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Targeting Hypoxia: Hypoxia-Activated Prodrugs in Cancer Therapy

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Hypoxia is an important characteristic of most solid malignancies, and is closely related to tumor prognosis and therapeutic resistance. Hypoxia is one of the most important factors associated with resistance to conventional radiotherapy and chemotherapy. Therapies targeting tumor hypoxia have attracted considerable attention. Hypoxia-activated prodrugs (HAPs) are bioreductive drugs that are selectively activated under hypoxic conditions and that can accurately target the hypoxic regions of solid tumors. Both single-agent and combined use with other drugs have shown promising antitumor effects. In this review, we discuss the mechanism of action and the current preclinical and clinical progress of several of the most widely used HAPs, summarize their existing problems and shortcomings, and discuss future research prospects.

Keywords: hypoxia, hypoxia-activated prodrugs, tirapazamine, AQ4N, PR-104, EO9, TH-302, SN30000

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INTRODUCTION

Hypoxia is a hallmark of a wide variety of solid tumors. In tumors, hypoxia arises due to a mismatch between oxygen delivery and consumption. Hypoxia is closely related to tumor progression, metastasis, therapeutic resistance, and poor prognosis (1). Hypoxia in tumor microenvironment leads to the transcriptional induction of a series of genes. The most important factor mediating this response is the hypoxia-inducible factor-1 (HIF-1), which extensively participates in glucose metabolism, angiogenesis, apoptosis, tumor metastasis and therapeutic resistance (2). Under hypoxic condition, HIF-1α regulates the switch from oxidative phosphorylation to anaerobic glycolysis, by activating the expression of glucose transporter 1 and 3 (GLUT-1 and GLUT-3) and related glycolytic enzymes (3). By regulating its downstream angiogenesis related genes, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMPs), HIF-1α is widely involved in every step of angiogenesis, including endothelial progenitor cells recruitment and their differentiation to endothelial cells and smooth muscle cells, degradation of extracellular matrix, and the stability of peripheral cells (4). HIF-1α could induce apoptosis by regulating p53, Bcl-2, BNIP-3 and other genes (5). Through induction of MMPs, E-cadherin, CXCR4, CA9, HIF could promote tumor invasion and metastasis by regulating epithelial-to-mesenchymal transition (EMT) (6).

Tumor cells response to hypoxia depends in part on the duration of exposure. Hypoxic tumor cells may undergo necrosis, but some of the tumor cells may adjust to hypoxic stress and survive, which is also mediated by HIF-1α, resulting in a more aggressive phenotype and therapeutic resistance (5). Hypoxia and HIF could induce cell cycle arrest and hypoxic tumor cells generally

have a relatively low proliferation rate (7, 8), while radiotherapy or chemotherapy mainly act on proliferating cells (9–11). Therefore, the hypoxic regions of tumors are usually insensitive to current radiotherapy and chemotherapy, and treatments targeting the hypoxic regions may provide additional clinical benefits. To this end, increasing efforts have been focused on the development of agents that selectively target and kill hypoxic tumor cells.

Hypoxia-activated prodrugs (HAPs), also referred to as bioreductive drugs, are compounds that can be selectively reduced by specific reductases under hypoxic conditions to form cytotoxic agents that precisely target hypoxic tumor cells and have little toxicity to normal tissue. At present, several classes of HAPs have been developed, including quinones, nitroaromatics, aliphatic N-oxides and hetero-aromatic N-oxides. The most representative ones are tirapazamine, AQ4N (banoxantrone), PR-104, EO9 (apaziquone), TH-302 (evofosfamide), and SN30000 (**Figure 1**). This review puts a special emphasis on the past achievements as well as limitations of HAPs and attempts to analyze the potential reasons for unsuccessful clinical trials, with the aim of guiding future investigations into optimizing the use of this therapeutic approach.

TIRAPAZAMINE

Tirapazamine (SR-4233, WIN 59075) [3-amino-1,2,4-benzotriazine-1,4 dioxide], the first hypoxia-activated prodrug, was reported in 1986 (12). Through one-electron reduction, the prodrug can generate an oxidative radical, which will diffuse into hypoxic regions and cause oxidative damage (13) (**Figure 2**). Cytochrome P-450 (CYP) is the main catalytic reductase involved in the reduction of tirapazamine (14). Although evidence showed that tirapazamine is a substrate for NAD(P)H: (quinone acceptor) oxidoreductase (DT-diaphorase) (15), the amount of DT-diaphorase expression in cells did not affect their sensitivity to tirapazamine (16).

Tirapazamine kills hypoxic cells by inducing chromosome aberrations and DNA double-strand breaks (17). Chromosome breaks caused by tirapazamine were more damaging and difficult to repair (18). Under hypoxic conditions, tirapazamine causes damage to both purine and pyrimidine residues in double-stranded DNA. DNA base damage was dominated by formation of formamidopyrimidine and 5-hydroxy-6-hydropyrimidine (19, 20). The DNA damaging activity of tirapazamine mainly results from radicals generated within the nucleus but not in the cytoplasm (21). Tirapazamine can induce acute changes in energy metabolism and intracellular pH in tumors (22). Skarsgard et al. (23) found that tirapazamine-induced DNA damage was pH-dependent (more effective at acidic pH) and could be repaired by certain gene products including uvrC and exonuclease III (24). The affinity of tirapazamine for hypoxic tissues was confirmed by many researchers but Durand and Olive demonstrated that this selectivity of tirapazamine was much lower *in vivo* (3 fold higher than aerobic) than that observed *in vitro* (50-500 fold) (25). Under aerobic conditions, tirapazamine can still induce cell cycle interruption and apoptosis, which may lead to its aerobic toxicity (26).

In preclinical studies, tirapazamine effectively inhibited tumor colony-forming *in vitro*, especially in hypoxic cells (27). Tirapazamine induced cell cycle arrest and apoptosis, and down-regulated HIF-1α, CA-IX and VEGF expression (28, 29). Brown (30) suggested that the activity of tirapazamine was p53-independent, but Yang's study on neuroblastoma revealed that tirapazamine had clinical activity only in p53-functional neuroblastoma (31). Zeman and Brown published a series of reports focusing on the radiosensitization effects of tirapazamine. They reported that tirapazamine enhanced radiation-induced

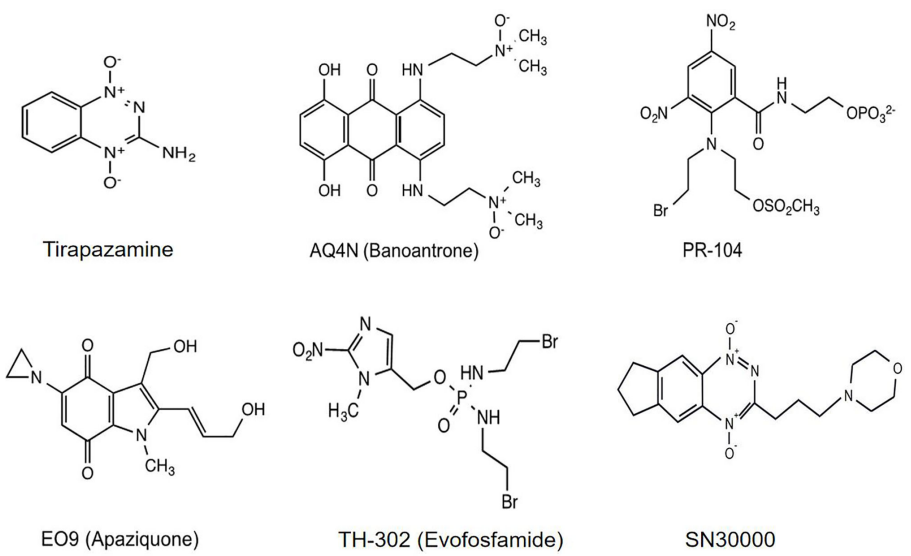

FIGURE 1 | Chemical structures of representative HAPs.

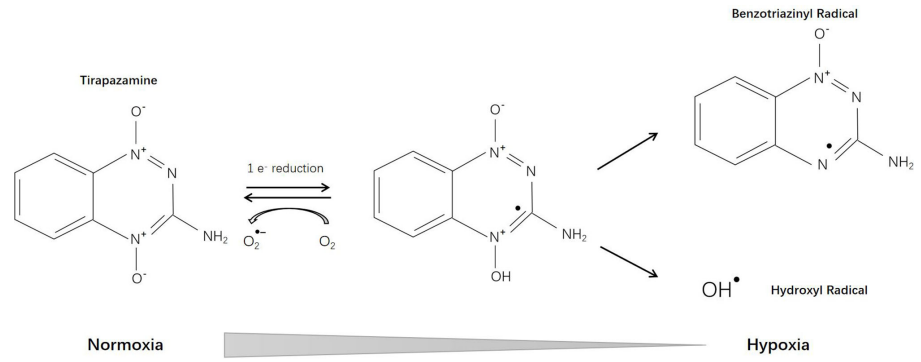

FIGURE 2 | Reductive reaction of tirapazamine.

antineoplastic effects while sparing normal tissues (12, 32–38). As flavone acetic acid (FAA) reduces the blood supply of tumors, tirapazamine in combination with FAA could significantly enhance the antineoplastic efficacy of both drugs (39). Many studies have investigated the synergistic effect of tirapazamine and chemotherapy (such as cyclophosphamide, cisplatin, paclitaxel, etc.) or radioimmunotherapy (40–45). Tirapazamine, together with hyperthermia, electric pulses, etc. also exhibited encouraging antineoplastic efficacy (46–49). However, studies conducted by Adam et al. (50, 51) demonstrated that tirapazamine plus cisplatin and/or irradiation significantly increased toxicity and mortality.

In clinical trials, the reported adverse events associated with tirapazamine included muscle cramping, ototoxicity, granulocytopenia, nausea and vomiting, etc. (52, 53). Most phase 1 and 2 clinical trials have shown encouraging antineoplastic efficacy and tolerable toxicity (54–60). However, others, as well as two phase 3 clinical studies showed little benefit or significant toxicity (61–65).

AQ4N

AQ4N [1,4-bis{[2-(dimethylamino-N-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione], an aliphatic N-oxide, was first reported in 1993 (66). Its prodrug has no intrinsic DNA binding affinity and thus is non-toxic. Under hypoxic conditions, AQ4N can be activated into AQ4 (with an intermediate product AQ4M) through a two-electron reduction mediated by CYP, which is DNA-affinic and possesses 1000-fold cytotoxic potency compared with its prodrug (**Figure 3**). During the subsequent decade, Patterson and his team deeply investigated the pharmacology of AQ4N. They demonstrated that AQ4N combined with radiotherapy or chemotherapy (cisplatin, cyclophosphamide, thiotepa, mitoxantrone) showed enhanced antineoplastic effects (67–70). In 2003, they proposed a gene-directed enzyme prodrug therapy (GDEPT) strategy using CYPs in order to facilitate the bioreduction of AQ4N (71). Other researchers also investigated the activation of AQ4N by different types of CYPs and nitric oxide synthase (NOS) (72–75).

Many researchers have confirmed that AQ4N exerts antitumor effects in preclinical models of pancreatic cancer (76), bladder cancer and lung cancer (77), prostate cancer (78), gliosarcoma (79), etc., in both single-agent and combined chemotherapy, and in radiotherapy. Gieling et al. (80) demonstrated that AQ4N was more effective toward metastases in a fibrosarcoma-bearing mouse model (subcutaneous KHT tumors). Trédan et al. compared the penetration capacity of AQ4N and mitoxantrone through multi-layer cell cultures and

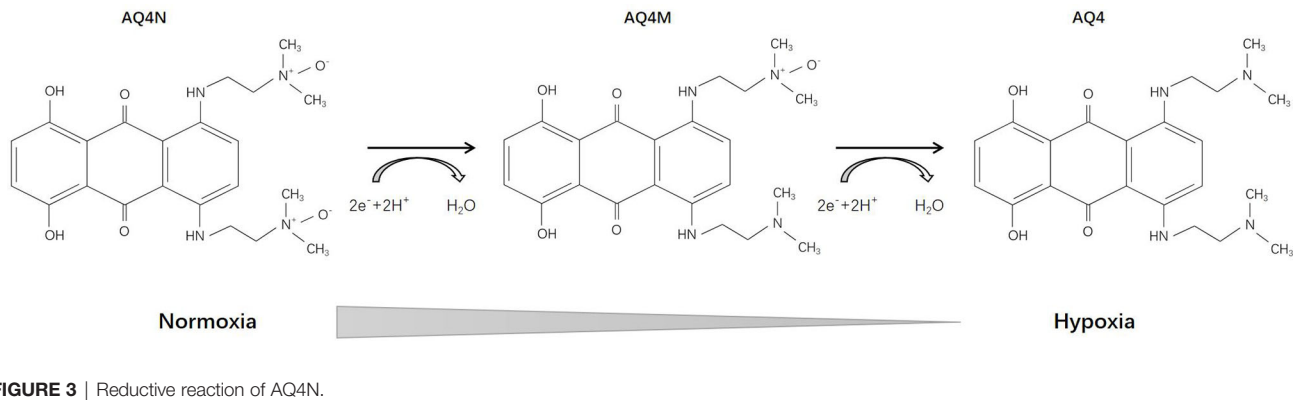

FIGURE 3 | Reductive reaction of AQ4N.

tumor xenografts, and found that AQ4N could penetrate deeply into the hypoxic regions of the tumor and that combination therapy of AQ4N with mitoxantrone showed decreased tumor growth (81). There is also evidence showed that AQ4N had anti-angiogenic effects (82, 83).

The first phase 1 study of AQ4N was reported in 2007, in which 22 esophageal carcinoma patients received an AQ4N infusion followed by fractionated radiotherapy (84). Three of 22 patients had > 50% reductions in tumor volume and 9 had stable disease without dose-limiting toxicity. Albertella et al. enrolled 32 patients with different malignancies in a phase 1 study, and demonstrated that AQ4N was activated selectively in hypoxic regions of tumors and that it can penetrate the blood-brain barrier (85). No objective antitumor effect was observed in another phase 1 clinical study conducted by Papadopoulos et al. (86).

In recent years, a series of new therapeutic strategies have been under development, including combination therapy with AQ4N and photodynamic therapy (PDT), vascular-targeted photodynamic therapy (VTP) (87–92). Feng et al. (93) developed a treatment strategy that combined PDT with AQ4N. Using an AQ4N-64Cu-hCe6-liposome *in vivo* PET probe, they were able to monitor tumor hypoxia status after illumination with light-emitting diode light and demonstrated that utilization of PDT-induced hypoxia to trigger hypoxia-targeted therapy achieved significant antineoplastic effects. Zhang et al. (94) showed that AQ4N combined with starvation therapy (by using stealth liposomes to deliver glucose oxidase together with prodrugs) exhibited similar enhancement of antitumor effects. These methodologies provide new insights for future cancer diagnosis and therapy.

PR-104

PR-104 is a 3,5-dinitrobenzamide-2-mustard. The water-soluble phosphate PR-104 can transform to a more lipophilic prodrug PR-104A (3,5-dinitrobenzamide-2-nitrogen mustard) systemically, and then, under hypoxic conditions, it can be further activated by reduction to PR-104H (5-hydroxylamine) and PR-104M (5-amine), allowing it to act as a DNA interstrand cross-linking agent in hypoxic cells and exert cytotoxic effects (95) (**Figure 4**). The reduction reaction is catalyzed anaerobically mainly by NADPH-cytochrome P450 reductase (96). There are studies demonstrating that PR-104 may also be reduced by aldo-keto reductase (AKR) 1C3 anaerobically, which might cause systemic toxicity (97, 98). The sensitivity of PR-104 depends on the oxygenation status, reductase activity, and DNA repair ability (99). Two studies have revealed the bystander effect of PR-104 (100, 101).

In *in vitro* studies, the antitumor efficacy of PR-104 has been investigated in cervical squamous cell carcinoma (SiHa cells), ovarian carcinoma (A2780 cells), non-small cell lung carcinoma (H1299 and A549 cells), colorectal carcinoma (RKO and HCT116 cells), hepatocellular carcinoma, etc., PR-104 as a single agent or in combination with radiotherapy or chemotherapy has shown different degrees of antineoplastic effects (95, 102–104).

In clinical trials, however, no or only partial responses were observed, but with obvious toxicities, mainly thrombocytopenia and neutropenia (105–108). However, PR-104 showed advantages in the treatment of leukemia. Evidence showed that in acute lymphoblastic leukemia, T-cell acute lymphoblastic leukemia, and acute myeloid leukemia, PR-104 decreased

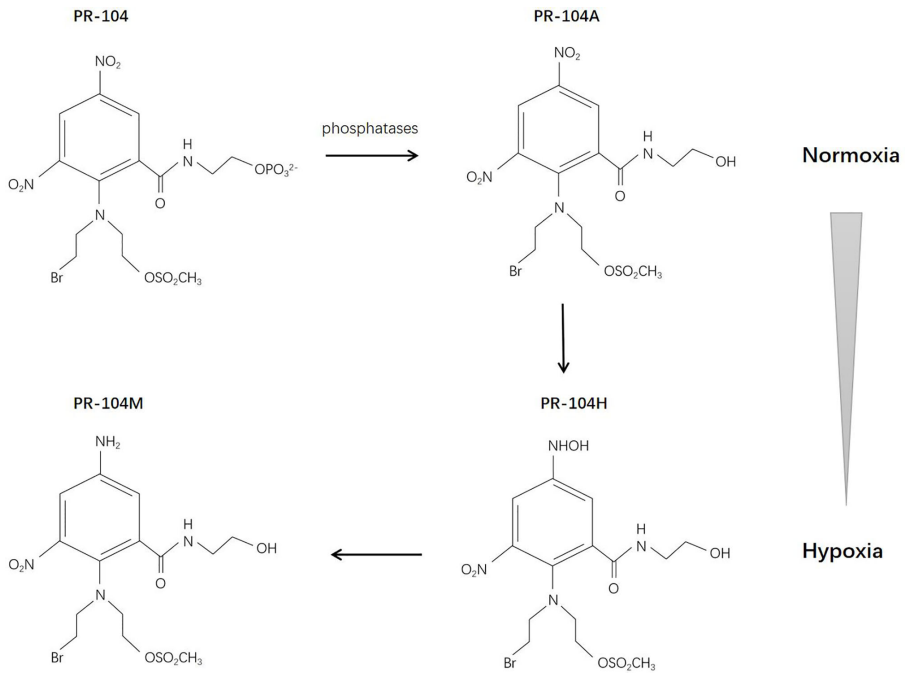

FIGURE 4 | Reductive reaction of PR-104.

tumor burden and prolonged survival in pre-clinical studies (109), and also was associated with disease response in a phase I/II clinical trial (110). The expression of AKR1C3 can be used as a biomarker to predict response to PR-104 and patients screening (111).

EO9 (APAZIQUONE)

EO9 (Apaziquone) [3-hydroxy-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-beta-en-alpha-ol], which is structurally related to mitomycin C, was first reported 1989 and has been deeply investigated since then. Pharmacological studies have shown that DT-diaphorase plays a vital role in the reduction of EO9 prodrug (112), implying that detection of DT-diaphorase activity might predict the sensitivity of certain tumors to EO9 (113–115) (**Figure 5**).

In vitro, EO9 was proved effective toward colon adenocarcinoma cells, melanoma cells, central nervous system tumors, renal cancer cells, oral squamous cell carcinoma, and lung cancer cells (including NSCLC and certain cell lines of small cell lung cancer). *In vivo*, gastric and colorectal adenocarcinoma, ovarian carcinoma, and breast carcinoma were sensitive while leukemia was found to be resistant to EO9 (116–118). Certain inducers such as 1,2-dithiole-3-thiones (D3T) could enhance DT-diaphorase activity, thereby increasing the sensitivity of EO9 (119, 120). However, some researchers pointed out that *in vitro* studies on DT-diaphorase activity are different from *in vivo* studies, and may result in different sensitivity measurements (121). Further pharmacological studies have shown that in the presence of oxygen, DT-diaphorase reduces EO9 through 2-electron reduction, and the product is hydroquinone; while under hypoxic conditions, EO9 undergoes 1-electron reduction, and the product is semiquinone, which is more toxic than hydroquinone (122, 123). Therefore, EO9 may be more effective for hypoxic solid tumors (124, 125). Studies have also shown that the anti-tumor effect of EO9 is pH-dependent, and may exert a tumor suppressor effect in tumor areas with low pH (pH5.5-7.0) (126).

For clinical trials, nephrotoxicity and proteinuria were observed in both phase 1 and phase 2 clinical studies, but only partial response or stable disease was achieved (127–131). The reason for these unsatisfactory results may be attributed to the instability of both semiquinone and hydroquinone, with a short half-life and poor permeability, which will be quickly removed *in vivo* (131–134). However, this special pharmacokinetic profile is ideal for local treatment (135, 136). Intravesical instillation of EO9 was well tolerated and effective for superficial bladder cancer, manifested by a higher complete remission rate and a lower recurrence rate (137–139). A recent study pointed out that EO9 may be inactivated by hematuria, which suggests that the timing of medication should be selected with this in mind in the design of future phase 3 clinical trials (140).

TH-302 (EVOFOSFAMIDE)

TH-302 (Evofosfamide), a second-generation HAP, consists of a 2-nitroimidazole moiety linked to bromo-iso-phosphoramide mustard (Br-IPM). Br-IPM is a DNA cross-linking agent. Under hypoxic conditions through a 2-nitroimidazole reduction reaction, TH-302 prodrug releases Br-IPM and perform cytotoxic effect (141) (**Figure 6**). Cytochrome P450 oxidoreductase (POR) also plays an important role in the reduction reaction and is the main determinant of cell sensitivity to TH-302 (142). Thus, the efficacy of TH-302 is highly dependent on the tumor type (143).

Many researchers have reported the antitumor efficacy of TH-302 as a single agent in malignancies including multiple myeloma, osteosarcoma, chondrosarcoma, neuroblastoma, rhabdomyosarcoma, breast cancer, non-small cell lung cancer, head and neck tumors, acute myeloid leukemia, etc. (144–152). The effect of TH-302 on spherical cells was significantly enhanced (153) and its activity was related to tumor hypoxic fractions (154), indicating that TH-302 had high hypoxic selectivity. The reported antineoplastic mechanisms include DNA fragmentation, cell cycle arrest, down-regulation of hypoxia-inducible factor-1α expression, etc.

In addition to monotherapy, TH-302 also showed synergistic effects with many traditional chemotherapy drugs, including doxorubicin, topotecan, paclitaxel, cisplatin, docetaxel, pemetrexed, irinotecan, gemcitabine, and temozolomide (155–157). TH-302 was able to inhibit the reoxygenation and

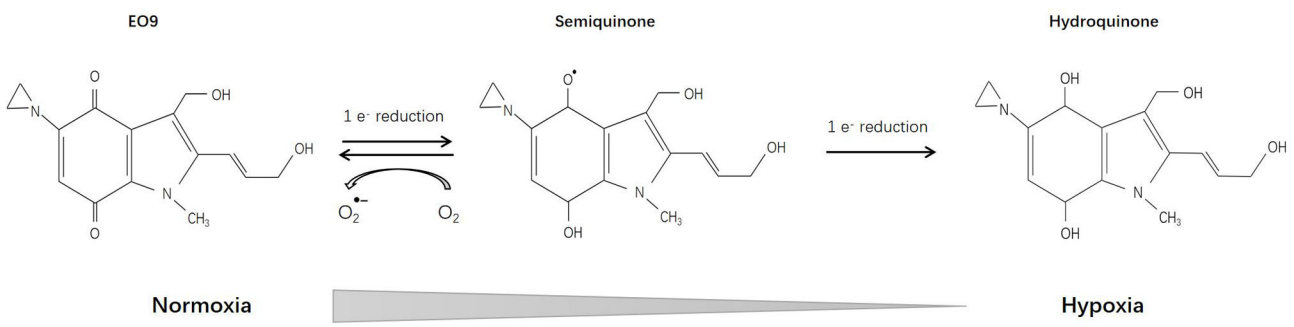

FIGURE 5 | Reductive reaction of EO9.

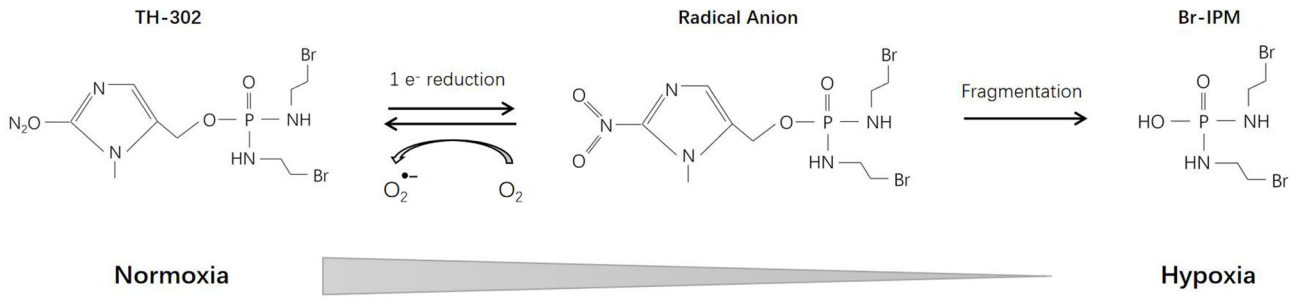

FIGURE 6 | Reductive reaction of TH-302.

proliferation of hypoxic tumor cells that survived chemotherapy (158). Studies also revealed that the application of hypoxia inducers, such as Chk1 inhibitor, mTOR inhibitor, hydralazine, and pyruvate, enhanced the efficacy of TH-302 (159–161). TH-302 also has a radiosensitization effect. It exerts a synergistic effect when combined with radiotherapy (162–164). TH-302 has been shown to be beneficial in combination with conventional transarterial chemoembolization (cTACE) (165); anti-angiogenic therapy, such as VEGF-A inhibitor, sunitinib, and pazopanib (166–168); molecular targeted therapy, such as sorafenib and erlotinib (169, 170); and immunotherapy, such as CTLA-4 and PD-1 blockade (171, 172), where it also exerted a significant tumor inhibition effect. Recent evidence suggests that TH-302 can not only kill hypoxic pancreatic cancer cells, but also has the ability to improve the oxygenation status of residual tumor cells, so it may be useful to enhance the effect of radiotherapy and chemotherapy (173).

Since 2007, TH-302 has been in clinical trials. The main toxicities reported were skin and/or mucosal toxicity, thrombocytopenia, neutropenia, and myelosuppression (174–177). Several phase 1/2 clinical trials have reported encouraging results. For several types of tumors, including soft tissue sarcoma, pancreatic cancer, glioblastoma, and papillomavirus-negative head and neck squamous cell carcinoma, etc, TH-302 alone or in combination with other therapies showed varying degrees of antineoplastic activity (171, 175–178). It showed limited efficacy in the treatment of leukemia and failed in two phase 3 clinical trials (179–181). Researchers analyzed the possible reasons, including the lack of patient screening based on tumor hypoxia status (182, 183), antagonism between drugs (184), and drug formulation changes (185). Further research is still in progress.

SN30000

SN30000 [3-(3-Morpholinopropyl)-7,8-dihydro-6H-indeno[5,6-e][1,2,4]triazine 1,4-dioxide], previously known as CEN-209, is a second-generation benzotriazine-N-oxide hypoxia-activated prodrug and a modified analogue of tirapazamine (**Figure 7**). Currently, it is still in the stage of preclinical research. Several studies have confirmed that SN30000 possesses similar pharmacological mechanisms (186) to tirapamine, but is superior in terms of antineoplastic effects and hypoxia selectivity (187).

Mao et al. (188) proved that, compared with monolayer tumor cells, SN30000 has higher activity on tumor spheroids, and when combined with radiation, it can cause significant tumor spheroid growth delay. Moreover, when used together with or before gemcitabine, SN30000 can effectively inhibit the proliferation of reoxygenated tumor cells (189). EF5 binding may be a promising biomarker for hypoxia stratification and SN30000 treatment response assessment (190, 191).

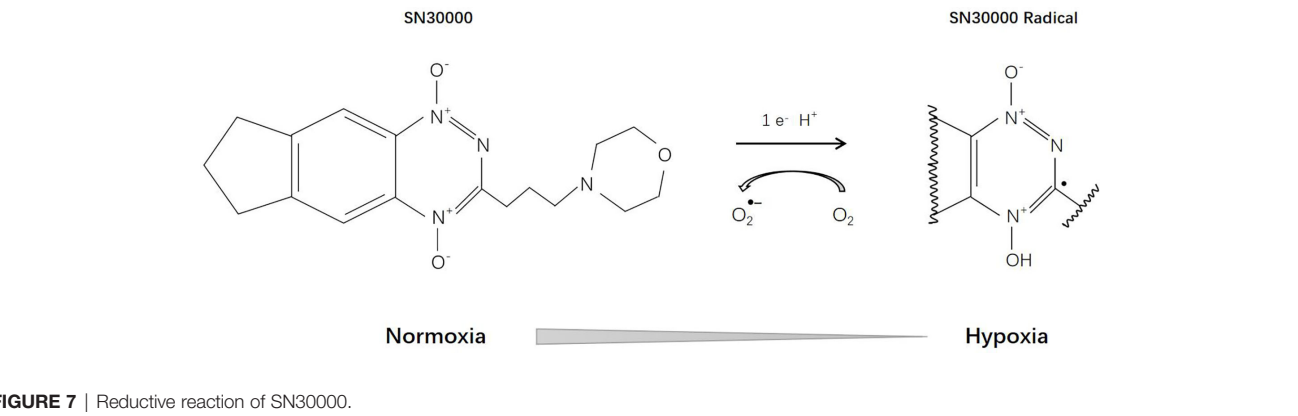

FIGURE 7 | Reductive reaction of SN30000.

TABLE 1 | Summary points.

Summary points
Current Research Status:
•At present, the research of HAPS is mostly limited to the curative effect of macroscopic solid tumors. However, the results are not satisfactory.
•Evidence showed that hypoxic tumor cells could only survive for 2-3 days *in vivo*, suggesting that *in vivo* hypoxic cells are destined to enter necrosis *in vivo* and that hypoxia-targeting therapy of macroscopic tumors should be revisited.
Suggestions For Future Investigations:
•Our experimental evidence showed that micro-metastases (< 1 mm in diameter) and tumor cells in ascites and pleural effusion were severely hypoxic and in low proliferation state.
•They were insensitivity to traditional radiotherapy and chemotherapy.
•Micro-metastases (< 1 mm in diameter) and tumor cells in ascites and pleural effusion are more suitable therapeutic targets for HAPs.

CONCLUSIONS AND SUGGESTIONS FOR FUTURE INVESTIGATIONS

Since the 1980s, HAPs have been developed and validated step by step, from preclinical to clinical. Despite their antineoplastic effects, their drawbacks and limitations have also been revealed by many studies. Here, we summarize the past experience and the latest research progress, and propose the following directions for future research (**Table 1**):

First, screening methods need to be developed based on tumor hypoxia to select the best candidates for this type of therapy. A growing number of studies have shown that PET/CT imaging can be an effective method to monitor HAPs uptake and therapeutic response (148, 190, 192). Second, biomarkers to predict drug sensitivity are needed. Since HAP is a bioreductive drug, it requires specific enzymes to complete the reduction reaction. Therefore, the detection of specific enzymes can play a role in predicting drug sensitivity (112, 142). In addition, experiments conducted by our group and others showed that hypoxic tumor cells could only survive for 2-3 days *in vivo* (193, 194), suggesting that *in vivo* hypoxic cells are destined to enter necrosis *in vivo* and that hypoxia-targeting therapy of macroscopic tumors should be revisited.

Hypoxia is not only a characteristic of macroscopic tumors. In 2007, Li et al. reported that peritoneal disseminated micrometastases (< 1 mm in diameter) were severely hypoxic and in low proliferation state (7, 8, 195–197). This hypoxic state of early micrometastases likely confers insensitivity to traditional radiotherapy and chemotherapy, making them suitable therapeutic targets for HAPs. HAPs may have the potential to prevent them from developing into macroscopic tumors, thereby reducing the metastatic rate of tumors. Our group is working to further confirm the efficacy of HAPs on such tumors and its effect on early tumor metastasis.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YL performed the literature search and wrote the manuscript. LZ performed the literature search and figure editing. X-FL contributed to write and revise the manuscript. All authors contributed to the article and approved the submitted version.

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