Activity of the hypoxia-activated prodrug, TH-302, in preclinical human acute myeloid leukemia (AML) models

Running Title: TH-302 in acute myeloid leukemia

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STATEMENT OF TRANSLATIONAL RELEVANCE

Acute myeloid leukemia (AML) is an aggressive hematological neoplasm affecting older adults. The primary site of disease initiation and expansion in AML patients 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 demonstrate that the hypoxia-activated prodrug, 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 hematological 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.
ABSTRACT

Purpose: Acute myeloid leukemia (AML) is an aggressive hematological neoplasm. Recent evidence has demonstrated the bone marrow microenvironment in AML patients 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 i.p. five 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 non-leukemic 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 acute myeloid leukemia and provide the rationale for further clinical studies of this and other hypoxia-activated agents for leukemia therapy.

Keywords: TH-302, acute myeloid leukemia, hypoxia, marrow microenvironment, chemoresistance
INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive immature hematological neoplasm primarily affecting older adults. Chemotherapy for AML has not changed since the 1980s and continues to result in long-term cure in only 20-30% of patients. Recent data has demonstrated that the bone marrow (BM) 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 non-perfused 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 HSC and decrease levels of potentially toxic reactive oxygen species. (8, 9)

The hypoxic bone marrow microenvironment has also been shown to contribute to AML disease. Prior data in multiple animal models demonstrated that acute leukemic progression within the BM is associated with further decreases in overall marrow oxygenation and expansion of hypoxic BM areas. (10-12) This has been attributed in part to potentially compromised blood flow within a restricted non-compliant 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 BM 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 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 biological
adaptations by AML cells to hypoxia also confer resistance to chemotherapy. We and other investigators have reported that overexpression of hypoxic inducible factor-1 α (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 prodrug of the cytotoxin 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 hematological cancers have demonstrated 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 (Manassas, VA). ML-2 cells containing MLL/11q23 were obtained from DSMZ (Braunschweig, Germany). (30) NB-4, MV4;11, LAMA, KG-1, and THP-1 were originally obtained from DSMZ and were kindly provided by Dr. Costa. G. 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%
fetal bovine serum, 2 mM L-glutamine, 4.5 g/L glucose, 1.5 gm sodium bicarbonate, 1 u/ml penicillin/ streptomycin, 10 mM HEPES, and 1 nM sodium pyruvate. TH-302 was provided by Threshold Pharmaceuticals, Inc (South San Francisco, CA). Cytarabine, doxorubicin, and the NADPH oxidase inhibitor, diphenyleneiodonium (DPI), were purchased from Sigma-Aldrich (St. Louis, MO). 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, New York) 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., Herndon, VA). Results were averaged among triplicate wells.

**Flow cytometry.** Leukemia cells were plated in triplicate in 6-well plates at a concentration of 5 x 10⁵ cells/ml in the presence of vehicle (DMSO) or varying concentrations of TH-302. Cells were then incubated for 24-72 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, San Diego, CA). Double-stranded DNA breaks were assessed via measurements of phosphorylated histone H2A.X (EMD Millipore, Billerica, MA) and with the APO-BrdU TUNEL Assay kit (Molecular Probes, Life Technologies, Grand Island, NY) following manufacturer's instructions. Cell cycle analysis was performed on cells treated for 24 hours and then washed, fixed with 70% ethyl alcohol for 30 minutes at 4° C, and centrifuged 3200 rpm for 3 minutes. Cells were then treated with RNAseA treatment (0.5 mg/ml) for another 30 minutes at 37° C. followed by the addition of propidium
iodide (1 mg/ml) (GenScript, Piscataway, NJ) for an additional 30 minutes at 4°C in the dark followed by analysis. To detect reactive oxygen species (ROS) in live cells, the fluorescein-based dye CM-H$_2$DCFDA (Invitrogen) was added to samples at a final concentration of 10uM 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 performed on a FacsCalibur instrument (BD, Franklin Lakes, NJ) followed by analysis using Winlist Version 6.0 (Verity Software House, Topsham, ME) and FCS Express software (De Novo Software, Los Angeles, CA). Experiments were performed independently at least twice.

**Protein analyses.** Cells (1 x 10$^6$/ml) were plated in 6-well plates for drug and/or hypoxic treatment followed by lysis in RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1mM 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, Hercules, CA). Lysates were separated by SDS-PAGE (12% for cleaved caspase-3, 8% for the rest) and detected by chemiluminescence (ECL plus, GE Healthcare, UK). Specific antibodies included anti-cleaved caspase 3 (#9661, Cell Signaling Technology, Danvers, MA) and anti–cleaved poly (ADP-ribose) polymerase (PARP) (#9546, Cell Signaling Technology) used at a final dilution ratio of 1:1000. Antibody to actin (#CP01) was obtained from Calbiochem (San Diego, CA). For HIF-1α immunoassays, protein samples were analyzed following the manufacturer’s instructions (R&D Systems). Cells treated with cobalt chloride 100 μM (Sigma Aldrich) for four hours served a positive control.
**Primary AML clonogenic assays.** Primary AML patient cells (2 x 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 3U/ml (HSC003, R&D Systems, Minneapolis, MN) and cultured at 37°C (21% O₂, 5% CO₂) for 14 days to allow for baseline growth. Following this, vehicle (PBS), cytarabine (0.01 nM), doxorubicin (0.5 nM), or TH-302 (0.1-1 nM) 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 (±SEM) leukemia colony formation units (CFU-L) were quantified using an inverted microscope.

**Leukemia xenograft experiments.** All animal experiments were performed under an approved IACUC protocol. Six to eight 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 (1x10^7 cells) injection of human HEL and HL60/VCR cells stably transfected with a pGL4 luciferase reporter vector (Promega, Madison, WI) as described previously. (31) Mice were evaluated weekly for evidence of leukemia engraftment and overall leukemia disease progression by serial bioluminescent imaging (BLI) (Xenogen IVIS 50 System, Caliper Life Science, Hopkinton, MA) 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 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 pimonidazole 60 mg/kg (Hypoxyprobe® Store, Burlington, MA, USA) 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, San Diego, CA) myeloid antigen expression as previously described.(31) Experiments evaluating in vivo TH-302 effects were performed in Leukemia-naive and systemic leukemia engrafted animals. Mice were divided into groups of 5-10 animals and treated with vehicle (PBS) or TH-302 (at doses ranging from 50-100 mg/kg i.p. once a week up to five times a week. Mice were monitored three 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 5µm, placed on charged slides, and dried in a 60°C oven for 1 hour. Room temperature slides were de-paraffinized in three changes of Xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H₂O₂ for 10 minutes and washed with phosphate buffered saline with Tween 20 (PBS/T). Antigen retrieval was performed 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, Carpinteria, CA). Casein 0.03% was used to block for 30 minutes, blown off, and then the primary antibody Hypoxyprobe™ was applied at 1.4µg/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, Burlingame, CA) 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 cover-slipped. Slides were evaluated using an Olympus Bx40 light
microscope with 10x/0.30 and 20x/0.50 lenses and photographed with a Hitachi HV-C20U camera using Image Pro Plus Software (Media Cybernetics, Silver Spring, MD).

**Statistical analysis.** Results of xenograft experiments are shown as mean BLI ± standard error of the mean (SEM) at each time point for groups of 10-20 mice. Statistical evaluation of differences in mean values was performed using two-tailed student’s t test analysis with unequal variance. All p values < 0.05 were considered significant. Log-rank tests were used to calculate the statistical significance of the difference in Kaplan-Meier survival curves. These analyses were performed using the GraphPad Prism software program (Prism v5.0, GraphPad Software, San Diego, CA).

**RESULTS**

**Marrow hypoxia increases with progressive leukemia disease**

Prior data in animal models demonstrated 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 prior to 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 vivo leukemia disease by BLI (**Supplemental Figure 1**) and marrow flow cytometry for human CD33 antigen expressed by HEL cells (**Supplemental Figure 2**). 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 were treated with single doses of vehicle (PBS), cytarabine, doxorubicin, or the vascular
endothelial growth factor (VEGF) inhibitor aflibercept (VEGF Trap, Regeneron/Sanofi Aventis, NJ)(31). Animals were sacrificed three 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 all contained pimonidazole-positive cells (representative images in Supplemental Figure 2).

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 nM) on growth of human AML cells (HEL, HL60, ML-2) under prolonged hypoxic (1% O2) or normoxic (21% O2) conditions for up to 72 hours. The selection of 1% O2 for the hypoxic condition was based on prior data that this oxygen tension may best reflect conditions within marrow hematopoietic niches. (3, 32) The cytarabine dose was selected based on prior dose escalation experiments (Supplemental Figure 3). As shown, AML cells (HEL, HL60, ML-2) propagated under chronic hypoxia for 48 to 72h exhibited less cytarabine-induced apoptosis than normoxic controls as determined by annexin-PE/7-AAD flow cytometry, western blot analyses for apoptotic markers, and cell viability assays with trypan blue exclusion (Figure 1A-D, Supplemental Figure 4). Of note, TH-302 treatment induced apoptosis and cell death of AML cells (HEL, HL60) under the same conditions (1% O2, 72h) 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 (Supplemental Table 2).

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 AML patients at our
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% O2) or hypoxic (1% O2) conditions and quantified. As shown, primary AML colonies were less responsive to the growth inhibitory effects of cytarabine treatment under hypoxia vs. normoxia (p=0.01). In contrast, TH-302 treatment significantly reduced the number of primary AML colonies under hypoxic vs. normoxic conditions (Figure 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 nM (Figure 2A). At higher doses of TH-302 (ranging from 0.5 to 50µM), 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 (Figure 2B, Supplemental Table 1).(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 µM) treatment of AML cells resulted in significant hypoxia-and dose-dependent DNA damage (Figure 2C, data not shown). These data were confirmed by measurement of DNA fragmentation via TUNEL assay in hypoxic AML cells under the same conditions (Figure 2D). TH-302 treatment in AML cells was also associated with dose-dependent decreases in HIF-1α protein, a master transcription factor typically upregulated in hypoxic cells (Figure 2E).
We observed that AML cells cultured under chronic hypoxia (1% O2, from 24 to 72h) generated increasing levels of reactive oxygen species (ROS), as detected by the fluorescent dye CM-H₂DCFDA (Supplemental Figure 5A). 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 dipehenyleneiodonium (DPI) (Supplemental Figure 5B). 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 µM). Production of ROS by normoxic AML cells was not affected by TH-302 treatment (Figure 2F-G, data not shown). Similar effects on hypoxia-induced ROS production were noted following cytarabine treatment of AML cells at these same time points (Supplemental Figure 5C).

**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 BM and other extramedullary sites. Animals were treated with vehicle (PBS) or TH-302 administered at three 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 five times a week for a total weekly dose of 250 mg/kg) resulted significantly prolonged overall survival in both models as compared with vehicle controls (Table 1, Figure 3A-C). Pimonidazole staining of BMs from leukemia-engrafted SCID mice following three weeks of TH-302 (50 mg/kg five times a week) therapy demonstrated fewer hypoxic cells than vehicle-treated mice. This difference was specifically seen in the diaphyses of leukemia-engrafted mice (Figure 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 under-perfused 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 non-leukemic or leukemia-engrafted mice following two weeks of vehicle vs. TH-302 treatment (100 mg/kg i.p. once a week or 50 mg/kg i.p. five times a week) (Table 2).

**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. Based on our finding that BM hypoxia increased with progressive AML disease in our models (Supplemental Figure 1), 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 five times a week) was begun either on Day 1 (one day prior to 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 determined by BLI measurement on day 20. Also of note, in these experiments, administration of the first dose of TH-302 up to 2 weeks prior to 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 (Figure 4, data not shown).
DISCUSSION

Emerging data has 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 demonstrated 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 BM microenvironment. Here we demonstrate 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, 72h) 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 reactive oxygen species (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 AML patients.

Our results show that TH-302 treatment inhibited AML disease and prolonged survival in two 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-naive SCID mice. After two weeks of therapy, no hematological toxicities were noted. These latter results are in line with prior clinical trials demonstrating 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 HSC 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 two weeks prior to 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 I trial of TH-302 in hematological cancers, provide the rationale for further clinical investigation of TH-302 for AML patients. (27-29) Our finding that chronic (five 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 anti-tumor activity also hold promise for AML. (41) Additional clinical studies are needed to verify these findings and potentially define those subsets of AML patients who might benefit from TH-302 therapy.
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AUTHORSHIP CONTRIBUTIONS
S.P., D.L., and R.V. performed research, and collected, analyzed, and interpreted data. Y-C.H. designed and performed research, collected data, and analyzed and interpreted results. M.W. provided primary patient samples and analyzed and interpreted data. C.P.H. provided reagent and analyzed and interpreted data. E.S. W. designed research, analyzed and interpreted data, and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

DISCLOSURES OF CONFLICTS OF INTEREST
C. P. H is an employee and stockholder of Threshold Pharmaceuticals, Inc. The other authors declare no competing financial interests in relation to the work described.
REFERENCES


Table 1: Summary of the anti-leukemic activity of different TH-302 regimens

<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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<tr>
<td>HEL</td>
<td>n=10</td>
<td>50 mg/kg (5x/week)</td>
<td>250 mg</td>
<td>83% (day 14, p=0.05)</td>
<td>Yes (p=0.0285)</td>
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<td>HEL</td>
<td>n=5</td>
<td>75 mg/kg (2x/week)</td>
<td>150 mg</td>
<td>93% (day 21, p=0.0398)</td>
<td>No (p=0.0875)</td>
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<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>
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<tr>
<td>HL60</td>
<td>n=10</td>
<td>50 mg/kg (5x/week)</td>
<td>250 mg</td>
<td>81% (day 19, p=0.0003)</td>
<td>Yes (p=0.0053)</td>
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<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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Table 2A. Peripheral blood counts following TH-302 treatment in non-leukemic SCID mice

<table>
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<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>TH-302</th>
<th>p-value</th>
<th>Normal range</th>
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<tr>
<td>White blood cells (x 10^8 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>
</tr>
<tr>
<td>Neutrophils (x 10^8 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>
</tr>
<tr>
<td>Lymphocytes (x 10^8 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 (gm/dl)</td>
<td>14 ± 0.3</td>
<td>13 ± 0.3</td>
<td>0.21</td>
<td>12.2 - 16.4</td>
</tr>
<tr>
<td>Platelets (x 10^9 cells/L)</td>
<td>477 ± 37</td>
<td>576 ± 38</td>
<td>0.09</td>
<td>100 - 1200</td>
</tr>
</tbody>
</table>

Mean ± SE values of 6 mice per treatment group.
Table 2B. Peripheral blood counts on treatment day 14 in leukemia-engrafted SCID mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>TH-302</th>
<th>p-value</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells</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>(x 10^8 cells/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils (x 10^8 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 (x 10^8 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 (gm/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 (x 10^9 cells/L)</td>
<td>790 ± 21</td>
<td>930 ± 50</td>
<td>0.80</td>
<td>100 - 1200</td>
</tr>
</tbody>
</table>

Mean ± SE values of 4 mice per treatment group.
Table Legends

Table 1. Anti-leukemic activity of different TH-302 regimens.

Table 2. (A) Peripheral blood counts following TH-302 treatment in non-leukemic SCID mice. 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 vs. TH-302 (50 mg/kg i.p. five times a week) in non-leukemic SCID mice (n=6 mice/group). (B) Peripheral blood counts on treatment day 14 in leukemia-engrafted SCID mice. 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 vs. TH-302 (100 mg/kg i.p. once a week) in HEL-luciferase engrafted SCID mice (n=4 mice/group).
**FIGURE LEGENDS**

**Figure 1. TH-302 preferentially inhibits in vitro growth of AML cells under hypoxia.** (A-B) Human AML cells (HL60, HEL) were cultured in the presence or absence of cytarabine 100 nM under normoxic (21% O₂) or hypoxic (1% O₂) conditions for up to 72 hours. Mean number of apoptotic cells ± SEM from triplicate samples under varying conditions as assessed by 7-AAD/annexin-V flow cytometry are shown. (B) Cytarabine-mediated apoptosis under hypoxic versus normoxic conditions was also measured by Western blot analysis for cleaved PARP in AML (HL60) cells. (C-D) TH-302 treatment resulted in cell death of AML cells (HL60, HEL) in a dose-dependent and hypoxia-specific manner. Shown are the mean ± SEM percentages of dead cells as determined by flow cytometry for 7-AAD/annexin-V following 72 hours of incubation with vehicle or TH-302 at doses ranging from 0.1-5 μM under normoxia (21% O₂) or hypoxia (1% O₂). (E) Primary AML cells (2 x 10⁵) from three AML patients were grown in methylcellulose culture for 10-14 days followed by treatment with vehicle (PBS), cytarabine (Ara-C 0.01 nM), or TH-302 (0.5 μM) for 72 hours under hypoxia or normoxia. Shown is the mean (± SEM) number of leukemia colony formation units (CFU) under each condition as determined in triplicate plates by inverted microscopy. For all results, white bars indicate normoxia; grey bars indicate hypoxia. All experiments were performed three separate times with triplicate samples per condition. P values <0.05 by t-test analysis were considered significant and were denoted by an asterisk.

**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 nM) 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 three 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 two 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. (E) Hypoxic AML cells treated with TH-302 (1-10 μM) 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-G) Production of reactive oxygen species (ROS) by HL60 AML cells following 48 hours of hypoxia (1% O₂) 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 performed 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 two independent experiments were performed for all of the above experiments. White bars indicate normoxia. Grey bars indicate hypoxia. Student’s t test analysis compared conditions versus controls. Significant p values are denoted as * for p<0.05, ** for p<0.001, and *** for p≤0.0001.

Figure 3. TH-302 treatment exerts 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 bioluminescent imaging (BLI) approximately 7-
10 days after tumor inoculation. Mice were then divided into groups of 5-10 animals and treated with vehicle (PBS) or TH-302 (50 mg/kg) i.p. five times a week for three weeks. (A-B) Representative whole animal bioluminescent imaging on successive treatment days (expressed as total average photon flux per animal in each treatment group) is shown. P values from student's t test analysis are denoted as * for p<0.05 and ** for p<0.001. (C) Overall survival analyses of HEL and HL60 engrafted mice by treatment group demonstrate significantly prolonged survival following TH-302 treatment versus vehicle controls (Kaplan-Meier analysis, p<0.05). (D) Bone marrow samples obtained on treatment day 14 from HEL-luciferase engrafted animals demonstrated reduced numbers of hypoxic (pimonidazole-positive brown-staining cells) marrow cells following TH-302 treatment as compared with vehicle controls (10-20x magnification, Olympus Bx40 light microscope). All experiments were independently repeated at least three times.

Figure 4. TH-302 treatment inhibits different stages of AML disease. (A) Experimental schema demonstrating 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. 5x/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 bioluminescent imaging (BLI). These animals received a total of 10 days of treatment. (B-C) Shown are representative bioluminescent imaging and total photon flux per animal per treatment group on Day 20. These results demonstrate similar inhibition of AML growth in both "early" and "late" TH-302 treatment groups as compared to vehicle controls by student's t-test analysis. P values <0.05 are denoted by an asterisk.
Figure 1.

A.

### HL60

<table>
<thead>
<tr>
<th>Condition</th>
<th>PBS (21% O₂)</th>
<th>PBS (1% O₂)</th>
<th>Ara-C (21% O₂)</th>
<th>Ara-C (1% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Annexin-V/7AAD positive cells by flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p</strong></td>
<td>0.0008</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### HEL

<table>
<thead>
<tr>
<th>Condition</th>
<th>PBS (21% O₂)</th>
<th>PBS (1% O₂)</th>
<th>Ara-C (21% O₂)</th>
<th>Ara-C (1% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Annexin-V/7AAD positive cells by flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p</strong></td>
<td>0.0015</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. **HL60**

<table>
<thead>
<tr>
<th>Cyta (100nM)</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nor</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypo</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Cleaved PARP**

Actin
Figure 1.

D. HEL

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean Percentage of dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (21% O2)</td>
<td>13</td>
</tr>
<tr>
<td>Vehicle (1% O2)</td>
<td>18</td>
</tr>
<tr>
<td>TH302 5μM (21% O2)</td>
<td>75</td>
</tr>
<tr>
<td>TH302 5μM (1% O2)</td>
<td>86</td>
</tr>
<tr>
<td>TH302 1μM (21% O2)</td>
<td>74</td>
</tr>
<tr>
<td>TH302 1μM (1% O2)</td>
<td>***</td>
</tr>
<tr>
<td>TH302 0.5μM (21% O2)</td>
<td>60</td>
</tr>
<tr>
<td>TH302 0.5μM (1% O2)</td>
<td>***</td>
</tr>
<tr>
<td>TH302 0.1μM (21% O2)</td>
<td>34</td>
</tr>
<tr>
<td>TH302 0.1μM (1% O2)</td>
<td>***</td>
</tr>
</tbody>
</table>
E. Primary AML samples

![Graph showing mean number of CFU-L per plate for different conditions.](image)

- **PBS (21% O₂)**
- **PBS (1% O₂)**
- **Ara-C (21% O₂)**
- **Ara-C (1% O₂)**

- **PBS (21% O₂)**
- **PBS (1% O₂)**
- **TH-302 (21% O₂)**
- **TH-302 (1% O₂)**

* *p=0.01

**p=0.74

* *p=0.01
Figure 2.

A. HL60

B. HEL

---

$^{3}$H-tritiated thymidine counts

- PBS (21% $O_2$)
- PBS (1% $O_2$)
- TH-302 (21% $O_2$)
- TH-302 (1% $O_2$)

---

$^{3}$H-tritiated thymidine counts

- PBS (21% $O_2$)
- PBS (1% $O_2$)
- TH-302 (21% $O_2$)
- TH-302 (1% $O_2$)

***
Figure 2.

B.

**Normoxia (21% O₂)**

- **Vehicle**
  - G₁
  - S
  - G₂
  - DNA content (PI)

- **TH-302 0.5 μM**
  - G₁
  - S
  - G₂
  - DNA content (PI)

- **TH-302 5 μM**
  - G₁
  - S
  - G₂
  - DNA content (PI)

**Hypoxia (1% O₂)**

- **Vehicle**
  - G₁
  - S
  - G₂
  - DNA content (PI)

- **TH-302 0.5 μM**
  - G₁
  - S
  - G₂
  - DNA content (PI)

- **TH-302 5 μM**
  - G₁
  - S
  - G₂
  - DNA content (PI)
Figure 2.

C. H2AX phosphorylation
Figure 2.

D. DNA fragmentation by TUNEL

![Graph showing DNA strand breaks under different conditions]

- Vehicle (21% O₂)
- Vehicle (1% O₂)
- TH-302 1 μM (21% O₂)
- TH-302 1 μM (1% O₂)
- TH-302 5 μM (21% O₂)
- TH-302 5 μM (1% O₂)

- Normoxia (21% O₂)
- Hypoxia (1% O₂)

*** statistically significant difference
Figure 2.

E. HIF-1α protein expression
Figure 2.

F. ROS generation

ROS levels (fluorescent intensity)

Vehicle (DMSO)
TH-302 0.05μM
TH302 0.5μM
TH-302 5μM

Vehicle (DMSO)
TH302 0.05μM
TH302 0.5μM
TH-302 5μM

Hypoxia (1% O2)
Normoxia (21% O2)

***
Figure 2.

G. Normoxia

- Vehicle
- TH-302 0.05 μM
- TH-302 0.5 μM
- TH-302 5 μM

Hypoxia

- Cell counts

Fluorescent intensity
Figure 3.

A. HL60

Day 11                Day 14              Day 18

Vehicle

TH-302

Total Flux (photons/s)

0                   1.0x10^09
                      2.0x10^09
                      3.0x10^09


* **
Figure 3.

B. HEL

Day 7  Day 11  Day 14

Vehicle

TH-302

TH-302 D7  TH-302 D11  TH-302 D14

Vehicle D7  Vehicle D11  Vehicle D14  TH-302 D14

Total Flux (photons/s)

0  2.0×10^8  4.0×10^8  6.0×10^8  8.0×10^8  1.0×10^9

Luminescence (photons/cm^2/s)

0.2  0.4  0.6  0.8  1.0

Color Scale
Min = 3.75E5
Max = 1.00E6
Figure 3.

C. HL60

- Vehicle
- TH-302

Percent survival

Days

p=0.005

HEL

- Vehicle
- TH-302

Days

p=0.024
Figure 3.

D. Vehicle TH-302

Pimonidazole

10x

20x
Figure 4.

A.

Vehicle (PBS)

Day 1: Vehicle (PBS)

Day 2: Inject AML cells

Days 3-20: Vehicle (PBS) treatment

Day 20: Image mice

Early TH-302

Day 1: TH-302

Day 2: Inject AML cells

Days 3-20: TH-302 treatment

Day 20: Image mice

Late TH-302

Day 2: Inject AML cells

Observation →

Days 10-20: TH-302 treatment

Day 20: Image mice
Figure 4.

B. Day 20

Vehicle

TH-302 Early

TH-302 Late

Day 20

Total Flux (photons/s)

0

2.0x10^7

4.0x10^7

6.0x10^7

8.0x10^7

Vehicle 20 days
TH-302 20 days
TH-302 10 days

* *
Activity of the hypoxia-activated prodrug, TH-302, in preclinical human acute myeloid leukemia (AML) models

Scott Portwood, Deepika Lal, Yung-Chun Hsu, et al.

Clin Cancer Res Published OnlineFirst October 2, 2013.