Molecular Pathways: Hypoxia-Activated Prodrugs in Cancer Therapy
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Abstract

Hypoxia is a known feature of aggressive solid tumors as well as a critical hallmark of the niche in aggressive hematologic malignances. Hypoxia is associated with insufficient response to standard therapy, resulting in disease progression and curtailed patients’ survival through maintenance of noncycling cancer stem-like cells. A better understanding of the mechanisms and signaling pathways induced by hypoxia is essential to overcoming these effects. Recent findings demonstrate that bone marrow in the setting of hematologic malignancies is highly hypoxic, and that progression of the disease is associated with expansion of hypoxic niches and stabilization of the suppressor activity (8). The role of HIFs in leukemogenesis and carcinogenesis was demonstrated in various models. Generally, suppression of HIF1α or HIF2α inhibited the proliferation and growth of hematologic malignancies, delaying disease progression (9, 10), whereas HIF overexpression triggered upregulation of hypoxia-driven genes in tumors, inducing activities promoting tumor growth, progression, and invasiveness (refs. 7, 8, 11–15; Figs. 1 and 2).

Background

Hypoxia and hypoxia-inducible factors in cancer

Hypoxia is a well-known feature of the microenvironment in solid tumors, usually attributed to rapid tumor growth and insufficient oxygen supply (1). Hypoxic tumors undergo proteomic, genomic, and epigenetic aberrations and are generally resistant to radiotherapy and chemotherapy (2). Hypoxia’s role in poor therapeutic responses and aggressive tumor biology has prompted ongoing efforts to develop diagnostic and therapeutic tools to target hypoxia or its key mediators, such as hypoxia-inducible factors (HIF; refs. 1, 3).

HIFs and their role in hypoxia. A hypoxic environment maintains changes in tumor cells via the HIF family (4, 5). In normoxic conditions, HIF1α undergoes degradation via catalysis by prolyl hydroxylases and the E3-ubiquitin ligase von Hippel–Lindau tumor suppressor protein complex and by the 26S proteasome (6). In turn, prolyl hydroxylase activity falls in hypoxia, leading to stabilization of HIF1α, translocation of HIF1α to the nucleus, and formation of HIF1α/β heterodimers. The response of HIF subtypes as transcription factors to hypoxia depends on duration of exposure; that is, whereas HIF1α is a response to acute hypoxia, increasing cell survival and chemoresistance, HIF2α participates in signal transmission during long-term hypoxia exposure (3), promoting apoptosis (7) and maintaining oncogenic or suppressor activity (8). The role of HIFs in leukemogenesis and carcinogenesis was demonstrated in various models. Generally, suppression of HIF1α or HIF2α inhibited the proliferation and growth of hematologic malignancies, delaying disease progression (9, 10), whereas HIF overexpression triggered upregulation of hypoxia-driven genes in tumors, inducing activities promoting tumor growth, progression, and invasiveness (refs. 7, 8, 11–15; Figs. 1 and 2).

Hypoxia, HIF, and the cell cycle. The hypoxic microenvironment is a crucial factor in cancer relapse because of its activities in regulation of the cell cycle, protection from apoptosis, maintenance and quiescence of stem cells, and selection of treatment-resistant noncycling cancer cells (8). Hypoxic tumors upregulate cell-cycle inhibitors as a protective mechanism, leading to cell dedifferentiation and arrest or quiescence of the cell cycle (11, 16). Chk1 is a central component of genome surveillance pathways required for the initiation of DNA damage checkpoints and is a key regulator of the cell cycle and cell survival. In response to replication stress such as hypoxia, the activation of Chk1 facilitates S and G2 cycle arrest, promoting tumor cell survival, which is maintained via phosphorylation of tumor suppressor p53. The majority of tumors are deficient in the G1–S DNA damage checkpoint because of mutations in p53, protecting tumor cells from apoptosis. HIFs further attenuate mTOR signaling, which triggers metabolic reprogramming and cancer stem cell quiescence (Fig. 1; refs. 5, 7, 12).

HIF1α and maintenance of immunosuppression. Hypoxia in the tumor microenvironment forms a barrier to T-cell infiltration and fosters resistance to chemotherapy and radiotherapy. Hypoxia likely also supports immune resistance in tumors by supporting development of suppressive myeloid and T-cell populations, by activating immunosuppressive signaling pathways in the tumor and stroma, and by creating a metabolically hostile environment for immune effector cells (17).
Markers of hypoxia
An important issue in targeting hypoxia is the identification of appropriate predictive markers. Tools based on immunohistochemical assessment of tumor biopsy specimens detect the distribution of hypoxia by EF5 or pimonidazole staining or by expression of particular hypoxia-associated molecules: HIF1α, LDH-5, GLUT1, MCT1, MCT4, or carbonic anhydrase IX (CAIX; ref. 18; Fig. 1). Specific tumor-imaging techniques such as oxygen-enhanced MRI or PET imaging with [18F]FAZA (NCT01542177), [18F]MISO (NCT02695628), or [18F]HX4 (NCT02233387) have been implemented in clinical trials with the goal of stratifying and identifying patients who would benefit from hypoxia-selective treatment (Table 1; ref. 19). The most common radiotracer is a derivative of nitroimidazole [18F] MISO (20). In hypoxia (<10 mm Hg of partial oxygen pressure), these molecules freely diffuse into cells and undergo reduction catalyzed by nitroreductases, serving as electron acceptors. Intracellular reduced species show a significant retention of radiolabeled metabolites cumulating through dechelation or covalent bond to thiol-rich proteins (19, 21).

In addition, hypoxia can be assessed indirectly by detecting mRNA or protein expression, incorporating single or multiple hypoxia-driven gene signatures (22, 23). Given the intratumor heterogeneity of oxygenation level and vascularization, understanding the distribution pattern of hypoxia and other biomarkers and its correlation with functional imaging, molecular profiling, and histopathology results might help in the selection of optimal therapy for an individual patient.

Targeting hypoxia and hypoxia-associated signaling pathways
One strategy for targeting hypoxia proposes the inhibition of HIF1α, HIF2α, and their downstream targets or upstream signaling partners. Although targeting HIF1α itself remains challenging, new approaches such as targeting the HIF1α/p300 complex with Chetomin, an inhibitor of HIF1α/p300 interaction, demonstrated antitumor activity in human myeloma cell lines and primary multiple myeloma cells from patients (24). Novel selective antagonists of HIF2α/HIF1β, PT2399, and its analogue PT2385 showed antitumor activity and selectivity in human clear-cell renal cell carcinoma (ccRCC) cell lines and xenografts with higher expression of HIF2α (HIF2α-dependent tumors) and improved progression-free survival in patients with metastatic, extensively pretreated RCC (NCT02293980; ref. 23). Another approach focuses on targeting HIF1 downstream targets such as CAIX via any of several antibodies or small molecules is a subject of ongoing preclinical and clinical studies (25). Another approach to eradicating hypoxic cells uses bioreductive or hypoxia-activated prodrugs.
prodrugs (HAP; Fig. 3). HAPs are activated by enzymatic reduction under low oxygen tension, sensing hypoxia in a tumor (1). The HAP molecules can be classified by chemical structure as aliphatic and aromatic N-oxides, nitro groups, quinones, and transition metals (1). HAPs can also be separated into two classes according to the hypoxic threshold required for activation by the specific reductase (e.g., POR). Class I requires relatively mild hypoxia for activation and includes, among others, benzotriazine, N-oxides, and tirapazamine. Class II depends on severe hypoxia for activation and encompasses nitro-compounds PR-104A and TH-302 (evofosfamide). Under extreme hypoxia, the prodrug radicals have longer lifetimes and can be reduced more easily to active drugs (1). Agents such as TH-302 have a stable effector molecule that can promote a bystander effect, diffusing from the targeted hypoxic cell to neighboring cells. The majority of HAP metabolites result in DNA damage by interfering with DNA replication.

Despite promising preclinical data, several HAPs have failed to show clinical efficacy. A few, however, such as TH-302, tarloxotinib bromide (TH-4000), and tirapazamine, are undergoing ongoing exploration in clinical trials for specific indications (refs. 1, 26, 27; Table 1).

**Clinical–Translational Advances**

**HAP monotherapy**

**TH-302 and PR-104.** TH-302 is a 2-nitroimidazole radical anion prodrug, activated in hypoxia by one-electron reductases such as POR to the active cytotoxic drug bromo-isophosphoramide mustard (Br-IPM; Fig. 3; ref. 28). TH-302 exhibits activity against a range of cell lines, especially those with deficiency in homology-directed DNA repair, BRCA1, BRCA2, or FANCA, as well as in H460 multicell spheroids and multicell layer...

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**Figure 2.**

HIFα maintenance in hypoxia. HIFα is stabilized and undergoes heterodimerization with subunit β and is then translocated to the nucleus, where it activates hypoxia-dependent gene transcription. HIF plays a key role in hypoxic cells, regulating genetic, epigenetic, and metabolomic reprogramming of cells to survive.
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models and a variety of preclinical models of solid tumors [pancreatic and osteolytic breast cancers, non–small cell lung cancer (NSCLC), osteosarcoma; refs. 28–39] and hematologic malignancies [multiple myeloma and acute myeloid leukemia (AML); refs. 27, 40, 41].

TH-302 induces γH2AX and generates intra- and interstrand DNA cross-links in hypoxic tissue, damaging DNA in both quiescent and proliferating cells and inhibiting cell proliferation and tumor growth through cell-cycle arrest and induction of apoptosis. In vivo studies in AML xenograft models demonstrated that TH-302 depleted hypoxic cells, prolonged survival, and reduced the leukemia stem cell pool (41). Administration of TH-302 to mice with residual AML following chemotherapy prolonged survival, suggesting that this approach may be suitable for eliminating chemotherapy-resistant leukemia cells (41) and, thus, demonstrating the feasibility of targeting hypoxic cells by hypoxia-activated cytotoxins. Because of these encouraging antitumor responses in xenograft models, TH-302 entered phase I/II clinical trials as monotherapy in solid tumors and acute leukemias (42). Its clinical activity was limited, however, and only a few objective responses, mostly transient, were observed (42, 43). These results make a

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Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoblastic leukemia; CML, chronic myeloid leukemia; 5-FU, 5-Fluorouracil; GCSF, granulocyte colony stimulating factor; GIST, gastrointestinal stromal tumors; HCC, hepatocellular carcinoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NR, not recruiting; NSCLC, non–small cell lung cancer; NYR, not yet recruiting; pNET, pancreatic neuroendocrine tumors; RCC, renal cell carcinoma; RT, radiotherapy; SCCHN, squamous cell carcinoma of head and neck; SCLC, small cell lung cancer; SLL, small lymphocytic leukemia; TACE, transcatheter arterial chemoembolization; TAE, transcatheter arterial embolization; TURB, transurethral resection of the bladder.

*Trial included biological or imaging study with FAZA and/or evaluation biomarkers.

*Polychemotherapy: vincristine + irinotecan + cyclophosphamide + doxorubicin + ifosfamide + etoposide + GCSF.
compelling case for combination therapies targeting both hypoxic and normoxic neoplastic cells.

PR-104 is a phosphate ester that is rapidly hydrolyzed to the corresponding alcohol PR104A, which acts as a HAP through its metabolic reduction to activated nitrogen mustards PR-104H and PR-104M (Fig. 3). PR-104 was shown to have significant activity in animal studies, inhibiting disease progression, decreasing tissue infiltration by tumor cells, reducing tumor growth, and prolonging mouse survival, especially in AKR1C3-expressing xenografts (44, 45). PR-104 monotherapy elicited significant reductions in growth of hepatocellular carcinoma xenografts, which was reduced even further in mice treated with both PR-104 and sorafenib (46). In phase I clinical trials, however, PR-104 showed little evidence of therapeutic activity in advanced solid tumors, with several hematologic toxic events (47). In a phase I/II study in patients with relapsed/refractory AML or acute lymphoblastic leukemia (48), PR-104 was tolerated at doses much higher than the solid tumor maximum tolerated dose; however, myelosuppression (neutropenia and thrombocytopenia) was prolonged at the higher doses. Despite reductions of hypoxia markers (HIF1α, CAIX, and distribution of hypoxia assessed by immunohistochemical staining with pimonidazole) after administration of PR-104, the treatment responses were transient. However, these biomarker studies provided evidence that hypoxia is a prevalent feature of the leukemic microenvironment.

HAPs in combination with chemotherapy or targeted therapy

The activity of TH-302 in combination with conventional chemotherapy or targeted therapy has been reported in multiple preclinical solid tumor models and hematologic malignancies (32, 35, 37, 38, 49, 50). In various xenograft models, the addition of TH-302 significantly increased DNA damage, apoptosis, and tumor necrosis and reduced stroma density and intratumoral hypoxia, without additive toxicity. In a xenograft model of pancreatic cancer, TH-302 showed promising activity with gemcitabine, decreasing the frequency of tumor-initiating cells from
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The same drug combination also increased the benefit (37). TH-302 also improved the efficacy of a gemcitabine and nab-paclitaxel combination in mouse xenograft models of human pancreatic ductal adenocarcinoma (PDAC; ref. 51). The addition of TH-302 to topotecan improved tumor response and prolonged survival in neuroblastoma and rhabdomyosarcoma xenograft models (50). In a murine model of multiple myeloma, the combination of TH-302 with bortezomib significantly prolonged survival (40). A combination of TH-302 with the VEGF-A inhibitor pazopanib showed high efficacy in a sarcoma xenograft model, blocking tumor growth, increasing DNA damage and total and endothelial cell–specific apoptosis, and decreasing HIF1α activity and percentage of sarcoma stem-like cells (52).

The preclinical results have been evaluated in phase I to III clinical trials in a variety of tumor entities. TH-302 has been evaluated in a phase III study in advanced unresectable or metastatic PDAC at the recommended phase II dose; however, the final result is pending. The efficacy of TH-302 and dexamethasone in combination with bortezomib or pomalidomide was investigated in patients with relapsed or refractory multiple myeloma. The preliminary data from this study showed an International Myeloma Working Group response (MR, minimal response; PR, partial response; or CR, complete response) in 29% of extensively pretreated patients at the recommended phase II dose (54). The combination of TH-302 with pemetrexed in advanced nonsquamous NSCLC yielded a mean overall survival duration of 14.9 months (55). In a placebo-controlled, multicenter phase II study in the same setting, however, this combination as second-line therapy was stopped because it showed no survival benefit compared with pemetrexed alone (NCT02093962).

A novel approach of adding TH-302 to neoadjuvant chemoradiotherapy (paclitaxel, carboplatin, and radiotherapy) was proposed for a clinical trial of untreated patients with esophageal cancer (30). This study will utilize several novel tools to monitor and predict the therapy response, including PET/CT scan with hypoxia tracer 18FHX4 and analysis of CAIX and osteopontin (30). Patients with a complete pathologic response after neoadjuvant treatment could then opt for a wait-and-see strategy to omit or postpone surgery. The effect of tumor hypoxia on the response to standard chemoradiation is being investigated by visualizing hypoxia with 18FHX4 imaging before treatment and 2 weeks after the start of treatment in another ongoing phase II clinical trial (30).

HAPs and immune checkpoint inhibition

Hypoxia drives the establishment of a highly interdependent network of immunosuppressive stromal cells, such as myeloid-derived suppressor cells and myofibroblasts. In the tumor, hypoxic zones might resist infiltration by T cells even in the context of robust T-cell infiltration in normoxic areas of the same tumor. To target the reprogrammed hypoxic immunoenvironment of a tumor, Ai and colleagues proposed a novel combination of immunotherapy and hypoxia-specific chemotherapy, suggesting the potential of such a combination to render some of the most therapeutically resistant cancers, such as prostate adenocarcinoma, sensitive to checkpoint inhibition (56). Studies in vitro and in a mouse model of prostate cancer suggest that combination of TH-302 and T-cell checkpoint blockade promotes an inside-out tumor destruction, with the drug killing at the core, releasing antigen, and diminishing immunosuppression, while the antibodies help expand and protect the activated T cells as a result.

Antibody blockade of CTLA-4 and PD-1 in conjunction with TH-302 promoted tumor rejection in a significantly larger percentage of mice than either single agent, promoting uniquely advantageous ratios of effector T cells to myeloid-derived suppressor cells within the tumor microenvironment. This finding provides a strategy for rendering some of the most therapy-resistant cancers sensitive to immunotherapy (56).

HAPs and DNA damage signaling

Chronic hypoxia has been found to induce activation of DNA-dependent protein kinase (DNA-PK) in the absence of applied DNA damage, and this activation was found to promote stabilization of HIF1α and to increase therapy resistance and treatment failure (57). To overcome resistance based on the DNA repair machinery, two approaches have been explored. Meng and colleagues showed that TH-302 cytotoxicity was greatly enhanced by Chk1 inhibition in p53-deficient human cancer cell lines. Chk1 inhibitors reduced TH-302–induced cell-cycle arrest by increasing histone H3 and Cdc2-Y15 (58). Combination of TH-302 with the Chk1 inhibitor AZD-7762 had greater efficacy in a colorectal xenograft model than either agent alone. This sensitization was shown to be due to disruption of the Chk1-mediated DNA damage checkpoint of the cell cycle and induction of apoptosis, providing additional support to the preclinical translational rationale for combining TH-302 with a Chk1 inhibitor (58).

Alternatively, the concept of hypoxia-activating Chk1 inhibitors was realized in the compound CH-01, a HAP that releases Chk1/Aurora A inhibitor following reduction of a 4-nitrobenzyl in hypoxic conditions (Fig. 3; ref. 39).

Another approach was proposed by Lidquist and colleagues in a study assessing the inhibition of DNA double-strand break repair in hypoxic cells by targeting DNA-PK with BCCA621C, a hypoxia-activated inhibitor of DNA-PK. BCCA621C is enzymatically activated, leading in severely hypoxic conditions to radiosensitization of NCI-H460 cells (60). Recent findings indicate that hypoxia induces resistance to alkylating agents via a distinct molecular pathway involving HIF1α, p53, and the mTOR target gene NDRG1, resulting in stabilization of O6-methylguanine-DNA methyltransferase (AGT), a key enzyme mediating resistance to alkylating agents in glioblastoma and melanoma (61). Hypoxia-selective 4-nitrobenzylxoxycarbonyl derivatives of O6-benzylguanine inhibit AGT and sensitized laromustine-resistant DU145 human prostate carcinoma cells to laromustine under hypoxic conditions (62). This approach could lead to selective depletion of AGT in tumor tissue and sensitization of tumors to O6 guanine–targeting cytotoxic drugs such as temozolomide.

Novel HAPs in ongoing in vitro and in vivo studies

Several novel hypoxia-induced cytotoxins have been generated, translating into hypoxia-activating DNA-damaging agents. Ikeda and colleagues developed a new doxorubicin produg with improved preclinical efficacy in pancreatic and colon cancers in vitro and in vivo (63). A novel tirapazamine analogue, Q6, showed hypoxia selectivity and topoisomerase II poisoning and has been proposed for treatment of human hepatocellular carcinoma (64). Schreiber-Brynzak and colleagues developed compound 2, a HAP of platinum(IV), which inhibited tumor growth in vivo significantly better than other satraplatin drugs (65). Kumar and colleagues

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have so far failed to show significant consequences of hypoxia and HIFs. Despite enormous progress on development of novel strategies to overcome the detrimental effects of chemoresistance and radioresistance supports continuing emphasis on the fundamental role of hypoxia in tumor biology and in the chemoresistance and radioresistance supports continuing emphasis on the development of novel strategies to overcome the detrimental effects of hypoxia and HIFs. Despite enormous progress in HAP development in preclinical settings, the existing strategies have so far failed to show significant clinical benefit either as monotherapy or in combination with standard chemotherapeutic agents, possibly because of their narrow therapeutic windows. Hypoxia-activated, molecularly targeted inhibitors might overcome this limitation and provide broader therapeutic efficacy for tumors with driver mutations (e.g., HER2, EGFR, VEGF, AGT, or CHK1). Alternatively, rational combinations with targeted agents, metabolic modulators, or immunotherapies could be highly effective and safe. Novel trial designs, such as the use of HAPs in the neoadjuvant setting or in the setting of minimal residual disease, should be further contemplated to address patients with specific profiles of hypoxia biomarkers. Finally, expansion of our knowledge of HIF signaling networks in normal cells, as well as our understanding of HIF-dependent pathways hijacked by cancer cells, is eagerly awaited.

### Summary and Future Perspectives

The fundamental role of hypoxia in tumor biology and in chemoresistance and radioresistance supports continuing emphasis on the development of novel strategies to overcome the detrimental consequences of hypoxia and HIFs. Despite enormous progress in HAP development in preclinical settings, the existing strategies have so far failed to show significant clinical benefit either as monotherapy or in combination with standard chemotherapeutic agents, possibly because of their narrow therapeutic windows. Hypoxia-activated, molecularly targeted inhibitors might overcome this limitation and provide broader therapeutic efficacy for tumors with driver mutations (e.g., HER2, EGFR, VEGF, AGT, or CHK1). Alternatively, rational combinations with targeted agents, metabolic modulators, or immunotherapies could be highly effective and safe. Novel trial designs, such as the use of HAPs in the neoadjuvant setting or in the setting of minimal residual disease, should be further contemplated to address patients with specific profiles of hypoxia biomarkers. Finally, expansion of our knowledge of HIF signaling networks in normal cells, as well as our understanding of HIF-dependent pathways hijacked by cancer cells, is eagerly awaited.

### References


### Disclosure of Potential Conflicts of Interest

M. Konopleva reports receiving commercial research grants from Proacta and Threshold. No potential conflicts of interest were disclosed by the other author.

### Authors’ Contributions

Conception and design: N. Baran, M. Konopleva

Writing, review, and/or revision of the manuscript: N. Baran, M. Konopleva

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