Leukemia Microenvironment and Pathologic Hypoxia: Sensitivity to Hypoxia-Activated Cytotoxin TH-302

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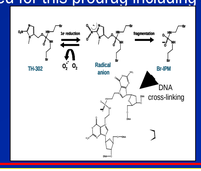
ABSTRACT

We have recently demonstrated that the leukemic bone marrow (BM) niche is highly hypoxic and that hypoxia promotes resistance of leukemic cells to chemotherapy (Benito et al., PLoS One 2011, e23108). Our preliminary data indicate that AML cells surviving chemotherapy in the human xenograft mouse models of leukemia reside within hypoxic areas of BM microenvironment as documented by staining with the hypoxia marker CAIX. These findings support utility of hypoxia-activated pro-drugs with the goal to eliminate leukemic blasts and leukemic stem cells residing in hypoxic BM microenvironment. TH-302 is a 2-nitroimidazole linked bromo-isophosphoramide mustardcytotoxin that upon hypoxia-dependent activation induces DNA cross-linking. TH-302 exhibited potent hypoxia-selective anti-leukemia activity in pre-B ALL (REH, NALM6), AML (OCI-AML3, MOLM-13, KG-1) and CML cell lines (KBM5), with IC50s at 1% O_2 ranging from 0.04μM to 2.3μM and hypoxia cytotoxicity ratio (HCR) ranging from 116 for KBM5 to 11 for REH cells. TH-302 at 5-7.5μM also exhibited hypoxia-dependent anti-leukemia activity in primary ALL and AML samples (N=3; normoxia, 2-8%; hypoxia, 28-65% apoptotic cells).To better recapitulate the multidimensional BM niche we utilized co-cultures of GFP-labeled leukemic cells with bone marrow-derived RFP-labeled mesenchymal stromal cells (MSC) immobilized within Matrigel. MSC and leukemic cells generated three-dimensional (3D) structures -"spheroids" -and co-proliferated over time with colonies of leukemic cells firmly attached to MSC, as monitored by confocal microscopy (Fig. 1). Pimonidazole staining shows that vast hypoxia is present in the MSC/AML spheroids grown at normal oxygen tension, in contrast to what is observed in plastic-based (2-D) stromal co-cultures. Anti-leukemia activity of TH-302 was next determined in 2D vs 3D co-cultures of MSCs plus MOLM-13 or OCI-AML3 cells. In 2D co-cultures, MSC protected MOLM-13 and OCI-AML3 cells from TH-302-induced cytotoxicity, while extensive apoptosis was documented in hypoxic spheroid co-cultures (at 50nM TH-302, reduction in viability 10-15% vs >60%, Fig. 1). These findings suggest that culture conditions faithfully mimicking BM microenvironment promote pathologic hypoxia generated by rapidly proliferating AML cells, which in turn leads to their increased sensitivity to hypoxia-activatedcytotoxins. To validate these findings *in vivo*, we next tested anti-leukemia efficacy of TH-302 in the *in vivo* model of primary AML established in NSG mice. TH-302 (50 mg/kg IP 3 times a week for three weeks) reduced the number of circulating AML cells (control, 13.2+/-5.7 x10^6/ml; TH-302, 2.5+/-2.1 x10^6/ml) and prolonged survival of NSG mice engrafted with primary AML cells compared to the vehicle treated mice (median survival time: TH-302=75 days; Control=56 days; P=0.003, n= 8 mice/group). To test the ability of hypoxia-activated prodrug to target leukemia-initiating cells, secondary transplant experiments were performed in which BM cells from control or TH-302 treated mice (collected after two weeks of therapy) were serially diluted and injected into secondary NSG recipient mice at 0.01, 0.005 or 0.0001x10^6 cells/mouse (N=5 mice/dilution). Although all mice transplanted with higher cell doses died from leukemia, we observed significantly prolonged survival of animals injected with 0.01X10^6 cells from TH-302-treated primary recipients compared with vehicle-treated controls (median survival control=68 days; TH-302=79 days; P=0.0031) or with 0.005x10^6 cells (control=79 days; TH-302=83 days; P=0.0462). In summary, our findings suggest that pathologic hypoxia is a prevalent condition of leukemic BM microenvironment that promotes survival of leukemic blasts and leukemia-initiating cells. The results support targeting hypoxia with hypoxia-activated cytotoxins such as TH-302 to enhance the efficacy of therapeutic regimens in AML. A Phase 1 single agent clinical trial of TH-302 in patients with relapsed/refractory hematologic malignancies is ongoing. Disclosures: Handisides: *Threshold Pharmaceuticals:* Employment. Hart: *Threshold Pharmaceuticals:* Employment.Konopleva: *Threshold Pharmaceuticals:* Research Funding.

BACKGROUND

- We have recently demonstrated a marked expansion of hypoxia in leukemia bone marrow (Benito et al., 2011).
- Hypoxia promotes resistance to radiation and chemotherapy.
- Hypoxia targeting bioreductive prodrugs potentially provide a direct mechanism for sensing and eliminating hypoxic cells.
- Hypoxia-activated prodrugs are a substrate for intracellular one-electron (1e-) reductases, such as cytochrome P450 reductase, which add an electron to the prodrug and therefore convert it to a radical anion.
- TH-302 is an inactive prodrug created by the covalent conjugation of 2-nitroimidazole as an oxygen sensor to bromo-isophosphoramide (Br-IPM). The 2-nitroimidazole sensor moiety undergoes reduction, and in the presence of severe hypoxia and near anoxia, the Br-IPM is released in situ and cross-links DNA. Bromo-IPM is the active alkylating moiety (Figure 1). Good tumor growth inhibition in pre-clinical cancer models has been reported for this prodrug including solid tumors and multiple myeloma (Hu et al., 2010; Sun et al., 2011).

Figure 1. TH-302: Hypoxia Targeted Drug
Mechanism of prodrug activation and MOA of released effector



GOAL: to characterize anti-leukemia activity of TH-302

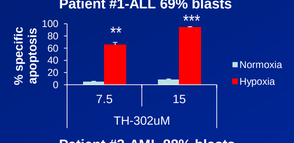
RESULTS

1-TH-302 exhibits potent anti-leukemia activity in vitro

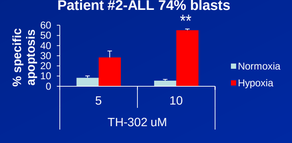
Cell line	TH-302 incubation (hr)	Total incubation time	Normoxic IC_{50}	Hypoxic IC_{50}	HCR
KBM5	6hr	72hr	16.5	0.14	115.8
KG-1	6hr	48hr	3.3	0.04	80.7
OCI-AML3	6hr	48hr	17	1.25	13.7
Molm-13	6hr	48hr	9.5	0.5	20.5
REH	6hr	48hr	26	2.31	11.3
Nalm6	6hr	48hr	6.9	0.07	92.3

Table 1. Cell lines were exposed to TH-302 for the indicated period of time under normoxic (21% O_2) or hypoxic (1%O_2) conditions and then washed and incubated for indicated time-period under normoxia. Effects on cell growth and apoptosis induction were determined by FACS. HCR: hypoxic cytotoxicity ratio. *P<0.05; ** P<0.01; *** P<0.001. IC_{50} values are uM.

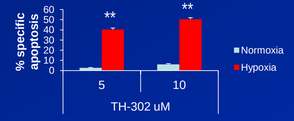
Patient #1-ALL 69% blasts



Patient #2-ALL 74% blasts



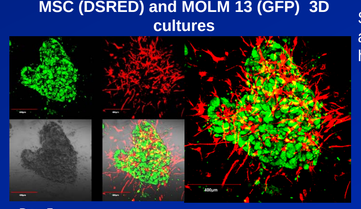
Patient #3-AML 88% blasts



TH-302 exposure for 6hr followed by wash-out and culture for 24 hrs at 21%O_2

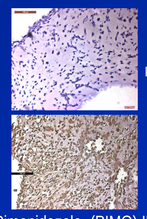
2- Establishment of a 3D in vitro co-culture system of leukemia cells and BM-derived marrow stromal cells (MSC) that recapitulates the multidimensional BM niche

MSC (DSRED) and MOLM 13 (GFP) 3D cultures



Day 5

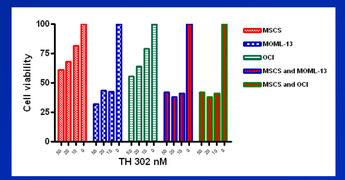
Spheroids are hypoxic



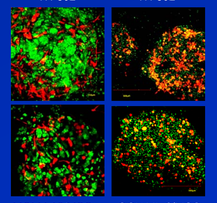
PIMO negative

Pimonidazole (PIMO) IHC

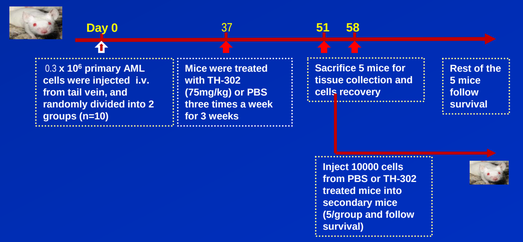
3- Potent TH-302 activity in 3D AML-MSC co-cultures



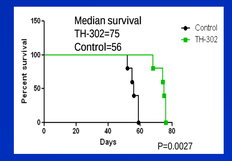
TH-302 for 4 days at 21%O_2. Effect on viability was determined by MTT assay

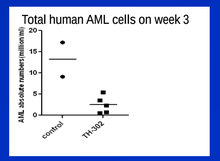


4- TH-302 in vivo anti-leukemia activity: effect on leukemia-initiating cells

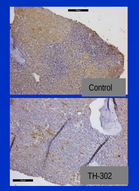


Primary transplants



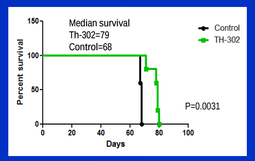


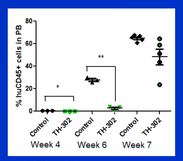
PIMO IHC



TH-302 reduces the hypoxic compartment

Secondary transplants





*: P<0.05; **: P<0.01

CONCLUSIONS

- Culture conditions faithfully mimicking BM microenvironment promote pathologic hypoxia generated by rapidly proliferating AML cells, which in turn leads to their increased sensitivity to hypoxia-activated cytotoxins
- The results support targeting hypoxia with hypoxia-activated cytotoxins such as TH-302 as a therapeutic regimen in AML