Targeting the multiple myeloma hypoxic niche with TH-302, a hypoxia-activated prodrug

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Running title: Hypoxia activated treatment of multiple myeloma.
Abstract

Hypoxia is associated with increased metastatic potential and poor prognosis in solid tumors. In this study, we demonstrated in the murine 5T33MM model that multiple myeloma (MM) cells localize in an extensively hypoxic niche compared to the naive bone marrow (BM). Next, we investigated whether hypoxia could be used as a treatment target for MM by evaluating the effects of a new hypoxia-activated prodrug TH-302 in vitro and in vivo. In severely hypoxic conditions, TH-302 induces Go/G1 cell cycle arrest by down-regulating cyclinD1/2/3, CDK4/6, p21cip1, p27kip1 and pRb expression, and triggers apoptosis in MM cells by up-regulating the cleaved pro-apoptotic caspase-3/8/9 and poly ADP-ribose polymerase (PARP), while having no significant effects under normoxic conditions. In vivo treatment of 5T33MM mice induces apoptosis of the MM cells within the BM microenvironment and decreases paraprotein secretion. Our data support that hypoxia-activated treatment with TH-302 provides a potential new treatment option for MM.

Keywords: Multiple myeloma, hypoxia, TH-302, cell cycle, apoptosis

Introduction

Multiple myeloma (MM) is an incurable clonal B cell malignancy characterized by the accumulation of neoplastic plasma cells in the bone marrow (BM). Studies have shown that the intimate reciprocal relationship between tumor cells and the cellular and noncellular microenvironment plays a pivotal role in MM growth and survival. Hypoxia, one of the important microenvironmental factors, is well known to be highly associated with increased angiogenesis and metastatic potential as well as poor prognosis in solid tumors. More recently, hypoxia has been demonstrated to be crucial for normal marrow hematopoiesis. However, the role of hypoxia in the etiology, pathogenesis, and possible treatment of hematological malignancies, such as MM, is still unknown.

Given very 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 also as a potential target for tumor-specific treatment. Currently, a number of hypoxia-targeted
therapeutics are under development. TH-302 is a new hypoxia-activated prodrug (HAP) that is being evaluated in Phase 1/2 clinical trials for the treatment of solid tumors as a monotherapy and in combination with four chemotherapeutic agents (gemcitabine, pemetrexed, doxorubicin and docetaxel). TH-302 is a 2-nitroimidazole (2-NI) prodrug of the cytotoxin bromo-isophosphoramidemustard (Br-IPM), with a favorable physico-chemical, metabolic, and pharmacokinetic profile and exhibits hypoxia-selective cytotoxicity across a broad spectrum of human cancer cell lines in vitro and in vivo efficacy in a large panel of human tumor xenografts. The doses employed in the clinical studies are in the same range as the doses demonstrating efficacy in both in vitro and in vivo preclinical models.

In this study, we investigated the hypoxic nature of MM by staining the BM of naive and 5T33MM mice with the exogenous hypoxia marker pimonidazole and endogenous hypoxia marker hypoxia inducible factor 1α (HIF1α), and demonstrated that MM cells reside in a more hypoxic BM environment. Furthermore, we evaluated the effects of TH-302 on MM cell lines in vitro, focusing on apoptosis and cell cycle as well as associated signaling pathways in MM, and evaluated the potential therapeutic effects in the 5T33vv mouse MM model.

Materials and Methods (see supplementary material)
All experiments were approved by the Ethical Committee for Animal Experiments at Vrije Universiteit Brussels.

Results and Discussion
Despite major therapeutic advances, MM still remains incurable. Compelling evidence suggests that the BM microenvironment favors MM progression by promoting MM cell growth and drug resistance. In solid tumors, hypoxia is one of the microenvironmental factors that drives tumor progression and treatment resistance. MM develops in a specialized BM microenvironment, which has been shown to be low in oxygen tension, however, our knowledge in this field is still in its infancy and many details are unknown. In contrast to solid tumors and other tissues or organs, the inaccessibility of the BM to direct noninvasive oxygen measurements is a major hurdle for formal experimental investigation. Moreover, the invasive needle oxygen electrode techniques for
detecting tissue pO2 measurement can not show the heterogeneity of oxygen tension in the BM, the gradients of O2 from hypoxic niches to the higher O2 tension in the sinusoidal cavity. Considering the potential role of hypoxia in hematopoiesis and MM progression in the BM, we investigated the oxygen level in the BM of naive and 5T33MM mice which closely mimics the human disease, by assessing the exogenous and endogenous hypoxia markers pimonidazole and HIF-1α. As shown in Figure 1A, our immunohistochemical staining results clearly delineate the hypoxic regions within the normal and MM BM. Both the exogenous marker pimonidazole and endogenous hypoxia marker HIF1α were dramatically increased in the BM of 5T33MM mice in contrast to the sporadic and weak positivity of hypoxia markers in the naive mice, strongly suggesting that the majority of MM cells localize in an extensively hypoxic niche. Our results provide tangible evidence and more detailed information regarding the hypoxic nature of normal and MM BM and confirm the results recently reported by the group of Nicola Giuliani. A role for hypoxic bone marrow in other hematological malignancies (lymphoma and leukemia) is also supported by the results of other recent studies.

The strategy of microenvironment-targeted treatment is gaining increased attention in hemato-oncology, and we evaluated whether the hypoxic niche of MM could also serve as a treatment target. Our data demonstrate that the hypoxia activated prodrug TH-302 exhibits potent in vitro cytotoxicity to MM cells with hypoxic selectivity and dose dependency. To study the growth inhibitory effects of TH-302 on MM cells, we analyzed the cell cycle phase distribution and apoptosis after drug treatment. Cell cycle analysis showed that TH-302 can induce Go/G1 cell cycle arrest under hypoxic conditions. Western blotting further revealed that the effect of TH-302 on cell cycle machinery was mediated by down-regulating cyclin D1/2/3, CDK4/6, p21\textsuperscript{cip1}, p27\textsuperscript{kip1} and pRb expression, whereas CDK2 expression remained undisturbed (Figure 1B, 1C), similar results were also found in RPMI-8226, LP-1, MMS1 and Karpas-707 MM cells (see Supplementary Figure 1 and 2). Furthermore, flow cytometry analysis demonstrated that TH-302 can induce dose-dependent apoptosis in both human and murine MM cells in hypoxic conditions (Figure 1D), similar results were also seen in RPMI-8226, 5T33vt, MMS1, Karpas-707 cells. (see Supplementary Figure 3). Western blotting further demonstrated that TH-302 activated apoptosis was mediated through down-regulating the anti-apoptotic proteins BCL-2 and BCL-xL, as well as
up-regulating the expression of cleaved proapoptotic protein caspase-3, 8, 9 and poly ADP-ribose polymerase (PARP). In contrast to the hypoxia-specific toxicity, TH-302 shows very low toxicity in normoxic condition even at high concentrations (Figure 1E), similar results were also found in RPMI-8226, 5T33vt, MMS1, Karpas-707 cells (see Supplementary Figure 4). In addition, we demonstrated that the production of HIF1α, the central regulator of the hypoxic response, is decreased with the treatment of TH-302. The expression of HIF1α in a hypoxic condition was reduced following exposure to TH-302 (Figure 1F, similar results were also found in 5T33vt, LP-1, Karpas-707 cells, see Supplementary Figure 5), accordingly, the secretion of VEGFa which is a downstream target gene of HIF1α was also significantly decreased (Figure 1G).

Furthermore, studies conducted in the 5T33MMvv mouse model demonstrated that in vivo treatment with TH-302 showed impressive improvements in multiple disease parameters. TH-302 induced significant MM cell apoptosis (12.5 mg/kg, 2.5 fold; 25mg/kg, 2.1 fold; 50mg/kg, 3.1 fold), decreased paraprotein secretion (12.5 mg/kg, 32% decrease; 25 mg/kg, 77% decrease; 50 mg/kg, 54% decrease), and significantly decreased microvessel density (MVD) (12.5 mg/kg, 19% decrease; 25 mg/kg, 20% decrease; 50 mg/kg, 26% decrease) in the BM of treated 5T33MMvv mouse, compared to vehicle-treated 5T33MMvv mice (Figure 2).

By employing a set of defined gas mixtures (0%, 1%, 1.25%, 1.5%, 2%, 3%, 20% O₂) for drug treatment of the MM cells, we evaluated the oxygen concentration dependent activation of TH-302. Our results suggest that the threshold of activating prodrug TH-302 is lower than 1.5% O₂ (Supplementary Figure 6). 2-nitroimidazole (the oxygen concentration sensing trigger for both pimonidazole and TH-302) has been shown to become activated in cells whose oxygen concentration is < 10 mmHg pO₂, it is reasonable to accept that the cytotoxic effect of TH-302 is tightly associated with the hypoxic nature of MM cells in the BM. In addition, the data from TH-302 treated naive mice show no significant toxicity in body weight, hemoglobin (HGB), red blood cell count (RBC), white blood cell count (WBC), hematocrit (HCT) and MVD, compared to the vehicle-treated naive mice, further indicating the specific hypoxia-activated effect of TH-302 and the limited hypoxia in the normal BM (Supplementary Figure 7).
Taken together, our results confirm that MM cells reside in an extensively hypoxic BM microenvironment. Hypoxia-activated treatment with TH-302 as a monotherapy shows efficacy in treatment of MM both in vitro and in vivo. The findings in this study indicate that targeting the hypoxic BM niche provides a potentially useful and novel treatment strategy for MM.

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Authorship Contributions

Contribution: Jinsong Hu designed research, wrote the paper, and participated in most of experiments; Damian R. Handisides, Qian Liu, Jessica D. Sun, Charles Hart participated in immunohistochemistry staining of hypoxia markers and in the writing of the paper; Hendrik De Raeve participated in H&E staining and in MVD analysis; Els Van Valckenborgh, Eline Menu, Isabelle Vande Broek contributed in performing 5T33MM treatment experiment and analyzing of the data. Ben Van Camp contributed to the paper in discussion. Karin Vanderkerken participated in the design of research, analyzing the data and in writing of the paper.

Disclosure of Conflicts of Interest


References


family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 alpha (HIF-1α) activity: involvement in myeloma-induced angiogenesis. *Blood*. 2007;110(13):4464-4475.


Figure 1. The hypoxia status of the BM and the in vitro effects of TH-302 on MM cells. A, immunohistochemistry staining of exogenous hypoxia marker and endogenous hypoxia marker in BM sections of naive and 5T33MM mice. Hypoxia was determined by the accumulation of Pimonidazole and HIF1α as described in Materials and Methods. Original magnification, ×40. Representative pictures, n=6/group. B, TH-302 induces Go/G1 cell cycle arrest in 5T33vt cells in a hypoxia selective manner. Similar results were also found in RPMI-8226, LP-1, MMS1 and Karpas-707 MM cells, data not shown. C, Effects of TH-302 on components of the 5T33vt MM cell cycle machinery. TH-302 induced Go/G1 cell cycle arrest depends on down-regulating cyclin D1/2/3, CDK4/6, p21, p27 and pRb expression. Similar results were also found in RPMI-8226, LP-1, MMS1, Karpas-707 MM cells, data not shown. D, TH-302 triggers specific apoptosis in a dose-dependent manner in LP-1 cells under hypoxia. *p<0.05, **p<0.01, ***p<0.001, compared to 20% O₂ (n=3). Similar results were also seen with RPMI-8226, 5T33vt, MMS1, Karpas-707 cells, data not shown. E, The mechanism of TH-302 induced apoptosis in LP-1 cells. Similar results were also found in RPMI-8226, 5T33vt, MMS1, Karpas-707 cells. TH-302 = 5μM. F, TH-302 decreases the accumulation of HIF1α in hypoxic RPMI-8226 cells. Similar results were also found in 5T33vt, LP-1, MMS1, Karpas-707 cells, data not shown. TH-302 = 5μM. G, VEGFa secretion was reduced by TH-302 in 5T33vt cells. *p<0.05, n=3.

Figure 2. In vivo therapeutic effects of TH-302 on 5T33MM model. 5T33MMvv mice were treated prophylactically with TH-302 for 3 weeks from day 1. A, serum paraprotein level (as determined by serum electrophoresis) was decreased after treatment with TH-302. B, Histomorphometric analysis of MVD. MVD was determined by CD31 staining as described in Materials and Methods. In the area with the highest blood vessel density (hot spot), the number of blood vessels was counted per 0.22 mm². C, H&E staining of bone marrow section. Nuclei from apoptotic cells show condensed, fragmented morphology. Original magnification, ×63, n=10/group. Quantitative data are shown the frequency of apoptotic multiple myeloma cells in bone marrow sections. *p<0.05, **p<0.01, n=10/group, significantly different from vehicle.
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