Hypoxia activated prodrug TH-302 for the treatment of multiple myeloma

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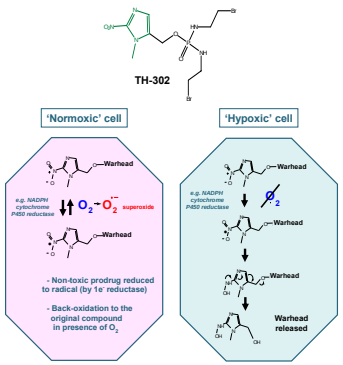
Introduction

Multiple myeloma (MM) is an incurable clonal B cell malignancy characterized by the accumulation of neoplastic plasma cells in the bone marrow (BM). The intimate reciprocal relationship between tumor cells and the cellular and noncellular microenvironment plays a pivotal role in MM growth and survival. As one of the important microenvironment factors, hypoxia is well known to be tightly associated with increased angiogenesis and metastatic potential as well as poor prognosis in solid tumors. In recent years there has been a great deal of progress in understanding the effects of hypoxia on hematopoietic stem cells in the BM. Several in vitro studies have shown that hypoxia is crucial for normal marrow hematopoiesis. Although it is popularly accepted that bone is a very low-oxygen tension environment, the few studies on oxygen level in BM still show differences.

In this study, we investigated the hypoxic status in the BM of naive and 5T33MM mice by comparing the staining with the exogenous marker Pimonidazole and endogenous hypoxia marker hypoxia inducible factor 1α (HIF1α), and found that almost all the MM cells are in a hypoxic BM enviroment. Given low oxygen levels, as found in tumors, are rarely observed in normal tissues, the presence of hypoxic tumor cells is therefore regarded not only as an adverse prognostic factor but as an opportunity for tumor-specific treatment. A number of hypoxia-targeted therapeutics are under development. TH-302 is a new hypoxia-activated prodrug (HAP) that is currently being evaluated in Phase 1/2 clinical trials both as monotherapy and in four different combinations with four chemotherpeutic agents for the treatment of solid tumors. Furthermore, we evaluated the effects of TH-302 on MM cells focusing on apoptosis, cell cycle, associated signaling pathways in MM cell lines, and evaluated the potential therapeutic effects in 5T33vv mouse MM model.



Postulated activation pathway of TH-302

Materials and methods

Animals and 5T33vv multiple myeloma model

C57BL/KaLwRij mice were purchased from Harlan CPB (Horst, The Netherlands). Male mice were 6 to 10 weeks old when used. Mice were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, VUB (license no. LA1230281). The animal ethics meet the standards required by the 1998 UKCCCR (United Kingdom Co-ordinating Committee on Cancer Research) Guidelines. The 5T33 MM originated spontaneously in aging C57BL/KaLwRij mice and has since been propagated in vivo by intravenous transfer of the diseased marrow in young syngeneic mice.

Assessment of oxygen tension in BM

To assess hypoxia, 6 naive mice and 6 5T33vv MM advanced burden mice were given intravenously (i.v.) 60mg/kg Hypoxyprobe (pimonidazole) from Hypoxyprobe Store 4 hours prior to sacrifice. Mouse legs were fixed in zinc fixative for 48 hours, decalcified for 48 hours, and embedded in paraffin. For detection of pimonidazole and HIF1α, the 1st Abs (Rabbit polyclonal Ab HP3-Kit, Hypoxyprobe Store; Rabbit polyclonal Ab to HIF1α, NB100-749,Novus) and, 2nd Ab (HRP-Goat anti Rabbit, #3051-1, Epitomics), Universal Negative Control (NC498AA, Biocare Medical), antigen retrieval reagent Pronase Reagent M31, Biomeda), Betazoid DAB Chromogen Kit (BDB2004H, Biocare Medical) were used.

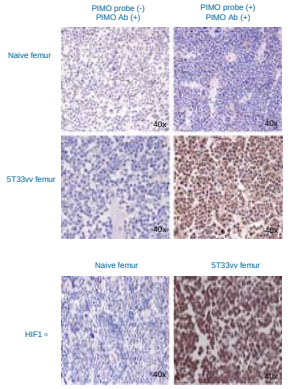


Fig. 1 Immunochemistry staining of exogenous hypoxia marker (Pimonidazole, PIMO) and endogenous hypoxia marker (HIF1α) on the BM section of naive and 5T33MM mice.

Cells and cell culture conditions

Human myeloma cell lines RPMI-8226, Karpas-707, LP-1, MMS1 were maintained in RPMI-1640 medium at 10% FCS, mouse myeloma cell line 5T33vt was maintained in RPMI-1640 medium supplemented with 10% FCI. Hypoxic (1%, 0% O_2) conditions were established by culturing myeloma cells in a sealed chamber, 1% serum and 20mM HEPES (Sigma) as supplements to RPMI-1640 medium. Unless otherwise mentioned, all the cells were cultured for 24 hours in normoxic and hypoxic conditions, when treated with TH-302 or vehicle.

Results

1. MM cells live in a hypoxic BM niche. (Fig. 1)

2. TH-302 induces G0/G1 cell cycle arrest in MM cell lines in hypoxic conditions. (Fig. 2)

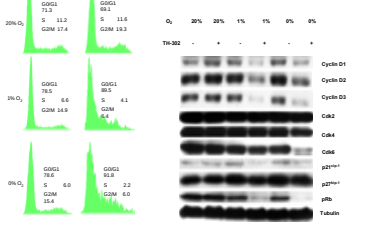


Fig 2B. The effects of TH-302 on the components of the 5T33vt MM cell cycle machinery. TH-302 can induce G₀/G1 cell cycle arrest in hypoxic condition, through down-regulating cyclin D1/2/3, CDK4/6, p21, p27 and pRb expressions. Similar results were also found in RPMI-8226, LP-1, MMS1, Karpas-707 cells.

Fig 2A. Cell cycle analysis of 5T33vt MM cells treated by TH-302. 1 x 10^6 cells per sample were washed once in cold PBS and resuspended in 500 μL staining solution containing 50 μg/mL propidium iodide (PI), 0.1% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium citrate. Cells were incubated at 4° C in the dark for 15 minutes and then analyzed by flow cytometry. Similar results were also found in RPMI-8226, LP-1, MMS1, Karpas-707 cells.

3. TH-302 triggers apoptosis in MM cell lines in hypoxic conditions. (Fig. 3)

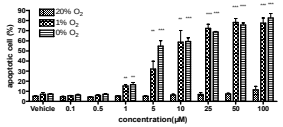


Fig.3A. TH-302 triggers specific apoptosis in a dose-dependent manner in LP-1 cells. One million cells were washed twice with (PBS and stained with 2μL PI and 5μLannexinV-FITC in 100 μLof binding buffer, and incubated at 4° C for 15 min. Then, cells were resuspended in 400 μl of binding buffer and immediately analyzed using a FACScan flow. Similar results were also found in RPMI-8226, 5T33vt, MMS1, Karpas-707 cells. *p<0.05, **p<0.01, ***p< 0.001, compared to 20% O_2.

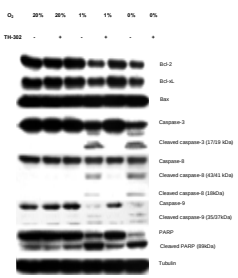


Fig.3B. The mechanism of TH-302 induced apoptosis in LP-1 cells. Western blotting results show that TH-302 triggers apoptosis in both human and murine MM cells in hypoxic condition, through down-regulating anti-apoptotic protein BCL-2 and BCL-xL, as well as up-regulating the expression of proapoptotic protein cleaved caspase-3,8,9 and PARP. Similar results were also found in RPMI-8226, 5T33vt, MMS1, Karpas-707 cells. TH-302 = 5 μ M.

4. HIF1α expression and VEGFa expression are reduced by TH-302. (Fig. 4)

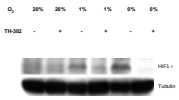


Fig. 4A. TH-302 decreased the accumulation of HIF1α in hypoxic RPMI-8226 cells. Similar results were also found in 5T33vt, LP-1, MMS1, Karpas-707 cells. TH-302 = 5 μ M.

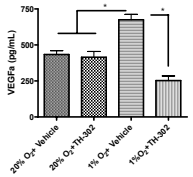
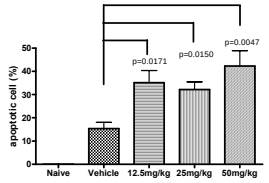


Fig. 4B. TH-302 decreased the secretion of VEGFa in hypoxic 5T33vt cells. TH-302 = 5 μ M. (* p<0.05)

5. 5T33MM Mice treated prophylactically with TH-302 showed significant improvements in multiple parameters. Mice were treated with vehicle, 12.5mg/kg, 25 mg/kg or 50 mg/kg TH-302 injected intraperitoneally prophylactically (from Day 1) for with a dosing schedule of 5 days on, 2 days off for 3 weeks, after which all animals were sacrificed and body, liver, spleen weights, serum paraprotein level as well as tumor load were measured. Treatment with TH-302 resulted in no adverse events, any observable detriment to the mice or weight loss. (Fig. 5).



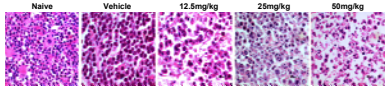


Fig. 5A Hematoxylin and Eosin (H&E) staining of bone marrow section. Nuclei from apoptotic cells show condensed, or fragmented morphology. Bar=20μm. The frequency of apoptotic multiple myeloma cells in bone marrow sections was significantly increased (12.5 mg/kg, 2.5 fold, p<0.05; 25mg/kg, 2.1 fold, p<0.05; 50mg/kg, 3.1 fold, p<0.01).

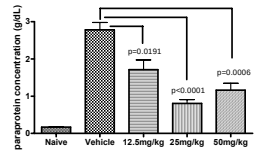


Fig.5B. Serum paraprotein level was decreased after treatment with TH-302. 5T33vv mice treated prophylactically with TH-302 (12.5 mg/kg, 25 mg/kg and 50 mg/kg, i.p.) for 3 weeks from day 1 after tumor inoculation showed decreased serum paraprotein (12.5 mg/kg, 32% decrease, p<0.05; 25 mg/kg, 77% decrease, p<0.0001; 50 mg/kg, 54% decrease, p<0.001), compared to vehicle-treated 5T33vv mice (n=10).

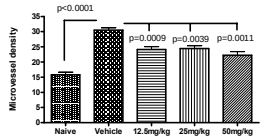


Fig.5C. TH-302 treatment decreased the microvessel density (MVD) , compared to vehicle-treated 5T33MMvv mice. MVD was determined by CD31 staining. In the area with the highest blood vessel density (hot spot), the number of blood vessels was counted per 0.22 mm^2.

Conclusions

◆ **MM cells exist in a hypoxic niche in bone marrow.**

◆ **Treatment with TH-302, a hypoxia-activated prodrug as monotherapy showed promise in treatment of MM cells in vitro and 5T33vv MM model in vivo.**

◆ **TH-302 combination therapy with conventional chemotherapeutics should be considered in further study of multiple myeloma.**

Conflict of interest

Handisides: *Threshold Pharmaceuticals:* Employment. **Liu:** *Threshold Pharmaceuticals:* Employment. **Sun:** *Threshold Pharmaceuticals:* Employment. **Hart:** *Threshold Pharmaceuticals:* Employment. **Vanderkerken:** *Threshold Pharmaceuticals:* Research Funding.

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