Cancer Therapy: Preclinical

Activity of the Hypoxia-Activated Prodrug, TH-302, in Preclinical Human Acute Myeloid Leukemia Models

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Abstract

Purpose: Acute myeloid leukemia (AML) is an aggressive hematologic neoplasm. Recent evidence has shown the bone marrow microenvironment in patients with AML to be intrinsically hypoxic. Adaptive cellular responses by leukemia cells to survive under low oxygenation also confer chemoresistance. We therefore asked whether therapeutic exploitation of marrow hypoxia via the hypoxia-activated nitrogen mustard prodrug, TH-302, could effectively inhibit AML growth.

Experimental Design: We assessed the effects of hypoxia and TH-302 on human AML cells, primary samples, and systemic xenograft models.

Results: We observed that human AML cells and primary AML colonies cultured under chronic hypoxia (1% O2, 72 hours) exhibited reduced sensitivity to cytarabine-induced apoptosis as compared with normoxic controls. TH-302 treatment resulted in dose- and hypoxia-dependent apoptosis and cell death in diverse AML cells. TH-302 preferentially decreased proliferation, reduced HIF-1α expression, induced cell-cycle arrest, and enhanced double-stranded DNA breaks in hypoxic AML cells. Hypoxia-induced reactive oxygen species by AML cells were also diminished. In systemic human AML xenografts (HEL, HL60), TH-302 [50 mg/kg intraperitoneally (i.p.) 5 times per week] inhibited disease progression and prolonged overall survival. TH-302 treatment reduced the number of hypoxic cells within leukemic bone marrows and was not associated with hematologic toxicities in nonleukemic or leukemic mice. Later initiation of TH-302 treatment in advanced AML disease was as effective as earlier TH-302 treatment in xenograft models.

Conclusions: Our results establish the preclinical activity of TH-302 in AML and provide the rationale for further clinical studies of this and other hypoxia-activated agents for leukemia therapy. Clin Cancer Res; 19(23); 6506–19. ©2013 AACR.

Introduction

Acute myeloid leukemia (AML) is an aggressive immature hematologic neoplasm primarily affecting older adults. Chemotherapy for AML has not changed since the 1980s and continues to result in long-term cure in only 20% to 30% of patients. Recent data have shown that the bone marrow microenvironment, the principal site of AML initiation and expansion, is characterized by intrinsically low oxygen tension. This relative hypoxia within the bone marrow functions as a key mediator of normal hematopoiesis, specifically hematopoietic stem cell biology (1–4). In fact, most primitive pluripotent hematopoietic stem cells (HSC) are preferentially located in distinct nonperfused specialized marrow niches characterized by the most severe hypoxia (5–7). The cytokine-rich milieu of these poorly oxygenated marrow niches has been shown to promote long-term cellular quiescence and self-renewal of HSCs and decrease levels of potentially toxic reactive oxygen species (ROS; refs. 8, 9).

The hypoxic bone marrow microenvironment has also been shown to contribute to AML disease. Prior data in multiple animal models showed that acute leukemic progression within the bone marrow is associated with further decreases in overall marrow oxygenation and expansion of hypoxic bone marrow areas (10–12). This has been attributed, in part, to potentially compromised blood flow within a restricted noncompliant bony cavity (13, 14). Poorly perfused hypoxic marrow niches could also affect the systemic exposure of AML cells to systemic chemotherapy agents as well as the circulation and recruitment of host immune effector cells mediating anti-tumor responses. Theoretically, such bone marrow microenvironmental changes may lead to the selective outgrowth of AML clones which are “better adapted” to survive within a severely hypoxic microenvironment than normal hematopoietic cells (15–17). Prolonged culturing of AML cells under hypoxia has been

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AML cells, primary samples, and xenograft models.

investigated the preclinical activity of TH-302 in human toxicities and evidence of clinical efficacy (27–29). Here, we with solid and hematologic cancers have shown limited under low oxygenation (24–26). Clinical trials of TH-302 have an active role in the treatment of AML and other bone resistance to chemotherapy (22, 23). For these reasons, has subsequently been shown to induce cell dormancy and biologic adaptations by AML cells to hypoxia also confers resistance to chemotherapy. We and other investigators have reported that overexpression of hypoxic-inducible factor-1 (HIF-1α) in primary AML and acute lymphocytic leukemia (ALL) marrow samples correlated with poor and biologic adaptations by AML cells to hypoxia also confers resistance to chemotherapy. We and other investigators have reported that overexpression of hypoxic-inducible factor-1α (HIF-1α) in primary AML and acute lymphocytic leukemia (ALL) marrow samples correlated with poor cell death. In systemic human leukemia xenografts, TH-302 reduces disease burden and prolongs overall survival without overt hematologic toxicity. Treatment with TH-302 was also effective in advanced AML disease, the setting in which marrow hypoxia would be expected to be greatest. These results support further clinical development of TH-302 as a novel approach for acute leukemia therapy.

**Translational Relevance**

Acute myeloid leukemia (AML) is an aggressive hematologic neoplasm affecting older adults. The primary site of disease initiation and expansion in patients with AML is the bone marrow. At baseline, the marrow microenvironment is known to be intrinsically hypoxic. One consequence of rapid leukemia growth is further marrow hypoxia. We show that the hypoxia-activated prodrg, TH-302, exerts significant anti-leukemic activity. TH-302 overcomes chemoresistance conferred by hypoxia in cultured human AML cells and primary AML samples. TH-302 induces hypoxic-dependent DNA damage, cell-cycle arrest, decreased reactive oxygen production, and cell death. In systemic human leukemia xenografts, TH-302 reduces disease burden and prolongs overall survival without overt hematologic toxicity. Treatment with TH-302 was also effective in advanced AML disease, the setting in which marrow hypoxia would be expected to be greatest. These results support further clinical development of TH-302 as a novel approach for acute leukemia therapy.

shown to favor quiescent immature stem cell–like clones as well as cells with higher expression of signaling pathways promoting leukemia survival and expansion (3, 9, 18, 19).

Similar to solid tumor cells, it now appears that molecular and biologic adaptations by AML cells to hypoxia also confer resistance to chemotherapy. We and other investigators have reported that overexpression of hypoxic-inducible factor-1α (HIF-1α) in primary AML and acute lymphocytic leukemia (ALL) marrow samples correlated with poor chemotherapy outcomes (20, 21). Hypoxia-induced upregulation of HIF-1α in human acute leukemia cells in vitro has subsequently been shown to induce cell dormancy and resistance to chemotherapy (22, 23). For these reasons, hypoxia-activated agents, novel cytotoxic drugs preferentially activated under low oxygenation conditions, may have an active role in the treatment of AML and other bone marrow malignancies.

TH-302 is a 2-nitroimidazole hypoxia-activated prodrg of the cytotoxic bromo-isophosphoramide mustard. TH-302 has been shown to exhibit broad-spectrum preclinical activity against solid tumor and multiple myeloma cells under low oxygenation (24–26). Clinical trials of TH-302 alone and in combination with chemotherapy in patients with solid and hematologic cancers have shown limited toxicities and evidence of clinical efficacy (27–29). Here, we investigated the preclinical activity of TH-302 in human AML cells, primary samples, and xenograft models.

**Methods**

**Cell lines, patient samples, and reagents**

Human AML cell lines (HEL, HL60, Kasumi) were purchased from the American Tissue Culture Collection. ML-2 cells containing MLL/11q23 were obtained from DSMZ (30). NB-4, MV4;11, LAMA, KG-1, and THP-1 were originally obtained from DSMZ and were kindly provided by Dr. C. Frangou, Department of Cancer Genetics, RPCI. Unless otherwise indicated, cells were grown in humidified incubators (37°C, 21% O2, 5% CO2) in RPMI-1640 medium containing 10% FBS, 2 mmol/L l-glutamine, 4.5 g/L glucose, 1.5 g sodium bicarbonate, 1 U/mL penicillin/streptomycin, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate. TH-302 was provided by Threshold Pharmaceuticals, Inc. Cytarabine, doxorubicin, and the NADPH oxidase inhibitor, diphenyleneiodonium (DPI), were purchased from Sigma-Aldrich. Concentration and duration of treatments are shown in individual figures and detailed in the figure legends.

**Hypoxic conditions**

Cells were plated in 6-well plates in an X-vivo hypoxia chamber (BioSpherix) at 1% O2/5% CO2 level for indicated time points before harvesting for further analysis.

**In vitro assays**

Viable cells were counted using a hemocytometer in triplicate following dye exclusion (1:1 dilution in 0.4% trypan blue solution; Mediatech Inc.). Results were averaged among triplicate wells.

**Flow cytometry**

Leukemia cells were plated in triplicate in 6-well plates at a concentration of 5 × 10⁶ cells/ml in the presence of vehicle [dimethyl sulfoxide (DMSO)] or varying concentrations of TH-302. Cells were then incubated for 24 to 72 hours under normoxic (20% O2) or hypoxic (1% O2) conditions followed by flow cytometric assays. Apoptosis was assessed in asynchronously growing cells via an Annexin V-PE Apoptosis Detection kit (BD Pharmingen). Double-stranded DNA breaks were assessed via measurements of phosphorylated histone H2AX (EMD Millipore) and with the APO-BrdU TUNEL Assay Kit (Molecular Probes, Life Technologies) following manufacturer’s instructions. Cell-cycle analysis was conducted on cells treated for 24 hours and then washed, fixed with 70% ethyl alcohol for 30 minutes at 4°C, and centrifuged at 3,200 rpm for 3 minutes. Cells were then treated with RNase A treatment (0.5 mg/mL) for another 30 minutes at 37°C followed by the addition of propidium iodide (1 mg/mL; GenScript) for an additional 30 minutes at 4°C in the dark followed by analysis. To detect ROS in live cells, the fluorescein-based dye CM-H₂DCFDA (Invitrogen) was added to samples at a final concentration of 10 μmol/L followed by incubation in the dark with agitation at 37°C for 30 minutes. Sample viability was assessed by 7-AAD (BD Pharmingen) and confirmed by trypan blue exclusion under light microscopy. All studies were conducted on a FACSCalibur instrument (BD) followed by analysis using Winlist Version 6.0 (Verity Software House) and FCS Express software (De Novo Software). Experiments were carried out independently at least twice.
Protein analyses

Cells (1 × 10^6/mL) were plated in 6-well plates for drug and/or hypoxic treatment followed by lysis in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v)], supplemented with phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich), and 1% (v/v) protease inhibitor cocktail 1 and 2 (Sigma-Aldrich). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad). Lysates were separated by SDS-PAGE (12% for cleaved caspase-3, 8% for the rest) and detected by chemiluminescence (ECL plus, GE Healthcare). Specific antibodies included anti-cleaved caspase-3 (#9661, Cell Signaling Technology) and anti-cleaved PARP (#9546, Cell Signaling Technology) used at a final dilution ratio of 1:1,000. Antibody to actin (#CP01) was obtained from Calbiochem. For HIF-1α immunoassays, protein samples were analyzed following...
the manufacturer’s instructions (R&D Systems). Cells treated with 100 μmol/L cobalt chloride (Sigma Aldrich) for 4 hours served as a positive control.

**Primary AML clonogenic assays**

Primary AML patient cells ($2 \times 10^5$) were plated in triplicate in methylcellulose complete media containing rhSCF 50 ng/mL, rhGM-CSF 10 ng/mL, rhIL-3 10 ng/mL, and rhEpo 3 U/mL (HSC003, R&D Systems) and cultured at 37°C (21% O2, 5% CO2) for 14 days to allow for baseline growth. Following this, vehicle (PBS), cytarabine (0.01 nmol/L), doxorubicin (0.5 nmol/L), or TH-302 (0.1–1 nmol/L) was added. Plates were cultured for an additional 72 hours under hypoxia or normoxia to determine the concomitant effects of environmental and pharmacologic interventions on established primary AML growth. Mean ($\pm$SEM) leukemia colony formation units (CFU-L) were quantified using an inverted microscope.

**Leukemia xenograft experiments**

All animal experiments were carried out under an approved IACUC protocol. Six- to 8-week-old female severe combined immunodeficient (SCID) mice (RPCI, Department of Laboratory Animal Research, Buffalo, NY) were sublethally irradiated (200 rad) followed by tail vein (1 × 10⁷ cells) injection of human HEL and HL60 cells stably transfected with a pGL4 luciferase reporter vector (Promega) as described previously (31). Mice were evaluated weekly for evidence of leukemia engraftment and overall leukemia disease progression by serial bioluminescent...
Figure 2. TH-302 induces hypoxia-dependent cell-cycle arrest and DNA damage in AML cells. A, low-dose TH-302 treatment (0.1–1 nmol/L) preferentially inhibited the proliferation of AML (HL60, HEL) cells under hypoxic conditions as determined by tritiated thymidine incorporation in triplicate wells. B, AML cells were treated with vehicle (PBS) or TH-302 at the indicated concentrations for 24 hours under hypoxic (1% O₂) or normoxic (21% O₂) conditions in triplicate wells. Cells were stained with propidium iodide staining and analyzed by flow cytometry. Representative cell-cycle effects and flow histograms are shown. Similar results were obtained in 3 independent experiments. C, HEL cells were plated in triplicate wells and treated with vehicle or TH-302 for 24 hours under hypoxia (1% O₂) or normoxia (21% O₂). Cells were then harvested and stained for H2A.X phosphorylation followed by flow cytometry. Shown are the mean (±SEM) percentages of H2A.X-positive cells under each condition. Representative results of 2 independent experiments are shown. D, similarly treated HEL cells were also assessed for expression of DNA fragmentation of apoptotic cells by TUNEL assay using flow cytometry. (Continued on the following page.)
imaging (BLI; Xenogen IVIS 50 System, Caliper Life Science) following intraperitoneal d-luciferin injection (75 mg/kg). Dorsal and ventral images were acquired from each animal. Data were expressed as the average photon emission = photons per second per cm$^2$ per steradian calculated from dorsal and ventral images of each animal (Living Imaging Software, Calipers Life Sciences). Peripheral blood was collected intermittently for complete blood cell measurements. For marrow hypoxia determinations, sublethally irradiated SCID mice were injected in the tail vein with HEL-luciferase AML cells and serially followed for disease progression by BLI at intervals after tumor inoculation. Twenty days after inoculation, cohorts of leukemia-engrafted animals ($n = 5–10$ mice/group) were treated with vehicle (PBS), cytarabine 2 mg/kg intraperitoneally (i.p.) daily for 3 days, doxorubicin 35 mg/kg i.p. single dose, or aflibercept (VEGF Trap) 5 mg/kg single injection (31). Three days later, mice were injected i.p. with

![Diagram of HIF-1α protein expression](image1)

**Figure 2.** (Continued.) E, hypoxic AML cells treated with TH-302 ($1–10 \mu$mol/L) for 24 hours also expressed significantly less HIF-1α protein as determined by immunoassay. Cobalt chloride-treated cells and normoxic AML cells cultured under the same conditions served as controls. F and G, production of ROS by HEL AML cells following 48 hours of hypoxia (1% O$_2$) were significantly abrogated by TH-302 treatment in a dose-dependent manner, as determined by flow cytometry for mean fluorescent intensity (CM-H2DCFDA staining). ROS levels in cells incubated under normoxia were unaffected by TH-302 treatment. Analyses were conducted on gated viable cells only in triplicate wells. Each histogram records the fluorescent intensity of gated viable cells in triplicate. Outliers are observed primarily at higher concentrations of TH-302 treatment. Similar results were seen for HEL cells. At least 2 independent experiments were carried out for all of the above experiments. White bars indicate normoxia, gray bars indicate hypoxia. Student t-test analysis compared conditions versus controls. *, $P < 0.05$; **, $P < 0.001$; *** $P < 0.0001$. 

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E: Relative HIF-1α protein expression

F: ROS generation

G: Cell counts

Vehicle

TH-302 0.05 µmol/L

TH-302 0.5 µmol/L

TH-302 5 µmol/L

Normoxia

Hypoxia

Fluorescent intensity

Fluorescent intensity

Cell counts

Cell counts

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pimonidazole 60 mg/kg (Hypoxyprobe Store) and sacrificed after 30 minutes. Femurs were flushed with normal saline with cell suspension evaluated by flow cytometry for human and mouse (hCD33-PE, mCD45-PE, BD Pharmingen) myeloid antigen expression as previously described (31). Experiments evaluating \textit{in vivo} TH-302

<table>
<thead>
<tr>
<th>Human AML xenograft model</th>
<th>Number of mice per group</th>
<th>TH-302 regimen</th>
<th>Total weekly dose</th>
<th>Optimal % total flux reduction vs. vehicle</th>
<th>Overall survival difference</th>
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</thead>
<tbody>
<tr>
<td>HEL</td>
<td>$n = 10$</td>
<td>50 mg/kg (5×/week)</td>
<td>250 mg</td>
<td>83% (day 14, $P = 0.05$)</td>
<td>Yes ($P = 0.0285$)</td>
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<tr>
<td>HEL</td>
<td>$n = 5$</td>
<td>75 mg/kg (2×/week)</td>
<td>150 mg</td>
<td>93% (day 21, $P = 0.0398$)</td>
<td>No ($P = 0.0875$)</td>
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<tr>
<td>HEL</td>
<td>$n = 15$</td>
<td>100 mg/kg (once/week)</td>
<td>100 mg</td>
<td>75% (day 7, $P = 0.046$)</td>
<td>No ($P = NS$)</td>
</tr>
<tr>
<td>HL60</td>
<td>$n = 10$</td>
<td>50 mg/kg (5×/week)</td>
<td>250 mg</td>
<td>81% (day 19, $P = 0.0003$)</td>
<td>Yes ($P = 0.0053$)</td>
</tr>
<tr>
<td>HL60</td>
<td>$n = 10$</td>
<td>100 mg/kg (once/week)</td>
<td>100 mg</td>
<td>73% (day 14, $P = 0.09$)</td>
<td>No ($P = NS$)</td>
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</tbody>
</table>

Figure 3. TH-302 treatment exerts \textit{in vivo} anti-leukemic activity. Sublethally irradiated SCID mice were injected via tail vein with human AML (HL60-luciferase, HEL-luciferase) cells. Systemic disease engraftment was confirmed by BLI approximately 7 to 10 days after tumor inoculation. Mice were then divided into groups of 5 to 10 animals and treated with vehicle (PBS) or TH-302 (50 mg/kg) i.p. 5 times a week for 3 weeks. A and B, representative whole-animal BLI on successive treatment days (expressed as total average photon flux per animal in each treatment group) is shown. $P$ values from Student t-test analysis are denoted as * for $P < 0.05$ and ** for $P < 0.001$. (Continued on the following page.)
effects were carried out in leukemia-naïve and systemic leukemia-engrafted animals. Mice were divided into groups of 5 to 10 animals and treated with vehicle (PBS) or TH-302 (at doses ranging from 50–100 mg/kg i.p. once a week up to 5 times a week). Mice were monitored 3 times per week for moribund signs (i.e., weight loss, paralysis, respiratory distress) prompting sacrifice.

**Immunohistochemistry**

Murine sternums were fixed in zinc fixative for 48 hours, decalcified for 48 hours, and embedded in paraffin. Sections were cut at 5 μm, placed on charged slides, and dried in a 60°C oven for 1 hour. Room temperature slides were deparaffinized in 3 changes of xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H2O2 for 10 minutes and washed with PBS with Tween 20 (PBS/T). Antigen retrieval was conducted in the microwave for 10 minutes in pH 6.0 citrate buffer, with a 15-minute cool down followed by a PBS/T wash. Slides were then loaded on the DAKO autostainer (Dako). Casein 0.03% was used to block for 30 minutes, blown off, and then the primary antibody Hypoxyprobe was applied at 1.4 μg/mL (1:50) to slides for 1 hour. An isotype-matched control (1.4 μg/mL mo IgG1) was used on a duplicate slide in place of the primary antibody as a negative control. A PBS/T wash was followed by anti-mouse biotinylated secondary antibody and ABC reagent (Vector Labs) for 30 minutes each with PBS/T washes between reagents. The chromagen DAB+ (Dako) was applied for 10 minutes. Slides were counterstained with hematoxylin, dehydrated, cleared, and coverslipped. Slides were evaluated using an Olympus Bx40 light microscope with 10×/0.30 and 20×/0.50 lenses and photographed with a Hitachi HV-C20U camera using Image Pro Plus Software (Media Cybernetics).

**Statistical analysis**

Results of xenograft experiments are shown as mean BLI (± SEM) at each time point for groups of 10 to 20 mice.
Statistical evaluation of differences in mean values was conducted using 2-tailed Student t test analysis with unequal variance. All \( P < 0.05 \) was considered significant. Log-rank tests were used to calculate the statistical significance of the difference in Kaplan–Meier survival curves. These analyses were conducted using the GraphPad Prism software program (Prism v5.0, GraphPad Software).

Results

Marrow hypoxia increases with progressive leukemia disease

Prior data in animal models showed that progressive growth of AML cells within the marrow space was associated with worsening hypoxia (10, 13, 14). To confirm this and the hypoxic nature of the marrow microenvironment in our models, we assessed the expression of the hypoxia marker pimonidazole in the bone marrow of irradiated SCID mice before and at fixed time points following intravenous (tail vein) inoculation of human AML (HEL-luciferase) cells. We noted increased numbers of pimonidazole-positive cells in the marrow over time which correlated with evidence of increasing in vitro leukemia by BLI (Supplementary Fig. S1) and marrow flow cytometry for human CD33 antigen expressed by HEL cells (Supplementary Fig. S1). To test whether residual AML cells resistant to chemotherapy also expressed hypoxic markers, we established cohorts of leukemia-engrafted mice. Twenty days after leukemia inoculation when all animals had clear evidence of systemic AML disease by BLI, mice were treated with single doses of vehicle (PBS), cytarabine, doxorubicin, or the VEGF inhibitor aflibercept (VEGF Trap, Regeneron/Sanofi Aventis; ref. 31). Animals were sacrificed 3 days later for marrow immunohistochemistry and flow cytometry. Leukemic bone marrows with evidence of residual AML disease (by flow cytometry for hCD33 expression) after treatment also contained pimonidazole-positive cells (representative images in Supplementary Fig. S2).

TH-302 preferentially inhibits growth of AML cells under hypoxia

To determine whether a hypoxic microenvironment contributes to the chemoresistance of AML cells, we then examined the effects of a fixed dose of cytarabine (100 nmol/L) on growth of human AML cells (HEL, HL60, ML-2) under prolonged hypoxic (1% \( O_2 \)) or normoxic (21% \( O_2 \)) conditions for up to 72 hours. The selection of 1% \( O_2 \) for the hypoxic condition was based on prior data that this oxygen tension may best reflect conditions within marrow hematopoietic niches (3, 32). The selection of 1% \( O_2 \) was confirmed by Annexin/PE/7-AAD flow cytometry, Western blot analyses for apoptotic markers, and cell viability assays with trypan blue exclusion (Fig. 1A–D; Supplementary Fig. S4). Of note, TH-302 treatment induced apoptosis and cell death of AML cells (HEL, HL60) under the same conditions (1% \( O_2 \), 72 hours) where resistance to cytarabine was noted. Additional experiments confirmed the dose- and hypoxia-dependent apoptotic effects of TH-302 against a panel of several other human AML cell lines representing diverse molecular and cytogenetic disease subtypes (Supplementary Table S2).

TH-302 inhibits primary AML colony formation under hypoxia

To assess the relevance of these results to primary AML disease, we established methylcellulose colonies using bone marrow samples from three relapsed patients with AML at our center. Patient 1 was a 78-year-old woman with secondary AML characterized by complex karyotype. Patient 2 was a 40-year-old woman with de novo normal karyotype AML characterized by NPM-1 mutations with wild-type FLT-3 status. Patient 3 was a 76-year-old woman with therapy-related normal karyotype AML with unknown mutation status. Colonies were treated with vehicle (PBS), cytarabine, or TH-302 for 72 hours under normoxic (21% \( O_2 \)) or hypoxic (1% \( O_2 \)) conditions and quantified. As shown, primary AML colonies were less responsive to the growth-inhibitory effects of cytarabine treatment under hypoxia versus normoxia (\( P = 0.01 \)). In contrast, TH-302 treatment significantly reduced the number of primary AML colonies under hypoxic versus normoxic conditions (Fig. 1E).

TH-302 inhibits growth of AML cells via multiple mechanisms

We then examined the potential mechanisms of action of TH-302 on AML cells. We found that the in vitro proliferation of human AML cells, as determined by tritiated thymidine incorporation, was highly sensitive to very low doses of TH-302 treatment, with hypoxia-specific differences detected after doses as low as 0.1 to 1 nmol/L (Fig. 2A). At higher doses of TH-302 (ranging from 0.5 to 50 \( \mu \)mol/L), AML cells (HEL, HL60) underwent progressive cell-cycle arrest in a dose-dependent manner with gradual accumulation of cells in the G0–G1 phase similar to prior reports in other tumors (Fig. 2B, Supplementary Table S1; ref. 26). We then examined TH-302-induced cross-linking of DNA as expressed by increased phosphorylation of the histone variant (H2AX) on AML cells (HEL, HL60). Under differing oxygen conditions, TH-302 (1–10 \( \mu \)mol/L) treatment of AML cells resulted in significant hypoxia- and dose-dependent DNA damage (Fig. 2C, data not shown). These data were confirmed by measurement of DNA fragmentation via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in hypoxic AML cells under the same conditions (Fig. 2D). TH-302 treatment in AML cells was also associated with dose-dependent decreases in HIF-1a protein, a master transcription factor typically upregulated in hypoxic cells (Fig. 2E).

We observed that AML cells cultured under chronic hypoxia (1% \( O_2 \), from 24 to 72 hours) generated increasing
levels of ROS, as detected by the fluorescent dye CM-H2DCFDA (Supplementary Fig. S5A). ROS production under these conditions appeared to be dependent on NADPH oxidase and/or other NAD(P) enzymes as they were attenuated in the presence of the inhibitor DPI (Supplementary Fig. S5B). We found that that TH-302 decreased hypoxia-induced ROS generation by viable AML cells (HEL, HL60) in a dose-dependent manner at drug concentrations where increased leukemia cell death was noted (0.5–5 μmol/L). Production of ROS by normoxic AML cells was not affected by TH-302 treatment (Fig. 2F and G, data not shown). Similar effects on hypoxia-induced ROS production were noted following cytarabine treatment of AML cells at these same time points (Supplementary Fig. SSC).

**TH-302 inhibits systemic AML growth in preclinical models**

We then evaluated the in vivo activity of TH-302 in SCID mice systemically engrafted with luciferase-transfected human AML (HEL, HL60) cells in the bone marrow and other extramedullary sites. Animals were treated with vehicle (PBS) or TH-302 administered at 3 differing dosing regimens based on prior reported literature (24–26). As summarized in Table 1, TH-302 treatment significantly reduced overall leukemia disease burden, as reflected by differences in BLI, in both animal models in 3 of 5 TH-302 treatment regimens (P < 0.05). However, only one regimen (TH-302 administered at 50 mg/kg 5 times a week for a total weekly dose of 250 mg/kg) resulted in significantly prolonged overall survival in both models as compared with vehicle controls (Table 1, Fig. 3A–C). Pimonidazole staining of bone marrow from leukemia-engrafted SCID mice following 3 weeks of TH-302 (50 mg/kg 5 times a week) therapy showed fewer hypoxic cells than vehicle-treated mice. This difference was specifically seen in the diaphyses of leukemia-engrafted mice (Fig. 3D). TH-302 exerted no obvious effects on marrow vasculature, as reflected by VEGFR-3 immunostaining of leukemic bone marrow sections (data not shown).

Because normal pluripotent hematopoietic stem cells also reside within underperfused hypoxic marrow niches, we specifically investigated the potential effects of prolonged in vivo TH-302 administration on normal hematopoiesis. We found no significant differences in mean white blood cell, neutrophil, lymphocyte, or platelet counts or hemoglobin levels of nonleukemic or leukemia-engrafted mice following 2 weeks of vehicle versus TH-302 treatment (100 mg/kg i.p. once a week) in HEL-luciferase-engrafted SCID mice (n = 4 mice/group).

**TH-302 treatment was effective in a model of advanced AML disease**

It has previously been reported that the in vivo efficacy of TH-302 correlated with the degree of tumor hypoxia, as measured by pimonidazole staining, across eleven solid tumor xenograft models (24). On the basis of our finding that bone marrow hypoxia increased with progressive AML disease in our models (Supplementary Fig. S1), we compared the relative in vivo efficacy of TH-302 in early (less hypoxic) and late (more hypoxic) AML disease. We therefore established cohorts of HEL-luciferase-engrafted mice via tail vein injection but varied the timing of treatment initiation with vehicle or TH-302. TH-302 therapy (50 mg/kg 5 times a week) was begun either on day 1 (1 day before Table 2. Peripheral blood counts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>TH-302</th>
<th>P</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Following TH-302 treatment in non-leukemic SCID mice&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cells (&lt;10&lt;sup&gt;8&lt;/sup&gt; cells/L)</td>
<td>2.0 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>0.10</td>
<td>1.8–15.0</td>
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<tr>
<td>Neutrophils (&lt;10&lt;sup&gt;6&lt;/sup&gt; cells/L)</td>
<td>1.1 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.21</td>
<td>0.1–5.7</td>
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<tr>
<td>Lymphocytes (&lt;10&lt;sup&gt;6&lt;/sup&gt; cells/L)</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.55</td>
<td>0.9–14.2</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>14 ± 0.3</td>
<td>13 ± 0.3</td>
<td>0.21</td>
<td>12.2–16.4</td>
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<tr>
<td>Platelets (&lt;10&lt;sup&gt;12&lt;/sup&gt; cells/L)</td>
<td>477 ± 37</td>
<td>576 ± 38</td>
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<td>100–1200</td>
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<tr>
<td>B. On treatment day 14 in leukemia-engrafted SCID mice&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>White blood cells (&lt;10&lt;sup&gt;8&lt;/sup&gt; cells/L)</td>
<td>8.2 ± 2.9</td>
<td>8.9 ± 3.5</td>
<td>0.90</td>
<td>1.8–15.0</td>
</tr>
<tr>
<td>Neutrophils (&lt;10&lt;sup&gt;6&lt;/sup&gt; cells/L)</td>
<td>4.7 ± 1.7</td>
<td>5.2 ± 2.2</td>
<td>0.86</td>
<td>0.1–5.7</td>
</tr>
<tr>
<td>Lymphocytes (&lt;10&lt;sup&gt;6&lt;/sup&gt; cells/L)</td>
<td>1.8 ± 0.37</td>
<td>2.4 ± 0.83</td>
<td>0.55</td>
<td>0.9–14.2</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>15 ± 0.7</td>
<td>13 ± 1.1</td>
<td>0.31</td>
<td>12.2–16.4</td>
</tr>
<tr>
<td>Platelets (&lt;10&lt;sup&gt;12&lt;/sup&gt; cells/L)</td>
<td>790 ± 21</td>
<td>930 ± 50</td>
<td>0.80</td>
<td>100–1200</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SE values of 6 mice per treatment group. No significant differences in white blood cell, hematocrit levels, platelet counts, neutrophil or lymphocyte percentages were noted in peripheral blood samples obtained after 7–14 days of treatment with vehicle versus TH-302 (50 mg/kg i.p. 5 times a week) in nonleukemic SCID mice (n = 6 mice/group).

<sup>b</sup>Mean ± SE values of 4 mice per treatment group. No significant differences in white blood cell, hematocrit levels, platelet counts, neutrophil or lymphocyte percentages were noted in peripheral blood samples obtained after 14 days of treatment with vehicle versus TH-302 (100 mg/kg i.p. once a week) in HEL-luciferase-engrafted SCID mice (n = 4 mice/group).
leukemia inoculation, termed “Early TH-302”) or on day 10 (8 days following leukemia inoculation, termed “Late TH-302”). Despite the differences in treatment duration, both “early” (20-day total duration) and “late” (10-day total duration) TH-302 regimens resulted in similar degrees of leukemia growth inhibition as assessed by BLI.

Figure 4. TH-302 treatment inhibits different stages of AML disease. A, experimental schema showing treatment regimens for different groups of irradiated SCID mice (n = 5). Vehicle controls (top row) received their first dose of PBS on day 1 followed by HEL-luciferase inoculation via tail vein on day 2 and an additional 19 days of treatment (total 20 days). "Early" TH-302–treated mice (middle row) received their first dose of TH-302 (50 mg/kg i.p. 5/C2/week) on day 1 followed on day 2 by leukemia inoculation via tail vein and an additional 19 days of treatment (total 20 days). "Late" TH-302–treated mice (bottom row) did not start TH-302 treatment until day 10 when systemic disease was documented by BLI. These animals received a total of 10 days of treatment. B and C, shown are representative BLI and total photon flux per animal per treatment group on day 20. These results show similar inhibition of AML growth in both "early" and "late" TH-302 treatment groups as compared to vehicle controls by Student t-test analysis. P < 0.05 is denoted by an asterisk.
measurement on day 20. Also of note, in these experiments, administration of the first dose of TH-302 up to 2 weeks before tumor cell inoculation did not appear to impact on the ability of HEL-luciferase AML cells to efficiently engraft and propagate in SCID mice as determined by successive BLI imaging (Fig. 4, data not shown).

Discussion
Emerging data have highlighted the intrinsic hypoxia of the bone marrow microenvironment as a critical in vivo modulator of malignant hematopoietic growth (2–4). Preclinical evidence has shown that expansion of acute leukemia cells within the bone marrow is associated with further decreases in local oxygenation (10, 13, 14). We and other groups have shown that acute leukemia cells placed under hypoxia upregulate HIF-1α, undergo cell-cycle arrest, and exhibit resistance to chemotherapy agents such as cytarabine (22, 23).

We postulated that TH-302, a hypoxia-activated nitrogen mustard derivative prodrug, would be effective in eradicating AML cells within a hypoxic bone marrow microenvironment. Here, we show that TH-302 induced hypoxia and dose-dependent cell death in human AML cell lines and primary AML samples under the same conditions (1% O2, 72 hours) where cytarabine resistance was observed. Mechanisms of action of TH-302 under hypoxia included cell-cycle arrest, DNA strand breakage, and inhibition of HIF-1α protein expression.

It has been proposed that a major benefit of a chronically hypoxic marrow microenvironment to hematopoietic cells is reduced exposure to potentially damaging ROS which can induce genomic instability and DNA damage (8). In fact, under normoxia, chemotherapeutic agents, such as cytarabine, typically induce ROS followed by cell death in AML cells (33). In our experiments, we noted that human AML cells culture under prolonged hypoxia (1% O2 for 48–72 hours) expressed progressively higher levels of ROS over time. Although the mechanisms underlying this ROS production are unclear, they appear to depend, in part, on NAPDH-oxidase. Treatment of AML with TH-302 (as well as cytarabine) under these same conditions markedly decreased ROS generation by hypoxic AML cells. Elevated oxidative stress in primary leukemia blasts has recently been linked to AML progression, relapse, and poor prognosis, specifically in FLT-3–mutated AML (34, 35). Increased ROS may also regulate leukemia self-renewal and survival. For instance, ROS generation contributes to leukemogenesis mediated by BCR-ABL in hematopoietic cells and promotes the growth and migration of cells expressing other oncogenic kinases (36). The negative effects of TH-302 on ROS generation therefore may be of therapeutic benefit in patients with AML.

Our results show that TH-302 treatment inhibited AML disease and prolonged survival in 2 systemic human AML models at drug doses similar to those previously evaluated in human patients (27, 28). Because hypoxia is critical in regulating normal HSC biology, we examined whether treatment induced any significant myelosuppression in leukemia-engrafted or leukemia-naïve SCID mice. After 2 weeks of therapy, no hematologic toxicities were noted. These latter results are in line with prior clinical trials showing limited hematological toxicity of TH-302 therapy in patients with solid tumors (27, 28). The differential effects of TH-302 on normal versus malignant hematopoietic cells could be explained by the selective hypoxic nature of this agent. Because acute leukemia growth markedly expands hypoxic marrow regions, TH-302 may preferentially eradicate tumor cells populating these low oxygenated regions while sparing HSCs in better perfused areas. TH-302 may also selectively target leukemia cells via hypoxia-independent mechanisms; for instance, it has been shown that solid tumor cells deficient in homology-dependent repair exhibit enhanced sensitivity to TH-302 (25).

Hypoxia has also increasingly been implicated in leukemia stem cell biology (37). We noted that in vitro TH-302 treatment decreased numbers of primary AML colonies under hypoxia. However, in contrast, in vivo TH-302 treatment for up to 2 weeks before the inoculation of human AML (HEL-luciferase) cells in irradiated SCID hosts did not affect subsequent disease engraftment or propagation. Because our experiments did not clearly distinguish between the effects of TH-302 on leukemia stem cells as opposed to bulk hypoxic AML cells, further studies are needed.

Overall the results presented here, together with early results of clinical activity in a phase 1 trial of TH-302 in hematologic cancers, provide the rationale for further clinical investigation of TH-302 for patients with AML (27–29). Our finding that chronic (5 times per week) administration of TH-302 was associated with optimal in vivo activity in preclinical AML models may be relevant for future dosing regimens. Further development of TH-302 and other hypoxia-activated agents for acute leukemia therapy, however, may need to take into account the unique mechanism of action of such drugs within the leukemic marrow microenvironment (11, 38–40). Because hypoxic regions within the marrow expand as a consequence of acute leukemia progression, TH-302 may be most effectively used in later stage disease (10, 13, 14). Moreover, the presence of residual cells expressing hypoxic markers in leukemic marrows following cytarabine or doxorubicin treatment suggests that the administering TH-302 together with or following standard chemotherapy may further improve outcomes. Newer combinations of TH-302 with other therapeutic agents (such as bortezomib) with potential synergistic antitumor activity also hold promise for AML (41). Additional clinical studies are needed to verify these findings and potentially define those subsets of patients with AML who might benefit from TH-302 therapy.

Disclosure of Potential Conflicts of Interest
C.P. Hart is an employee and stockholder of Threshold Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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References


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