasion and metastasis. The cooperative interaction between CXCR3 and CXCR4 plays a crucial role in regulating CRC cell invasion. Recent studies (Cannon et al., 2021) have found that CRC cells expressing higher levels of CXCR3 and CXCR4 exhibit increased invasiveness. CXCR3 enhances CXCR4 functionality in CRC cells by forming heterodimers at the cell surface and preventing CXCR4 internalization. Taken together, targeting CXCR3 may represent a promising strategy for the clinical treatment of CRC cell invasion and metastasis.

4.1.7 Other cancers

CXCR3-A expression is elevated in ovarian cancer and endometriosis tissues compared to normal ovarian tissue, while CXCR3-alt and CXCR3-B show upregulation and downregulation, respectively, in ovarian cancer tissues (Cannon et al., 2021). CXCR3-A is predominantly expressed on cancer cells and infiltrating lymphocytes, whereas CXCR3-B and CXCR3-alt are expressed in microvessels. The expression of CXCL4 and CXCL4L1 in ovarian cancer associated with endometriosis is significantly lower than in endometriosis alone (Windmüller et al., 2017). CXCR3-positive tumor cells are strongly associated with immune reactivity in tumors with thickness greater than 1 mm or with invasive, lethal melanoma (Jenkins et al., 2015). In multiple myeloma, CXCR3 expression increases across different pathological stages, with higher expression observed in stage III compared to stage I (Ma et al., 2015). The presence of both CXCR3-A and CXCR3-B subtypes is associated with the regulation of the cell cycle and apoptosis.

Glioblastoma multiforme (GBM), a highly invasive brain tumor, has limited treatment options and poor prognosis (Chan et al., 2023). CXCR3, a chemokine receptor, functions with both autocrine and paracrine signaling mechanisms. Current clinical treatments targeting CXCR3 ligands, which are associated with the CXCR3 axis, have demonstrated antitumor effects in GBM (Guo and Gao, 2015).

4.2 Inflammatory diseases

CXCR3 plays a crucial role in various inflammatory diseases. Karkada et al. demonstrated the key role of CXCR3 in T cell migration to inflamed joints and the progression of arthritis in an adjuvant-induced arthritis model in Lewis rats. The study found that the use of anti-CXCR3 monoclonal antibodies significantly reduced T cell migration and alleviated the severity of arthritis, including reductions in clinical symptoms, weight loss, and neutrophil accumulation in the joints. These findings suggest that blocking CXCR3 could offer a novel therapeutic strategy for rheumatoid arthritis (RA). Furthermore, serum CXCL4 has been identified as a general biomarker for inflammatory bowel disease (IBD). IFN- γ -induced chemokines may contribute to the progression of IBD and exacerbate the associated gastrointestinal inflammation (Meuwis et al., 2007).

The inflammation in chronic obstructive pulmonary disease (COPD) involves both innate and adaptive immunity. CXCR3, through binding with its specific ligands, plays an important role

in regulating tissue inflammation and damage. In acute animal models subjected to cigarette smoke (CS) or pathogen challenges, CXCR3 knockout (KO) alleviated lung inflammation and pathology (Li et al., 2021).

4.3 Neurodegenerative diseases

CXCR3 plays a significant role in neurodegenerative diseases. CXCR3-positive T cells are increased in the blood of patients with multiple sclerosis (MS), and its ligand CXCL10 is expressed by astrocytes in MS brain lesions, suggesting that CXCR3 may be involved in the immune response and lesion development in MS (Zhou et al., 2019). The expression of CXCR3 is elevated in glioma cells and is associated with tumor malignancy. CXCR3 antagonists have been shown to inhibit glioma growth and prolong survival. Additionally, CXCR3 expression in neurons and neural processes in Alzheimer's disease (AD) patients is correlated with AD-related pathological changes. CXCR3-deficient mice exhibit improved spatial memory performance. CXCR3 antagonists have demonstrated therapeutic effects in various neurodegenerative disease models, indicating that CXCR3 may represent a potential therapeutic target for these disorders (Müller et al., 2010).

4.4 Diabetes

CXCR3 is a chemokine receptor expressed on activated T cells and natural killer (NK) cells. By binding to ligands such as CXCL10, it promotes the migration of immune cells to the pancreatic β cells, exacerbating the immune attack on these cells and playing a critical role in the development of type 1 diabetes (T1D). The activation of this receptor also stimulates the production of cytokines like interferon- γ , further enhancing the immune response and inflammation. Moreover, CXCR3 is involved in the development of insulinitis, affecting the function and survival of β cells, as β cells themselves can produce CXCL10, which in turn inhibits their proliferation. Certain viral infections can also induce β cells to produce CXCL10, attracting immune cells and exacerbating inflammation. Thus, CXCR3 not only promotes autoimmune attacks on pancreatic β cells but may also contribute to disease progression by influencing cytokine production and β cell self-regulation. This makes CXCR3 a potential therapeutic target, and blocking CXCR3 may slow or prevent the development of type 1 diabetes (Antonelli et al., 2014).

4.5 Cardiovascular diseases

CXCR3 plays a key role in cardiovascular diseases, such as atherosclerosis, cardiac hypertrophy, heart failure, cardiac transplant rejection, and myocardial infarction, through its interaction with various ligands. In atherosclerosis, CXCR3 and its ligands influence plaque formation and rupture. In cardiac hypertrophy and heart failure, elevated levels of CXCR3 ligands are associated with disease progression. In cardiac transplantation, increased expression of CXCR3 ligands is linked to transplant rejection, while in myocardial infarction, the recruitment of

CXCR3-positive cells is critical for cardiac repair (Altara et al., 2018). Additionally, CXCR3 and its ligands are involved in other cardiovascular conditions such as hypertension and myocarditis, indicating that they not only play a significant role in the onset and progression of these diseases, but also may serve as therapeutic targets, offering potential new strategies for the treatment of cardiovascular diseases (Satarkar and Patra, 2022).

5 CXCR3 small molecular inhibitors

In mouse disease models, antibodies or small molecule antagonists of CXCR3 have been shown to significantly delay the progression of diseases such as atherosclerosis, transplant rejection, and cancer, making CXCR3 antagonists a key focus in drug discovery. Several classes of small molecule CXCR3 antagonists have been developed, three of which have demonstrated efficacy in preclinical models, though only two have progressed to clinical trials (Yuan, 2024). These antagonists include piperazine-based piperidine compounds (e.g., SCH 546738) and 8-aziquinazoline derivatives (e.g., AMG487). The diversity of CXCR3 antagonists complicates the establishment of a universal pharmacophore model, and newly emerging ligands do not always contain highly basic

groups. Structural and spatial differences between small molecule antagonists and chemokines may lead to distinct binding sites, such as the binding site of TAK-779 with CCR5 [130]. Antagonists with different structures may act as inverse agonists on active mutants of CXCR3, providing new directions for future drug design (Ahmad et al., 2023).

5.1 Specific antagonists

5.1.1 Piperazine-based piperidine compounds

Ligands based on piperazine-piperidine scaffolds were identified as CXCR3 antagonists between 2006 and 2009, leading to the development of compounds 1 through 6, as shown in Figure 4A.

SCH 546738 (Compound 1, Table 2) is a piperazine-piperidine-based CXCR3 antagonist with a high affinity of 0.4 nM. It effectively non-competitively displaces radiolabeled CXCL10 and CXCL11 ($IC_{50} = 10$ nM) and significantly inhibits CXCR3-mediated chemotaxis in activated T cells. In autoimmune disease models in mice and rats, compound 1 alleviated disease symptoms and demonstrated favorable pharmacokinetic properties (Poulet et al., 2010). These findings suggest that compound 1 has potential as a therapeutic agent for autoimmune diseases and the prevention of

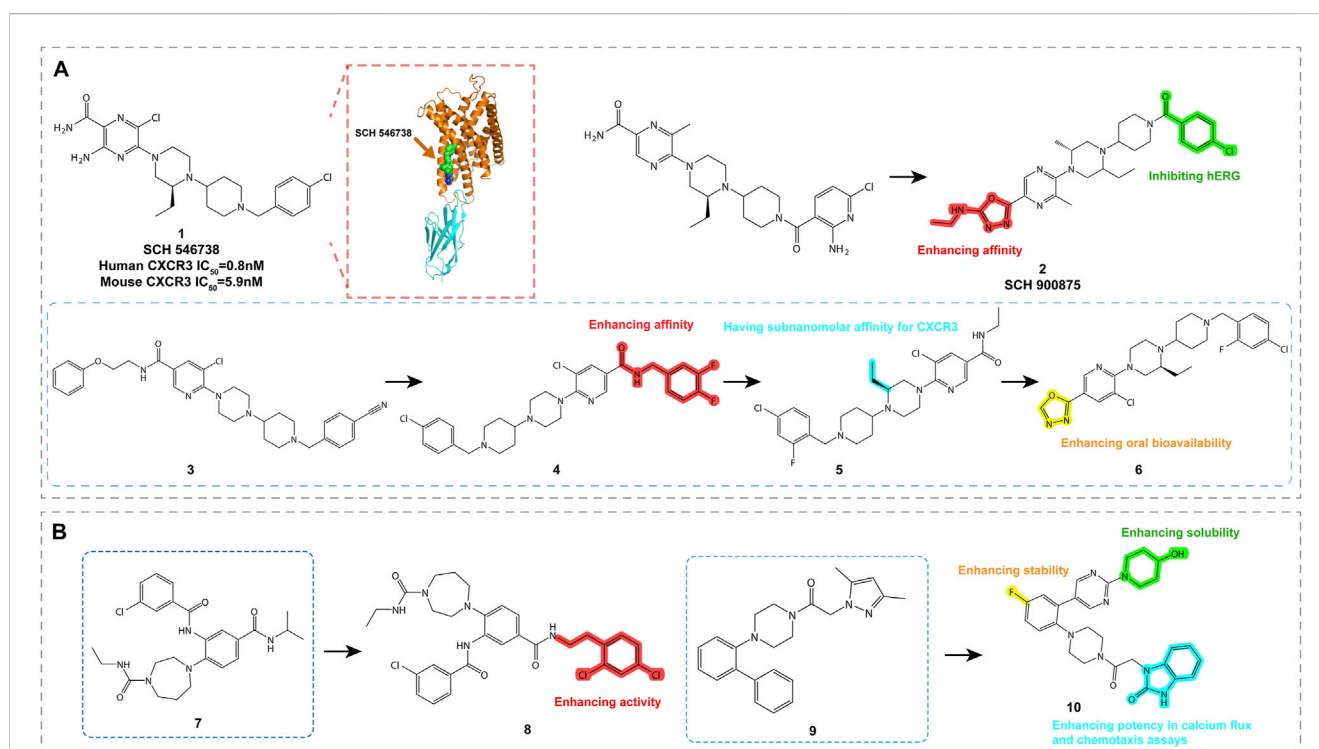


FIGURE 4

Structures and Optimization Pathways of Piperazine-based Piperidine Compounds. (A) Structure and optimization of piperazine-based piperidine compounds. For compound 3, the chloro-benzyl group is a key moiety for enhancing the compound's affinity. While shortening the N-benzylamide group decreases its affinity, introducing small alkyl groups, particularly (S)-substituents on the piperazine ring, significantly improves its *in vitro* activity. Halogenation of the benzamide group in compound 4 strengthens its binding affinity to CXCR3, and substituting the amide group with heterocyclic moieties increases its oral bioavailability. (B) Structure and optimization of arylpiperazine compounds. Compounds 7 and 8 contain amide or aryl groups at the ortho position of homopiperazine, which is crucial for maintaining their activity. SAR studies of compound 9 indicate that the central phenyl ring can accommodate a third substituent, such as an amide group, similar to compounds studied by Ligand Pharmaceuticals, or a halogen atom, and can also be modified to include a pyridine ring. The central phenyl ring of compound 10 can tolerate additional substituents, such as halogens or amide groups, which provide opportunities to fine-tune the pharmacological properties of the compound.

TABLE 2 Summary of CXCR3 specific antagonists.

Structural class	Compound	Disease focus	Study setting	PK/PD characteristics	Key results
Piperazine-based Piperidine	SCH 546738 (1)	Autoimmune diseases, transplant rejection	<i>In vivo</i>	High affinity (0.4 nM), non-competitive ligand displacement (IC ₅₀ = 10 nM), favorable PK in rodents	Inhibiting T-cell chemotaxis; alleviating autoimmune symptoms; requiring safety/efficacy optimization for clinical trials
	SCH 900875 (2)	Autoimmune diseases (arthritis, EAE)	<i>In vivo</i>	High affinity, improved metabolic stability, moderate oral bioavailability	Effective in collagen-induced arthritis and EAE models; species-specific side effects in animals
	Compound 3	General CXCR3-related diseases	<i>In vitro</i>	High affinity (pK _i = 7.0), pIC ₅₀ values: 7.3 (CXCL9), 7.7 (CXCL10), 7.1 (CXCL11)	Chlorobenzyl group critical for affinity; low oral bioavailability requires optimization
	Compound 4	General CXCR3-related diseases ^a	<i>In vitro</i>	High affinity (pK _i = 7.4), poor oral PK (AUC = 0.92 μM h), significant hERG inhibition	Enhancing binding but poor metabolic stability; cardiac toxicity concerns
	Compound 5	General CXCR3-related diseases	<i>In vitro</i>	Extremely high affinity (pK _i = 9.5), poor oral bioavailability (AUC = 0.95 μM h)	Improving affinity via ethyl substitution, but PK remains suboptimal
	Compound 6	General CXCR3-related diseases	<i>In vitro</i>	Improved oral bioavailability (AUC = 1.8 μM h), high affinity (pK _i = 8.9), high hERG inhibition	Heterocyclic substituents enhanced PK; cardiac toxicity unresolved
Arylpiperazines	Compound 7	Inflammatory/ Autoimmune	<i>In vitro</i>	Amide group at ortho position; CXCR3 antagonistic activity	Foundational activity but lacks optimization
	Compound 8	Inflammatory/ Autoimmune	<i>In vitro</i>	Aryl group at ortho position; high activity (pIC ₅₀ = 7.2), selectivity over 14 GPCRs	Optimized version of Compound 7; improving activity and selectivity
	Compound 9	Inflammatory/ Autoimmune	<i>In vitro</i>	Submicromolar activity; low metabolic stability (human liver microsomes), high permeability (PAMPA)	Demonstrating permeability but requiring metabolic stability optimization
	Compound 10	Inflammatory/ Autoimmune	<i>In vitro</i>	Improved solubility and bioavailability; pIC ₅₀ = 8.0 (calcium flux), 7.7 (chemotaxis)	Enhancing PK properties compared to Compound 9; remaining unstable in human liver microsomes
Azaquinazolinone	Compound 11	Inflammatory diseases	<i>In vitro/in vivo</i>	Submicromolar activity, low oral bioavailability in rats	Initial lead; foundational for optimization
	AMG 487 (12)	Psoriasis, inflammatory diseases	Phase II clinical trial	Time-dependent CYP3A4 inhibition (via M4 metabolite), variable human exposure	Failed Phase II due to inconsistent PK; inhibited CXCR3-mediated migration
	Compound 13	Inflammatory diseases	<i>In vitro/in vivo</i>	Improved stability and bioavailability, avoided CYP3A4 inhibition	Potent CXCR3 antagonism; advanced from PK optimization
	Compound 14	CXCR3-related diseases	<i>In vivo</i>	Good oral bioavailability, efficacy in preclinical models	Promising candidate for CXCR3-related diseases
	Compound 15	Transplant rejection	<i>In vivo</i>	Prolonged graft survival, reduced IFN-γ-producing T cells	Synergistic with anti-CD154 mAbs; potential for immunoregulation
	Compound 16	Inflammatory diseases	<i>In vitro/in vivo</i>	Strong affinity (IC ₅₀ = 1.0 nM), low clearance, good oral bioavailability	Inhibiting bleomycin-induced inflammation; lower plasma concentration vs AMG487
	Compound 17	Inflammatory diseases	<i>In vitro/in vivo</i>	High affinity (IC ₅₀ = 0.9 nM), low clearance, moderate oral bioavailability	Effective in pulmonary inflammation models; low drug-drug interaction risk
	Compound 18	CXCR3-related diseases	<i>In vitro</i>	High CXCR3 affinity, inverse agonistic effects	Inhibiting constitutive receptor signaling; novel mechanism

(Continued on following page)

TABLE 2 (Continued) Summary of CXCR3 specific antagonists.

Structural class	Compound	Disease focus	Study setting	PK/PD characteristics	Key results
	Compound 19	Autoimmune/inflammatory diseases	<i>In vitro/in vivo</i>	High affinity (IC ₅₀ = 12 nM), low clearance, reduced metabolic risk	Improving solubility/stability; reducing glutathione binding for safety
	Compound 20	Autoimmune/inflammatory diseases	<i>In vitro/in vivo</i>	High affinity (IC ₅₀ = 7.8 nM), low clearance, reduced metabolic risk	Improving solubility/stability; reducing glutathione binding for safety
	JN-2 (21)	Breast cancer bone metastasis	<i>In vitro/in vivo</i>	Inhibiting CXCL10/CXCR3/NF-κB axis	Reducing breast cancer cell motility and bone destruction
	NBI-74330 (22)	Rheumatoid arthritis	<i>In vitro</i>	Binding activity to CXCR3, unresolved PK/safety issues	Tool compound for design insights; not advanced clinically
Arylsulfonamide	Compound 23	CXCR3-related diseases	<i>In vitro</i>	Sub-micromolar activity; optimized to amide (pIC ₅₀ = 7.9), poor stability	Structural optimization improved activity; intermediates enhanced stability
	Compound 24	CXCR3-related diseases	<i>In vivo</i>	Favorable PK in mice; improved stability via isoelectronic analogs	Promising PK profile but lacking further development data
	Compound 25	CXCR3-related diseases	<i>In vitro</i>	High activity (IC ₅₀ = 13 nM in chemotaxis), poor metabolic stability	Key SAR insights: N-substituent (R3) and sulfonamide ring (R1) critical; tetrazole derivatives improved stability/solubility. Not clinically viable but a lead for future optimization
Spiropiperidine	Compound 26	Inflammatory/Immune-related diseases	<i>In vitro</i>	pIC ₅₀ = 6.9 (human CXCR3 calcium flux assay); limited <i>in vivo</i> PK/PD data	High antagonistic potency; SAR insights for future design
Benzimidazole	Compound 27	Inflammatory diseases	<i>In vitro</i>	Sub-micromolar affinity; substituents critical for affinity/solubility	Identified key SAR; foundational for optimization
	Compound 28	Inflammatory diseases	<i>In vitro</i>	IC ₅₀ = 80 nM; good oral bioavailability, limited stability in rat liver microsomes	Functional antagonist; improving PK but metabolic instability
	Compound 29	Inflammatory diseases	<i>In vitro</i>	Higher affinity (human/mouse CXCR3); metabolically unstable	Effective functional antagonist; guiding further optimization
Phenylethylpiperidine Derivatives	Compound 30	Inflammatory diseases	<i>In vitro</i>	Most active in HTS; enantiomer-specific muscarinic receptor activity	SAR revealed optimal aromatic ring substitution; retaining activity
	Compound 31	Inflammatory diseases	<i>In vitro</i>	Derived from SAR of Compound 30; selectivity undisclosed	Structural insights for CXCR3 antagonist design
1-Phenyl-3-piperidin-4-ylurea Derivatives	Compound 32	Inflammatory diseases	<i>In vitro</i>	High lipophilicity; prioritized for analog synthesis	Key lead molecule; enabling rapid structural optimization
	Compound 33	Inflammatory diseases	<i>In vitro</i>	IC ₅₀ = 16 nM; improved solubility, <i>in vitro</i> stability, low CYP inhibition	Promising drug template with balanced PK/PD.
	Compound 34	Inflammatory diseases	<i>In vitro/in vivo</i>	Antagonistic activity in CXCR3 internalization assays; good <i>in vivo</i> characteristics	Validated in receptor internalization models; potential for clinical translation
	Compound 35	Inflammatory diseases	<i>In vivo</i>	Good PK in mice; complete inhibition of CXCR3 internalization	Advanced candidate with robust <i>in vivo</i> efficacy

*CXCR3-related diseases: Includes cancers, inflammatory diseases, autoimmune disorders, neurodegenerative conditions, diabetes, cardiovascular diseases, and transplant rejection.

transplant rejection. However, despite its positive effects in preclinical models, compound 1 requires further optimization before clinical trials to ensure its safety and efficacy in humans, as well as to explore potential combinations with other therapeutic modalities (Jenh et al., 2012).

Through structure-activity relationship (SAR) studies and chemical modifications, including changes to the benzyl group

(Nair et al., 2014) and optimization of halogenated benzamide derivatives, SCH 900875 (compound 2, Table 2) was developed. Compound 2 exhibits high affinity and good *in vitro* activity. In animal models, compound 2 effectively treats various autoimmune diseases, including collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE). Despite showing potential side effects in animal models, possibly due to alterations in the

membrane lipid composition of red blood cells and platelets, its effects in humans may vary due to species differences.

Compound **3** (Table 2) was identified through high-throughput screening and demonstrated high affinity in radioligand binding assays ($pK_i = 7.0$). It effectively antagonizes the binding of CXCL9, CXCL10, and CXCL11 to CXCR3, with pIC_{50} values of 7.3, 7.7, and 7.1, respectively (McGuinness et al., 2009; Shao et al., 2010; Kim et al., 2011; Nair et al., 2014). SAR studies of this compound indicated that the chlorobenzyl group is critical for its high affinity. Despite its strong *in vitro* activity, the oral bioavailability of compound **3** needs further improvement. These findings provide important insights for the further optimization of CXCR3 antagonists.

Through halogenated benzylamide substitution on compound **3**, compound **4** (Table 2) was developed, which exhibited high affinity ($pK_i = 7.4$), but poor pharmacokinetics upon oral administration ($AUC = 0.92 \mu M h$) (Shao et al., 2010). Although structural modification enhanced binding to CXCR3, metabolic stability was not improved, and the compound exhibited significant hERG inhibition. Further shortening the benzylamide group to an ethyl group resulted in compound **5** (Table 2), which displayed extremely high affinity ($pK_i = 9.5$) *in vitro*, but no improvement in oral bioavailability ($AUC = 0.95 \mu M h$) (Mladic et al., 2015). Compound **6** (Table 2), achieved by replacing the amide group with heterocyclic substituents, improved both oral bioavailability ($AUC = 1.8 \mu M h$) and affinity ($pK_i = 8.9$), but still exhibited high hERG inhibitory activity (Kim et al., 2011). These compounds provide promising candidates for the treatment of CXCR3-related diseases, although further optimization is required to balance metabolic stability and cardiac toxicity.

5.1.2 Arylpiperazines

Aryl piperazine compounds and their derivatives have been developed as CXCR3 antagonists, with these compounds featuring amide or aryl groups at the ortho position of the piperazine ring. High-throughput screening identified compounds **7** and **8** (Figure 4B; Table 2), which belong to the arylpiperazine class and contain either an amide or aryl group. Compound **7** demonstrated CXCR3 antagonistic activity, while compound **8**, as an optimized version, showed high activity in *in vitro* assays ($pIC_{50} = 7.2$) and exhibited good selectivity over 14 other GPCRs (Cole et al., 2006; Afantitis et al., 2009).

Compound **9** (Figure 4B; Table 2), an aryl piperazine derivative discovered by Boehringer Ingelheim, exhibited submicromolar activity. Although its stability in human liver microsomes was low, it demonstrated high permeability in PAMPA (parallel artificial membrane permeability assay) screening (Prokopowicz et al., 2024). Compound **10** (Table 2), an improved version of compound **9** (Figure 4B), exhibited better solubility and bioavailability, as well as good potency in calcium flux and chemotaxis assays (pIC_{50} values of 8.0 and 7.7, respectively). These characteristics make compound **10** a promising candidate for CXCR3 antagonism research, despite its lower stability in human liver microsomes (Gottschling et al., 2009).

5.1.3 Azaquinazolinone compounds

Quinazolinone-based compounds are a significant area of research for CXCR3 antagonists, with compounds **11**, **12**, and **13**

(Figure 5, Table 2) discovered and optimized by ChemoCentryx and Amgen. These 8-azaquinazolinone compounds were identified through high-throughput screening and optimized for CXCR3 receptor antagonism.

Compound **11** was the initial active compound identified through high-throughput screening, exhibiting submicromolar activity and effectively blocking the binding of CXCR3 to its ligands. Although its oral bioavailability in rats was low, its discovery provided a starting point for further optimization (Johnson et al., 2007). After several rounds of optimization and efficacy validation in a bleomycin-induced murine model of pulmonary cell recruitment, 8-nitroquinazolinone, AMG487 (compound **12**), was selected as the clinical candidate. It demonstrated inhibition of CXCR3-mediated cell migration in both *in vitro* and animal models, showing potential for the treatment of various inflammatory diseases (Tonn et al., 2009). Further studies revealed that the M2 phenolic metabolite, M4, played a key role in the time-dependent inhibition (TDI) of CYP3A4, covalently binding to the Cys239 residue of CYP3A4 and leading to enzyme inactivation. Additionally, M4 was further metabolized to form M5, and these complex metabolic pathways collectively influenced the human pharmacokinetics of compound **12** (Henne et al., 2012). However, despite promising results in preclinical models, compound **12** failed to demonstrate efficacy in a Phase IIa clinical trial for psoriasis, possibly due to its time-dependent pharmacokinetic characteristics leading to variable exposure. As a result, further development of the compound was discontinued (Horuk, 2009).

To address the pharmacokinetic issues associated with compound **12**, researchers optimized compounds containing ethylsulfonyl groups and explored new core structures to improve stability. This led to the discovery of cyanide-substituted compounds that effectively avoided CYP3A4 inhibition, resulting in the development of compounds **13** and **14** (Figure 5; Table 2) (Chen et al., 2012). Compound **13** exhibited potent CXCR3 antagonistic activity in *in vitro* assays and, through structural optimization, demonstrated improved *in vivo* stability and bioavailability. Compound **14**, on the other hand, showed good oral bioavailability and efficacy in preclinical models, supporting its potential as a candidate for the treatment of CXCR3-related diseases.

Compound **15** (Figure 5; Table 2), a new non-peptide small molecule CXCR3 antagonist, prolonged the survival time of allogeneic hearts in a mouse heart transplant model, potentially offering therapeutic effects in preventing acute transplant rejection (Rosenblum et al., 2009). Moreover, compound **15** reduced the frequency of interferon- γ -producing donor-reactive T cells, and when used in combination with anti-CD154 monoclonal antibodies, significantly extended graft survival. These findings provide new insights into the role of CXCR3 in transplant immunology and offer a scientific basis for the development of novel immunoregulatory therapeutic strategies. Future research should further investigate the mechanism of action of compound **15** and its potential application in clinical transplantation, particularly regarding its efficacy and safety when used in combination with other immunosuppressive agents. Additionally, compound **15** was shown to inhibit CXCL9, CXCL10, and CXCL11-induced cell migration *in vitro*, disrupt the development of donor-

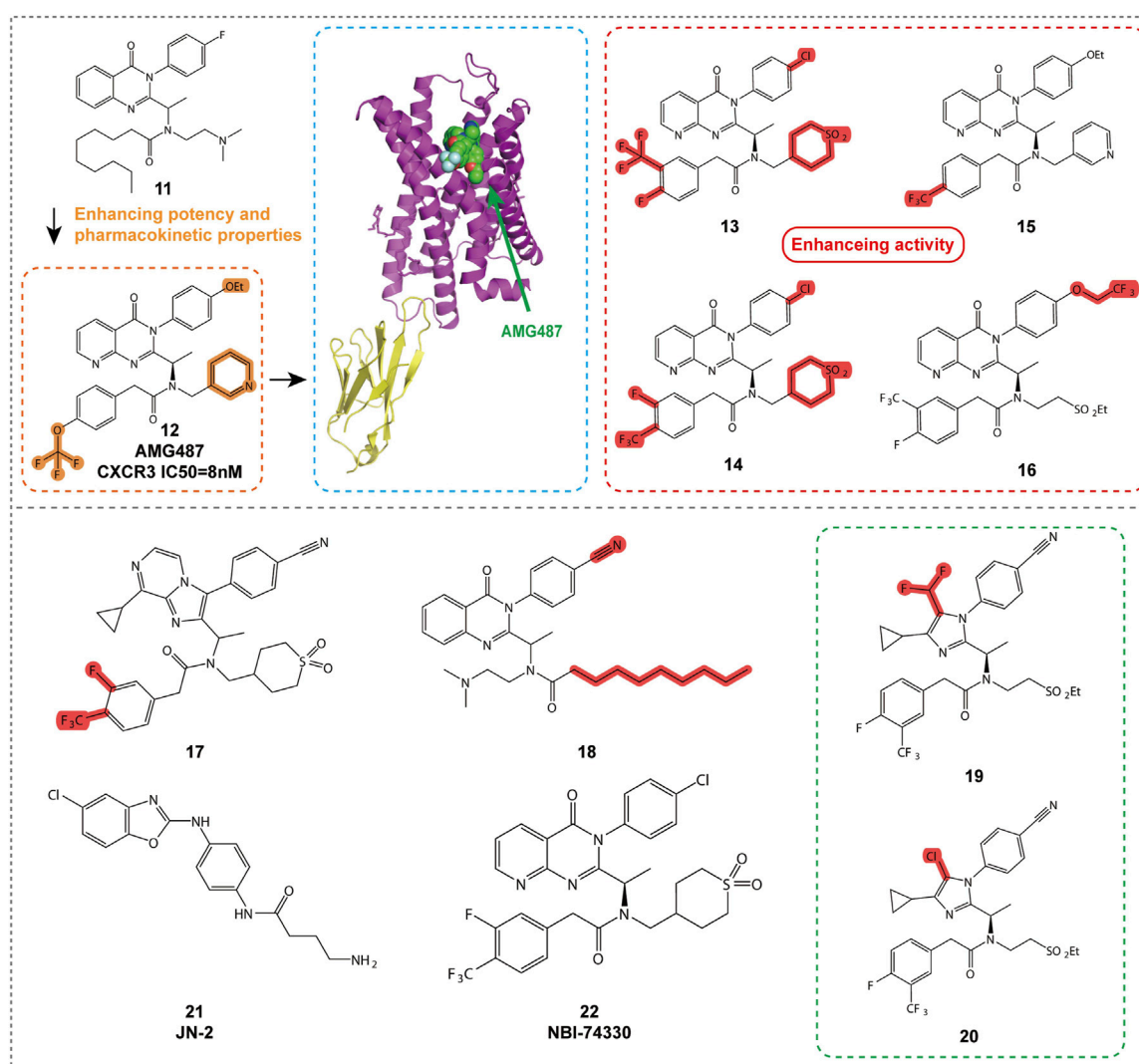


FIGURE 5
Structures and Optimization Pathways of 8-Azaquinazolinone Compounds.

specific responses and IFN- γ production, and prolong graft survival. These results suggest that CXCR3 may serve as a viable therapeutic target for preventing acute organ rejection.

Compound 16 (Figure 5; Table 2) demonstrates excellent pharmacological properties and favorable pharmacokinetic characteristics. *In vitro* studies show strong affinity for the CXCR3 receptor ($IC_{50} = 1.0$ nM) and excellent inhibition of cell migration ($IC_{50} = 0.8$ nM) (Liu et al., 2009). In multiple species, compound 16 exhibits low to moderate clearance rates and good oral bioavailability. It effectively inhibits bleomycin-induced cell infiltration and shows similar inhibitory effects to AMG 487, but with lower plasma concentrations.

Compound 17, with an imidazopyrazine core structure, demonstrates high affinity *in vitro* ($IC_{50} = 0.9$ nM) and strong inhibition of cell migration ($IC_{50} = 18.9$ nM). In pharmacokinetic studies in rats, compound 17 (Figure 5; Table 2) shows low clearance and moderate oral bioavailability, and it exhibits significant *in vivo* efficacy in a mouse model of pulmonary inflammation. It also presents a low risk of drug-drug interactions, making it a strong

candidate for the treatment of various inflammatory diseases (Du et al., 2009).

Compound 18 (Figure 5; Table 2) is a derivative of 3-phenyl-3H-quinazolin-4-one, exhibiting the highest CXCR3 receptor affinity among its class. It effectively blocks CXCR3-mediated calcium ion release, demonstrating moderate inhibitory activity ($K_i = VUF5834$). As a constitutively active mutant of compound 18, it shows complete inverse agonistic effects, inhibiting constitutive receptor signaling. This opens up new directions for the treatment of CXCR3-related diseases (Storelli et al., 2005).

Compounds 19 and 20 are imidazole derivatives, optimized to show high pharmacological activity and favorable pharmacokinetic properties. By introducing substituents on the imidazole ring, these compounds enhance affinity for the CXCR3 receptor and improve *in vivo* stability and solubility, while reducing metabolic activation risks. *In vitro* assays demonstrate that compounds 19 and 20 (Figure 5; Table 2) possess high affinity (IC_{50} values of 12 nM and 7.8 nM, respectively) and effectively inhibit CXCR3-mediated cell migration (Du et al., 2008). Pharmacokinetic studies show low

clearance in rats, moderate mean residence time, and good oral bioavailability, making them potential candidates for oral administration. The structural modifications of compounds **19** and **20** also reduce binding to glutathione, decreasing the risk of metabolic activation and improving drug safety. These compounds exhibit excellent characteristics both *in vitro* and *in vivo*, making them ideal tools for studying the role of CXCR3 in disease and valuable candidates for the development of new therapies for autoimmune and inflammatory diseases.

CXCL10 and its receptor CXCR3 play a crucial role in breast cancer bone metastasis and osteoclast activation. Researchers developed the CXCR3 antagonist JN-2 (compound **21**, Figure 5; Table 2), which was found to inhibit CXCL10 expression and the motility of breast cancer cells, reducing bone destruction. These findings reveal the role of the CXCL10/CXCR3/NF- κ B signaling pathway in breast cancer progression, providing a potential target for new therapeutic strategies (Kim et al., 2018; Jin et al., 2017).

Compound **22** (NBI-74330, Figure 5; Table 2), although showing binding activity to the CXCR3 receptor, was not advanced to clinical trials due to pharmacokinetic properties or potential side effects. Nevertheless, the research on this neutral 8-azaquinazolinone derivative provides important insights for the design of CXCR3 antagonists and may represent a new therapy for rheumatoid arthritis (Ahmad et al., 2023).

The introduction of small molecular substituents at the adjacent nitrogen atom on the left side of the core structure significantly enhances the CXCR3 antagonistic activity of compound **17**. Compound **19**, which contains a difluoromethyl substituent at position 5 of the imidazole ring, and compound **20**, which contains a chlorine substituent, both reduce binding to glutathione (GSH), thereby decreasing the risk of metabolic activation and improving the safety profile of the drugs. Compound **22** primarily occupies the minor binding site of the receptor, located around transmembrane regions (TMs) 2, 3, and 7.

5.1.4 Arylsulfonamide compounds

Compounds **23** and **24** (Figure 6A; Table 2) are CXCR3 antagonists discovered by Merck Serono, belonging to the aryloxy sulfonamide class of derivatives (Crosignani et al., 2010). During screening of 90,000 compounds, compound **23** exhibited sub-micromolar activity, and its structure was optimized into an amide without affecting activity, achieving a pIC₅₀ value of 7.9 for the optimized compound. Although these compounds have relatively poor stability, intermediate acids and their isoelectronic analogs (such as tetrazole) exhibited improved stability and activity. Compound **24** showed favorable pharmacokinetic properties in mice, although further studies have not been reported to date.

Compound **25** (Table 2) represents another important CXCR3 antagonist discovery, with an IC₅₀ value of 13 nM in chemotaxis assays, demonstrating excellent activity. This compound has specific requirements for the position of the amide N-substituent (R3) and the sulfonamide ring (R1), while the R2 position prefers aromatic or heteroaromatic groups with an unsubstituted methylene chain. Although compound **25** exhibits good receptor activity, its metabolic stability is poor. The research team employed various strategies to improve its metabolic stability, resulting in tetrazole derivatives with enhanced stability and solubility. Compound **25** provides valuable SAR insights for the design of CXCR3 antagonists.

Although it may not be suitable for direct clinical use, it offers a promising starting point for the discovery of new CXCR3 antagonists.

5.1.5 Spiropiperidine compounds

As shown in Figure 6B, compound **26** (Table 2) is a CXCR3 antagonist developed by Ono Pharmaceutical Company, belonging to the spiropiperidine class of compounds (Habashita and Shibayama, 2011). This compound exhibits a pIC₅₀ value of 6.9 in human CXCR3 calcium flux assays, indicating a high level of antagonistic potency. Although the *in vivo* pharmacodynamics and pharmacokinetic properties of these compounds have not been extensively reported, the discovery of compound **26** provides valuable SAR insights for the design of CXCR3 antagonists, which may aid in the development of drugs for the treatment of inflammatory and immune-related diseases in the future.

5.1.6 Benzimidazole compounds

Benzimidazole compounds are shown in Figure 6C. Compound **27**, (Table 2) discovered by Abbott Laboratories, is a CXCR3 antagonist and belongs to the benzimidazole derivative class, exhibiting sub-micromolar affinity. SAR studies reveal that certain substituents on the benzimidazole core are critical for maintaining both affinity and solubility (Hayes et al., 2008a). Compound **28** (Table 2), a derivative of compound **27**, serves as a functional antagonist with an IC₅₀ of 80 nM, demonstrating good oral bioavailability and half-life, but its stability in rat liver microsomes is limited (Hayes et al., 2008b). Further optimization led to the identification of compound **29** (Table 2), which exhibits higher affinity for both human and mouse CXCR3 and is an effective functional antagonist, although it remains metabolically unstable. These findings provide valuable information for the design of CXCR3 antagonists and the research of treatments for related inflammatory diseases (Hayes et al., 2008b).

5.1.7 Phenylethylpiperidine derivatives

Compounds **30** and **31** (Table 2) are CXCR3 antagonists discovered by Johnson & Johnson through high-throughput screening in CXCR3-transfected Chinese hamster ovary (CHO) cells, belonging to the phenylethylpiperidine derivative class, as shown in Figure 6D (Pierre Bongartz et al., 2008). After screening 256,000 compounds, these compounds exhibited activity against CXCR3, with compound **30** showing the most significant activity. Both enantiomers of compound **30** demonstrated CXCR3 inhibitory activity, but only one exhibited muscarinic receptor activity. SAR studies revealed the optimal substitution pattern for the two aromatic rings and indicated that the oxazine ring, when devoid of a carbonyl group, could retain activity as long as the nitrogen atom was substituted with an acyl or sulfonyl group. Compound **31** is a derivative derived from these conditions. Although compounds **30** and **31** (Table 2) exhibited CXCR3 activity and selectivity, other characteristics have not been disclosed. These compounds provide valuable structural insights for the drug design and development of CXCR3 antagonists, contributing to future research on the treatment of CXCR3-related inflammatory diseases.

5.1.8 1-Phenyl-3-piperidin-4-ylurea derivatives

Compound **32** (Figure 6E; Table 2) is a CXCR3 antagonist discovered by UCB after screening 15,000 compounds, representing

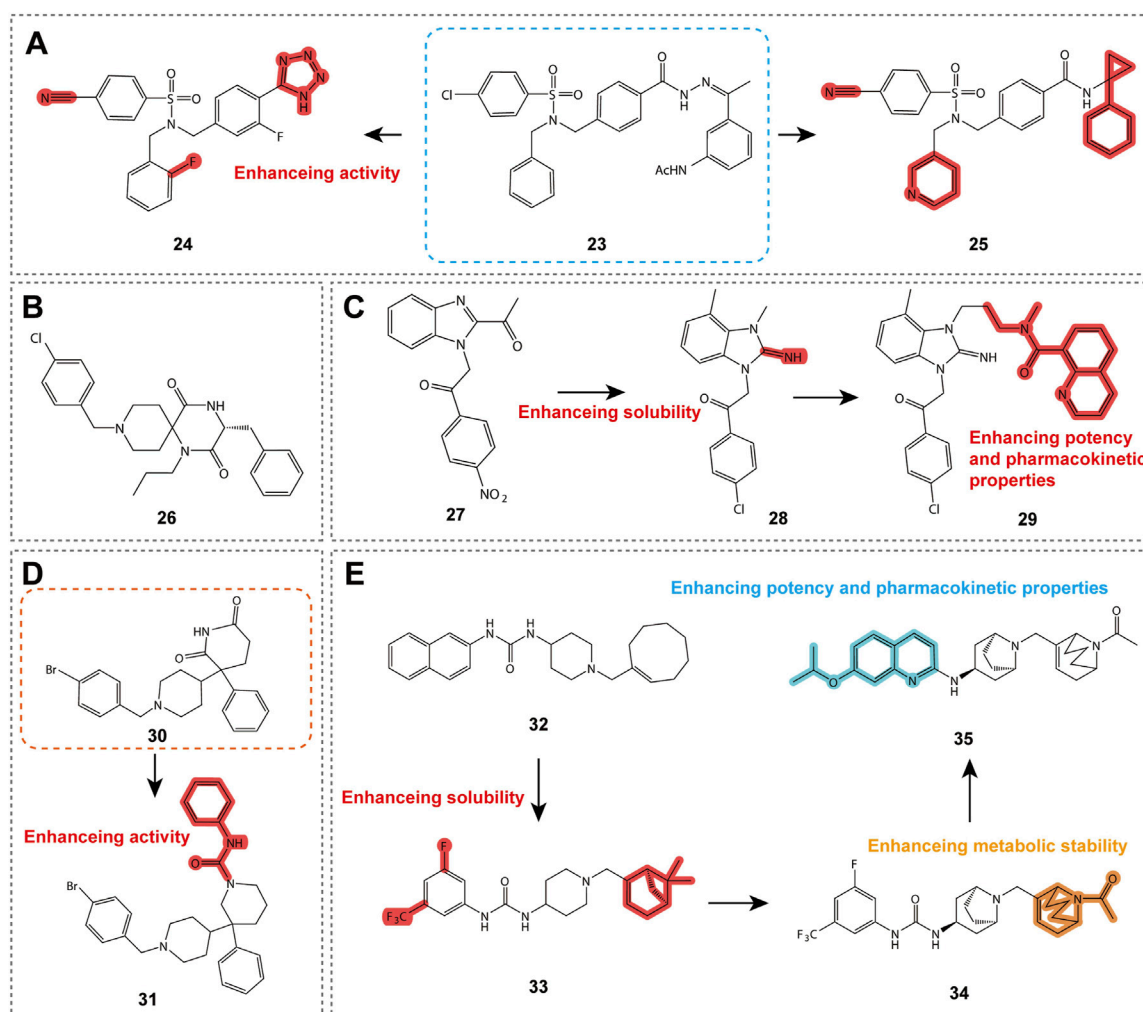


FIGURE 6

Structures and optimization pathways of aryl sulfonamide compounds, spiropiperidine compounds, benzimidazole derivatives, phenylethylpiperidine derivatives, and 1-phenyl-3-piperidin-4-ylurea derivatives. **(A)** Structure and optimization of aryl sulfonamide compounds. Replacing the acetohydrazone structure of compound **23** with an amide does not affect its CXCR3 antagonistic activity. In the design of compound **25**, a diversity exploration was conducted on the amide N-substituent R3, revealing a preference for more hydrophobic groups, particularly thiophene methylene and 1-phenyl-cycloprop-1-yl groups. Additionally, compound **25** contains a crucial chlorine atom at the R1 position on the sulfonamide ring, which is essential for maintaining the activity of the compound. Furthermore, a second benzyl substituent R2 on the sulfonamide nitrogen also shows some tolerance, with a preference for aromatic or heteroaromatic groups containing an unsubstituted methylene chain, especially the 2-pyridyl group. **(B)** Spiropiperidine compound structure. The different cyclic structures on the piperidine ring of compound **26** may be related to its potential binding to the CXCR3 receptor. **(C)** Structure and optimization of benzimidazole compounds. SAR studies show that the small aliphatic substituent at the C-4 position of compound **27** enhances its potency, leading to the discovery of compound **28**. **(D)** Structure and optimization of phenylethylpiperidine derivatives. **(E)** Structure and optimization of 1-phenyl-3-piperidin-4-ylurea derivatives. For compound **34**, acylated piperidine exhibits superior CXCR3 activity compared to the corresponding piperidine.

a key molecule within a series of compounds (Allen et al., 2007). Despite its high lipophilicity, compound **32** was selected for further study due to its molecular properties and potential for rapid analog synthesis. The improved compound **33** (Figure 6E; Table 2) demonstrated high activity ($IC_{50} = 16$ nM), along with improved physicochemical properties, good *in vitro* stability, and low CYP inhibition, making it a promising template for drug development (Watson et al., 2007). Further research led to the discovery of compound **34** (Figure 6E; Table 2), which exhibited good characteristics both *in vitro* and *in vivo*, and demonstrated antagonistic activity in CXCR3 receptor internalization assays *in vivo*. The latest findings include compound **35** (Table 2), which showed good pharmacokinetic properties in mice and completely

inhibited CXCR3 internalization following administration. These compounds provide valuable information for the drug design of CXCR3 antagonists and the study of treatments for related inflammatory diseases.

5.2 Non-Specific Antagonists (broad-spectrum antagonists)

5.2.1 Quaternary ammonium derivatives

As shown in Figure 7A, TAK-779 (compound **36**, Table 3) is a broad-spectrum small-molecule chemokine receptor antagonist that

antagonizes CXCR3, CCR5, and CCR2b. Initially developed as a CCR5 antagonist to inhibit HIV-1 entry, TAK-779 also antagonizes CCR2 and CXCR3, demonstrating potential for treating a range of Th1 cell-mediated diseases such as rheumatoid arthritis and cancer (Nedjai et al., 2015). In a rat small intestine transplantation model, TAK-779 significantly prolonged graft survival, reduced the number of infiltrating cells at the transplant site, and decreased the expression of CCR5 and CXCR3. Additionally, it reduced the total number of T cells involved in transplant rejection (Xu et al., 2008). Furthermore, TAK-779 was shown to reduce the migration of inflammatory cells to the central nervous system, decreasing the

incidence and severity of experimental autoimmune encephalomyelitis, without affecting T cell function (Ni et al., 2009). When used in combination with FK506, TAK-779 significantly prolonged allogeneic small intestine graft survival, with TAK-779 inhibiting T cell migration to the graft by blocking CCR5 and CXCR3 (Takama et al., 2011). These characteristics make TAK-779 a promising therapeutic strategy.

5.2.2 Camphor sulfonamide derivatives

As shown in Figure 7B, compounds 37 and 38 (Table 3) are CXCR3 antagonists discovered by GlaxoSmithKline, with compound

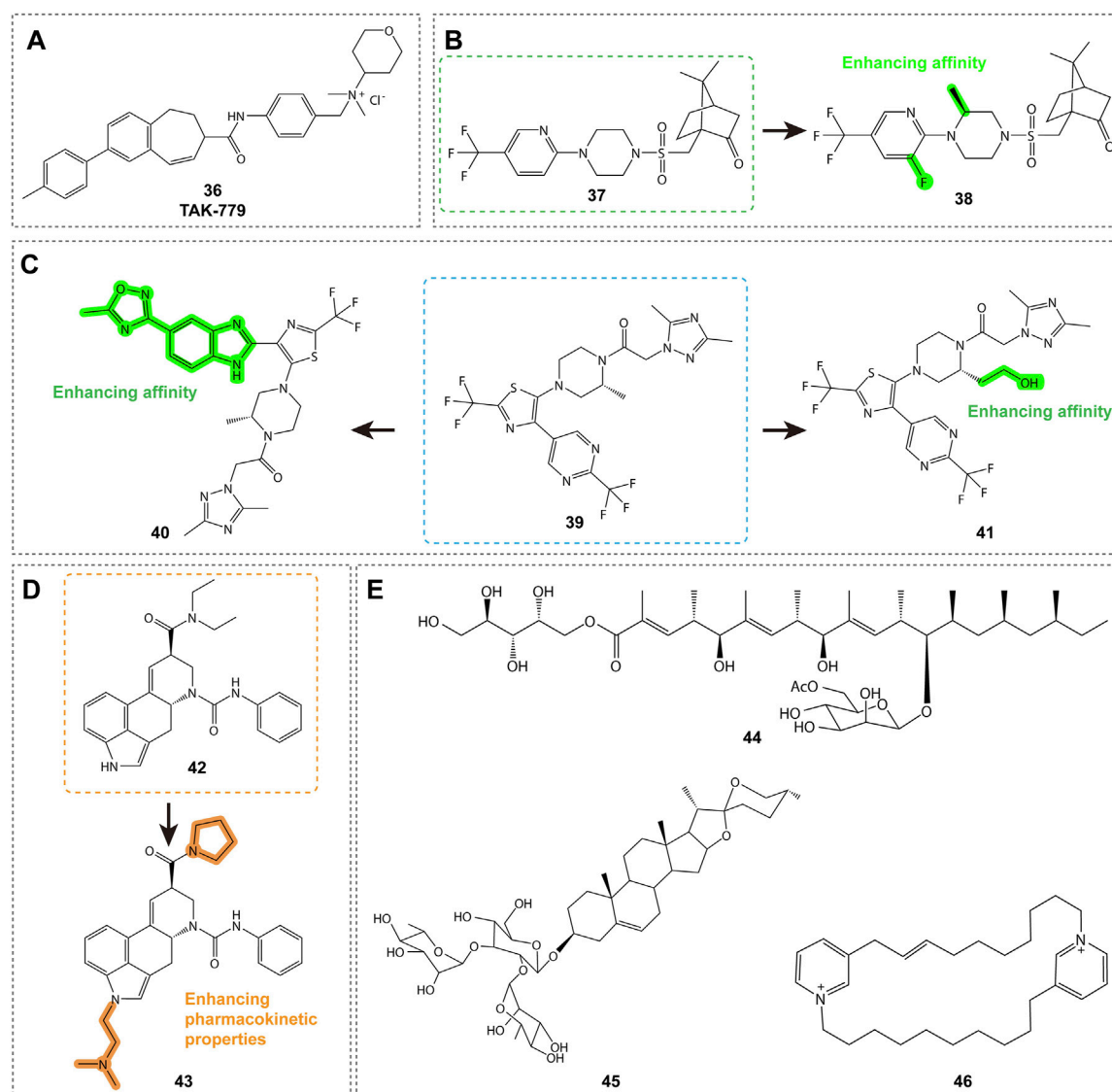


FIGURE 7

Structures and Optimization Pathways of Non-Specific Antagonists and Natural Products. **(A)** Quaternary Ammonium Salt Derivatives: The binding site of compound 36 to CXCR3 differs from that of another antagonist, VUF10085, in that it does not rely on the key amino acid residues that are essential for VUF10085 binding. **(B)** Camphor Sulfonamide Compounds: The structure and optimization of camphor sulfonamide derivatives. Compound 38 was discovered by systematically modifying the phenyl ring, piperidine ring, and camphor bicyclic structure of compound 37, incorporating optimal substituents. **(C)** Benzimidazole Derivatives: Structure and optimization of benzimidazole-based CXCR3 antagonists. **(D-E)** Natural Products: Structure and optimization pathway of natural products. For compound 42, replacing the N,N-diethylamide group with a pyrrolidinamide resulted in a tenfold increase in activity. Introducing hydrophobic substituents on the indole nitrogen of compound 42 led to reduced activity, while incorporating polar substituents not only maintained activity in binding and cell assays but also enhanced activity in whole blood experiments.

TABLE 3 Summary of CXCR3 non-specific antagonists (broad-spectrum antagonists).

Structural class	Compound	Disease focus	Study setting	PK/PD characteristics	Key results
Quaternary Ammonium Derivatives	TAK-779 (36)	HIV-1 entry; Th1 cell-mediated diseases (rheumatoid arthritis, cancer); transplant rejection; autoimmune encephalomyelitis	<i>In vitro/in vivo</i>	Not explicitly described	Prolonging graft survival; reducing infiltrating cells and CCR5/CXCR3 expression; suppressing T cell migration; reducing EAE severity; synergy with FK506
Camphor Sulfonamide Derivatives	Compound 37	Th1 cell-mediated diseases (implied via CXCR3 antagonism)	<i>In vitro</i>	Not described	Initial hit from HTS; structural precursor to compound 38
	Compound 38	Th1 cell-mediated diseases	<i>In vivo</i>	Low solubility; short half-life; low bioavailability	Moderate CXCR3 antagonism; good selectivity; unfavorable PK in rats; potential for structural optimization
Benzimidazole Derivatives	ACT-660602 (39)	Lung inflammation	<i>In vitro</i>	Improved metabolic stability; low hERG inhibition; primarily metabolized by CYP2D6 (polymorphic)	Reduced CXCR3+ CD8 ⁺ T cell recruitment; halted due to variable plasma exposure from CYP2D6 metabolism
	ACT-672125 (40)	Lung inflammation	<i>In vivo</i>	Not described	Inhibited CXCR3+ T cell recruitment
	ACT-777991 (41)	Recent-onset type 1 diabetes	<i>In vivo/in vitro</i>	Excellent safety profile; favorable pharmacokinetics	Dose-dependent efficacy and target engagement; progressed to Phase I with strong safety profile

37 being a camphor sulfonamide derivative identified through high-throughput screening (Wang et al., 2009). Compound 38 is an optimized version of compound 37, showing moderate CXCR3 antagonistic activity and good selectivity, but exhibiting unfavorable pharmacokinetic properties in rats, including low solubility, short half-life, and low bioavailability. While there is potential for improving these properties through structural modifications, specific details have not yet been disclosed.

5.2.3 Benzimidazole derivatives

Compound 39 (ACT-660602, Figure 7C; Table 3) is a CXCR3 antagonist with high biological activity, effectively inhibiting cell migration while improving metabolic stability and cardiac safety (low hERG channel inhibition) (Meyer et al., 2022). In a mouse lung inflammation model, a 30 mg/kg dose of ACT-660602 significantly reduced the recruitment of CXCR3+ CD8⁺ T cells. Despite favorable results from *in vitro* cardiac safety and toxicity assessments, the preclinical development of compound 39 was halted due to its primary metabolism via the polymorphic cytochrome P450 enzyme 2D6, which can lead to highly variable plasma exposure.

CXCR3 antagonists 40 (ACT-672125) and 41 (ACT-777991) were shown to inhibit the recruitment of CXCR3+ T cells in a lung inflammation model (Figure 7C; Table 3) (Caroff et al., 2022). Notably, compound 41 has progressed to Phase I clinical trials as a clinical candidate for the treatment of recent-onset type 1 diabetes. It demonstrated dose-dependent efficacy and target engagement, with excellent characteristics and safety profile.

5.3 Natural products

Natural products are shown in Figures 7D-E. Compounds 42 and 43 (Table 4) are promising CXCR3 antagonists discovered by

Novartis (Thoma et al., 2009; Rolf et al., 2006). Compound 42 is an ergot alkaloid derivative with high selectivity, showing no binding to other GPCRs, and demonstrating metabolic stability in human microsomes (Auberson et al., 2014). Compound 43 exhibits favorable pharmacokinetic properties and is a promising tool compound for exploring the role of CXCR3 in disease models (Thoma et al., 2011).

Merck identified three potential CXCR3 antagonist molecules through screening 51,000 extracts: compound 44 (roselipins, Table 4) (Tomoda et al., 2003), compound 45 (a diosgenin glycoside, Table 4) (Watson et al., 2007), and compound 46 (a cyclic 3-alkylpyridinium salt natural product, Table 4) (Turk et al., 2008). These compounds exhibit binding activity to CXCR3, offering new directions for the design of CXCR3 antagonists and the study of related inflammatory diseases.

6 Conclusion

In recent decades, the role of CXCR3 and its ligands in oncology and inflammatory diseases has attracted extensive attention. CXCR3 not only plays a key role in the development and metastasis of various tumors, but it is also critically involved in the pathogenesis of inflammatory responses and autoimmune diseases. With a deeper understanding of the CXCR3 signaling pathway, small molecule antagonists targeting CXCR3 have emerged as a potential therapeutic strategy. Despite some success in preclinical models, the clinical application of CXCR3 antagonists still faces several challenges.

First, the selectivity and specificity of CXCR3 antagonists remain critical issues in drug development. Non-selective antagonists may interfere with other biological processes, leading to adverse effects. Therefore, developing highly selective CXCR3 antagonists is crucial to minimizing off-target effects and improving therapeutic efficacy.

TABLE 4 Summary of CXCR3 natural products.

Structural class	Compound	Disease focus	Study setting	PK/PD characteristics	Key results
Ergot Alkaloid Derivative	Compound 42	CXCR3-related diseases*	<i>In vitro</i>	High selectivity (no binding to other GPCRs); High metabolic stability	Highly selective CXCR3 antagonist with metabolic stability; potential tool compound for research
Natural Product	Compound 43	CXCR3-related diseases	<i>In vivo/ in vitro</i>	Favorable pharmacokinetic properties	Promising tool compound for investigating CXCR3's role in disease models
Natural Product	Compound 44	CXCR3-related diseases	<i>In vitro</i>	Not described	Exhibiting CXCR3 binding activity; providing novel insights for antagonist design and inflammatory disease research
Diosgenin Glycoside	Compound 45	CXCR3-related diseases	<i>In vivo/ in vitro</i>	Not described	Binding to CXCR3; supports development of CXCR3 antagonists and inflammatory disease studies
Cyclic 3-Alkylpyridinium Salt	Compound 46	CXCR3-related diseases	<i>In vivo/ in vitro</i>	Not described	Demonstrating CXCR3 binding activity; offering new directions for antagonist design and inflammation research

*CXCR3-related diseases: Includes cancers, inflammatory diseases, autoimmune disorders, neurodegenerative conditions, diabetes, cardiovascular diseases, and transplant rejection.

In addition, the pharmacokinetic and pharmacodynamic properties of the drugs are also important factors that determine their clinical application. Many promising candidate drugs fail in clinical trials due to poor pharmacokinetic properties or insufficient efficacy.

Looking to the future, the research on CXCR3 antagonists needs to progress in several key areas: 1) Target Validation and Disease Models: Further studies are needed to validate the role of CXCR3 in various diseases and to develop more relevant animal models and preclinical models that better mimic human diseases. 2) Drug Design and Optimization: Through structure-based drug design and computational chemistry methods, more precise small molecules can be designed to bind with the CXCR3 receptor, improving their affinity and selectivity. Additionally, optimizing the chemical structure of the drugs can improve their pharmacokinetic properties, enhance oral bioavailability, and address metabolic stability issues. 3) Combination Therapy: Considering the complex roles of CXCR3 in multiple cell types and signaling pathways, single-drug therapies may struggle to achieve optimal therapeutic outcomes. Therefore, exploring combination therapies involving CXCR3 antagonists and other drugs may enhance therapeutic efficacy, particularly in the treatment of tumors and autoimmune diseases.

Notably, recent preclinical studies have shown that β -arrestin-biased inhibitors such as ACT-777991 can specifically block the infiltration of CXCR3+ CD8⁺ T cells into the heart, significantly improving survival rates in models of immune checkpoint inhibitor-induced myocarditis (Wang et al., 2022). In contrast, Gai-biased compounds may be more suitable for inhibiting tumor metastasis. Future efforts should focus on combining cryo-electron microscopy structures of the CXCR3-B isoform to design subtype-specific biased inhibitors (Rajagopal et al., 2013).

In conclusion, while the research and development of CXCR3 antagonists face challenges, the continued advancement of science and technology, along with a deeper understanding of CXCR3's biological functions, may lead to more CXCR3 antagonists entering clinical application. This could offer new therapeutic options for patients with tumors and inflammatory diseases. The search for effective chemokine receptor candidates continues, and

the concept of receptor allosteric targeting may be a promising direction for future research.

Author contributions

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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.

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