Synergistic Induction of Apoptosis in Multiple Myeloma Cells by Bortezomib and Hypoxia-Activated Prodrug TH-302, In Vivo and In Vitro

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Abstract

Recently, we showed that hypoxia is a critical microenvironmental factor in multiple myeloma, and that the hypoxia-activated prodrug TH-302 selectively targets hypoxic multiple myeloma cells and improves multiple disease parameters in vivo. To explore approaches for sensitizing multiple myeloma cells to TH-302, we evaluated in this study the antitumor effect of TH-302 in combination with the clinically used proteasome inhibitor bortezomib. First, we show that TH-302 and bortezomib synergistically induce apoptosis in multiple myeloma cell lines in vitro. Second, we confirm that this synergism is related to the activation of caspase cascades and is mediated by changes of Bcl-2 family proteins. The combination treatment induces enhanced cleavage of caspase-3/8/9 and PARP, and therefore triggers apoptosis and enhances the cleavage of proapoptotic BH3-only protein BAD and BID as well as the antiapoptotic protein Mcl-1. In particular, TH-302 can abrogate the accumulation of antiapoptotic Mcl-1 induced by bortezomib, and decreases the expression of the prosurvival proteins Bcl-2 and Bcl-xL. Furthermore, we found that the induction of the proapoptotic BH3-only proteins PUMA (p53-upregulated modulator of apoptosis) and NOXA is associated with this synergism. In response to the genotoxic and endoplasmic reticulum stresses by TH-302 and bortezomib, the expression of PUMA and NOXA were upregulated in p53-dependent and -independent manners. Finally, in the murine 5T33MMvv model, we showed that the combination of TH-302 and bortezomib can improve multiple disease parameters and significantly prolong the survival of diseased mice. In conclusion, our studies provide a rationale for clinical evaluation of the combination of TH-302 and bortezomib in patients with multiple myeloma.

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Introduction

Multiple myeloma is a malignant plasma-cell disorder that accounts for 1% of all cancers and 10% to 15% of all hematologic malignancies (1). In the past decade, there have been major advances in the treatment of multiple myeloma. The introduction of novel agents such as bortezomib (proteasome inhibitor) and thalidomide (immunomodulatory drug) has dramatically improved the outcome of patients with multiple myeloma (2–4). Moreover, significant advances in both basic and translational research have enhanced understanding of disease pathogenesis and guided the development of new and more effective therapies (5). It has been shown previously by us and other groups that hypoxia is a critical microenvironmental factor in multiple myeloma (6–11). Our data further support that targeting the hypoxic niche with hypoxia-activated prodrug (HAP) TH-302 (Fig. 1A) is a potential new treatment option for multiple myeloma (6). TH-302 as a 2-nitroimidazole prodrug of the cytotoxic bromo-isophosphoramide mustard (Br-IPM), exhibits hypoxia-selective cytotoxicity against a broad spectrum of human cancer cell lines in vitro, and shows efficacy across a large panel of human tumor xenografts in vivo. TH-302 is being evaluated in clinical trials for the treatment of solid tumors as a monotherapy and in combination with chemotherapeutic agents (12).

Bortezomib (PS-341), the first therapeutic proteasome inhibitor, was approved for refractory, relapsed, and newly diagnosed multiple myeloma (13). Treatment of multiple myeloma with bortezomib has dramatically improved survival for patients with multiple myeloma; however, some patients do not benefit from this treatment, and those responding ultimately acquire resistance...
On the other hand, severe treatment-related toxicities such as peripheral neuropathy have been observed in conjunction with the use of bortezomib (15). Combination therapy using lower doses of bortezomib is an approach to increase efficacy and reduce side effects, thereby enhancing the likelihood of survival for patients with multiple myeloma (16).

In the current study, we investigated the anti–multiple myeloma effects of TH-302 combined with bortezomib in hypoxic conditions. Our results show that the combination of TH-302 and bortezomib synergistically induced apoptosis in multiple myeloma cells in vitro, associated with the activation of caspases, the decrease of antiapoptotic Bcl-2 family members, and importantly, the increase of proapoptotic BH3-only proteins PUMA (p53-upregulated modulator of apoptosis) and NOXA. Further experiments in the 5T33MMvt mouse model in vivo showed that the combination of TH-302 and bortezomib can improve multiple disease parameters and result in a significant prolonged survival. Taken together, these findings provide preclinical rationale for further evaluation of the combination of TH-302 and bortezomib in clinical trials.

Materials and Methods

Drugs
TH-302 was provided by Threshold Pharmaceuticals. TH-302 was dissolved in sterile PBS at 10 mmol/L for in vitro studies and at 5 mg/mL for in vivo studies. Bortezomib was from LC Laboratories. For in vitro studies, bortezomib was reconstituted in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mmol/L and stored at −20°C until use, and this stock was diluted in medium just before use so that the concentration of DMSO never exceeded 0.1%. For in vivo studies, clinical-grade vials of bortezomib were reconstituted using a sterile NaCl 0.9% solution. Solutions were sterilized by filtration through a 0.22-µm syringe filter.

Cells and cell culture conditions
The human LP1, OPM2 (both from American Type Culture Collection), MMS1 (a kind gift from Y. Okuno, Kyoto University, Kyoto, Japan) and murine myeloma cell line 5T33MMvt (a kind gift of J. Radl, TNO, Leiden, the Netherlands) were used in this study. A stock was
prepared of all authenticated cell lines and experiments were carried out within 2 months after thawing vials. The murine cell line was authenticated by its expression of a specific idotype. The multiple myeloma cells were cultured in normoxic (20% O₂) and hypoxic conditions (1% O₂) as described previously (6).

Quantification of apoptosis
A total of 1 × 10⁶ cells were washed twice with PBS and stained with 2 μL 7-amino-actinomycin D (7-AAD; BD Biosciences) and 5 μL Annexin V–fluorescein isothiocyanate (FITC; BD Biosciences) in 100 μL of binding buffer, and incubated at 4°C for 15 minutes. Then, cells were resuspended in 400 μL of binding buffer and immediately analyzed using a FACSCanto flow cytometer (Becton Dickinson).

Synergy study of the combination treatment
Multiple myeloma cells were treated simultaneously with various concentrations of TH-302 and bortezomib for 16 hours in hypoxic conditions. Apoptosis induced by each agent alone and by the combination treatment was monitored by flow-cytometric analysis of multiple myeloma cells stained with Annexin V–FITC and 7-AAD as described earlier. The degree of synergy between the two compounds was determined using the CompuSyn Software (ComboSyn Inc.) developed by Chou (17). The combination index (CI) for each combination was calculated at a nonconstant ratio.

Western blotting
Western blot analysis was conducted as previously described (6). The primary antibodies against Bel-2, Bel-xL, Mcl-1, BAD, BID, BAX, caspase-3, caspase-8, caspase-9, PARP, phospho-p53 (Ser15), and β-actin were from Cell Signaling Technology and diluted at 1:1,000. The primary antibodies against PUMA, NOXA, p53, p21(WAF1/CIP1), ATF4, GRP-78, and CHOP (C/EBP homologous protein; also known as DNA damage–inducible gene 153) were from Santa Cruz Biotechnology and diluted at 1:500.

Quantitative real-time PCR (qRT-PCR)
RNA extraction was conducted using the RNeasy Kit (Qiagen). Total RNA was reverse transcribed using the Verso cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s instructions. Real-time PCR was conducted with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) on an ABI Prism 7900 Fast instrument using gene-specific primers. Primer sequences used for amplifications are as follows: β-actin, forward: 5'-TCTGATATGAGGACAGGAGAAG-3'; reverse: 5'-GAGGAGAAGGAGAATCGCTTGAACC-3'. RAD51, forward: 5'-TGAATACGATGAGGACAGGAGAAG-3'; reverse: 5'-GAGGAGGAGAATCGCTTGAACC-3'. p53, forward: 5'-TTGCGTGTGGAGTATTTGGATGAC-3'; reverse: 5'-TGAAGGAGCACCAGAGAGAG-3'. NOXA, forward: 5'-ACCGTGTGAGGATTTGGATGAC-3'; reverse: 5'-TGAAGGAGCACCAGAGAGAG-3'. ATP4, forward: 5'-AGGATGAGGACAGGAGAAG-3'; reverse: 5'-CAGGAGAGGACAGGAGAAG-3'. GRP-78, forward: 5'-AGGAGGAGAATCGCTTGAACC-3'; reverse: 5'-CAGGAGGAGAATCGCTTGAACC-3'. RAD51, forward: 5'-TCTGATATGAGGACAGGAGAAG-3'; reverse: 5'-GAGGAGAAGGAGAATCGCTTGAACC-3'. RAD51, forward: 5'-TGAATACGATGAGGACAGGAGAAG-3'; reverse: 5'-GAGGAGGAGAATCGCTTGAACC-3'. p53, forward: 5'-TTGCGTGTGGAGTATTTGGATGAC-3'; reverse: 5'-TGAAGGAGCACCAGAGAGAG-3'. NOXA, forward: 5'-ACCGTGTGAGGATTTGGATGAC-3'; reverse: 5'-TGAAGGAGCACCAGAGAGAG-3'. ATP4, forward: 5'-AGGATGAGGACAGGAGAAG-3'; reverse: 5'-CAGGAGAGGACAGGAGAAG-3'. GRP-78, forward: 5'-AGGAGGAGAATCGCTTGAACC-3'; reverse: 5'-CAGGAGGAGAATCGCTTGAACC-3'. RAD51, forward: 5'-TGAATACGATGAGGACAGGAGAAG-3'; reverse: 5'-GAGGAGGAGAATCGCTTGAACC-3'. p53, forward: 5'-TTGCGTGTGGAGTATTTGGATGAC-3'; reverse: 5'-TGAAGGAGCACCAGAGAGAG-3'. NOXA, forward: 5'-ACCGTGTGAGGATTTGGATGAC-3'; reverse: 5'-TGAAGGAGCACCAGAGAGAG-3'.

Animals and 5T33MMv multiple myeloma model
The 5T33MM model 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 (18). C57BL/KaLwRij mice were purchased from Harlan CPB. Male mice were 6 to 10 weeks old when used, and housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, VUB (license no. LA1230281).

In vivo analysis of tumor burden
To study the effects of TH-302 alone, bortezomib alone, and the combination on myeloma progression, four groups of C57BL/KaLwRij mice (n = 10) were injected intravenously with 0.5 × 10⁶ 5T33MMv cells; one group of 10 naïve mice was included as negative control. One week after tumor cell inoculation, mice were treated with either TH-302 alone [100 mg/kg, twice weekly, intraperitoneally (i.p.) injection], or bortezomib alone (0.8 mg/kg, twice weekly, s.c. injection), or bortezomib in combination with TH-302 (twice weekly, TH-302 was injected 4 hours after bortezomib), or vehicle (0.9% NaCl) until the first mouse showed signs of morbidity. Serum paraprotein concentration was assessed using standard electrophoretic techniques. Bone marrow tumor burden was assessed by determining plasmacytosis on cytosmears. Bone marrow angiogenesis was assessed by determining microvessel density (MVD) as previously described (19).

Survival analysis
Five days after tumor cell inoculation, four groups (n = 10) of mice injected with 5T33MMv cells were treated with either TH-302 alone (100 mg/kg, twice
weekly, i.p. injection), or bortezomib alone (0.8 mg/kg, twice weekly, s.c. injection), or bortezomib in combination with TH-302 (twice weekly, TH-302 was injected 4 hours after bortezomib), or vehicle (0.9% NaCl). Kaplan–Meier analysis was used for analyzing overall survival.

Statistical analysis
For statistical analysis of the in vivo data, differences between two groups were determined by Mann–Whitney test. Kaplan–Meier analysis was used to determine the effects on the survival time of the tumor-bearing mice, the differences between groups were tested for statistical significance using the two-tailed log-rank test. For qRT-PCR data analysis, a two-tailed paired t test was conducted for the analysis of differences in cells with or without treatment with a software package GraphPad Prism (GraphPad software). P < 0.05 was considered statistically significant.

Results
Combination effects of bortezomib and TH-302 on multiple myeloma cell apoptosis
In our previous study, we showed that the TH-302 exhibits hypoxia-selective cytotoxicity against multiple myeloma cells in vitro and shows attractive efficacy in multiple myeloma in vivo (6). In the present study, we investigated whether bortezomib can potentiate TH-302–induced apoptosis of multiple myeloma cells in hypoxic conditions. To examine the effects of the combination, three multiple myeloma cell lines were exposed to increasing concentrations of the two agents. Cell apoptosis was measured after 16 hours by flow cytometry. As shown in Fig. 1, bortezomib in combination with TH-302 increased the apoptosis levels in 5T33MMvt, OPM2, and MMS1 cells. The interaction between bortezomib and TH-302 was further analyzed using the Chou–Talalay method (17), to determine whether this combination exhibited additive or synergistic cytotoxicity. Using CompuSyn software, we calculated the CIs of varying concentrations of TH-302 with bortezomib. In 5T33MMvt cells, at doses ranging from 2.5 to 10 μmol/L TH-302 combined with 5 to 20 nmol/L bortezomib, CI ranged from 0.308 to 0.891, suggesting that this combination was synergistic (Table 1). Similar results were also observed in OPM2 and MMS1 cells (Supplementary Tables S1 and S2).

Combination bortezomib and TH-302 induces apoptosis through activation of the caspase cascade
Caspase activation plays a central role in the execution of apoptosis. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and thus leads to rapid cell death (20). To determine whether caspase activation is involved in the augmented apoptosis by the combination treatment with bortezomib and TH-302, two cell lines 5T33MMvt and OPM2, exhibiting good and moderate response to the combination treatment, were chosen for further studies.

Table 1. CI analysis of TH-302 combined with bortezomib at a nonconstant ratio in 5T33vt cells

<table>
<thead>
<tr>
<th>TH-302 (μmol/L)</th>
<th>Bortezomib (nmol/L)</th>
<th>Fa</th>
<th>CI</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>5</td>
<td>0.382</td>
<td>0.638</td>
<td>Synergism</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
<td>0.556</td>
<td>0.569</td>
<td>Synergism</td>
</tr>
<tr>
<td>2.5</td>
<td>20</td>
<td>0.605</td>
<td>0.891</td>
<td>Slight synergism</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.535</td>
<td>0.452</td>
<td>Synergism</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.603</td>
<td>0.550</td>
<td>Synergism</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0.828</td>
<td>0.370</td>
<td>Synergism</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0.576</td>
<td>0.538</td>
<td>Synergism</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>0.777</td>
<td>0.308</td>
<td>Synergism</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>0.826</td>
<td>0.404</td>
<td>Synergism</td>
</tr>
</tbody>
</table>

NOTE: Analysis was conducted using the CompuSyn software (CompuSyn, Inc.). Descriptions are based on CI values and the recommendations of CompuSyn: <0.1, very strong synergism; 0.1–0.3, strong synergism; 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism. Abbreviation: Fa, fraction affected as tested by the flow cytometry analysis of 5T33vt cells stained with FITC-labeled Annexin V and 7-AAD after 16 hours with each indicated treatment.

We examined the activation of caspase cascades as defined by their cleavage at specific aspartate residues by Western blot analysis. As seen in Fig. 2, treating the cells with the combination induced a greater processing of caspase-3, -8, and -9 and PARP in both 5T33MMvt and OPM2 cells, coincided with the appearance of bands representing cleavage products of caspase-3, -8, and -9 and PARP.

Effects of TH-302 and bortezomib on the expression of Bcl-2 family proteins
To characterize the mechanism responsible for bortezomib–TH-302–mediated synergism in multiple myeloma, we next investigated the changes of Bcl-2 family members at the protein level. In Fig. 3A and B, the changes of antiapoptotic protein Bcl-2, Bcl-xL, and Mcl-1 in two multiple myeloma cell lines are shown. TH-302 alone causes a decrease in the expression levels of all three antiapoptotic proteins, whereas bortezomib alone does not affect the expression of Bcl-xL, slightly decreases the expression of Bcl-2, and markedly increases the expression of Mcl-1. When treated with the combination, the expression of Bcl-xL is further decreased as compared with TH-302 alone, whereas Mcl-1 and Bcl-2 are similarly decreased as compared with TH-302 alone.

In addition, the effects of TH-302 and bortezomib on proapoptotic Bcl-2 family proteins were investigated. As shown in Fig. 3C and D, no significant changes were observed in BAX proteins. However, among the...
proapoptotic BH3-only members, both TH-302 and bortezomib induced the cleavage of BAD and BID, thereby consequently producing the truncated BAD and BID proteins, which are stronger inducer of apoptosis than the full-length proteins (21–23). In contrast to BAD and BID, the expression levels of the proapoptotic BH3-only

Figure 2. Bortezomib/TH-302 induces apoptosis through activation of the caspase cascade in multiple myeloma cells. 5T33MMvt (A) and OPM2 (B) cells were cultured with TH-302 in the presence or absence of bortezomib (Btz) for 16 hours under hypoxic conditions (1% O2). The concentrations of drugs, either alone or in combination, were as follows: 5 nmol/L bortezomib, 5 µmol/L TH-302. Whole-cell lysates were subjected to immunoblot analysis with anti-caspase-3, -8, -9, anti-PARP, and anti-β-actin antibodies. Blots shown are representative of three independent experiments.

Figure 3. Changes of Bcl-2 family members induced by TH-302 and bortezomib (Btz) under hypoxic conditions by Western blot analysis. A and B, changes of antiapoptotic Bcl-2, Bcl-xL, and Mcl-1 in TH-302- and bortezomib-treated 5T33MMvt (A) and OPM2 (B) cells. Multiple myeloma cells were treated with vehicle, TH-302 (5 µmol/L), bortezomib (5 nmol/L), or combination therapy for 16 hours under hypoxic conditions (1% O2). Whole-cell lysates were immunoblotted with the indicated antibodies. C and D, the effects of TH-302 and bortezomib on the expression of proapoptotic Bcl-2 family members. 5T33MMvt (C) and OPM2 (D) multiple myeloma cells were treated with TH-302 (5 µmol/L), bortezomib (5 nmol/L), or combination therapy for 16 hours under hypoxic conditions (1% O2). Whole-cell lysates were immunoblotted with the indicated antibodies. The results are representative of three independent experiments.
Figure 4. The role of p53 and ER stress in upregulating of pro-apoptotic PUMA and NOXA by TH-302 and bortezomib (Btz). A and B, TH-302 upregulates and activates p53 in OPM2 cells. A, OPM2 cells were cultured with indicated doses of TH-302 for 16 hours under hypoxic conditions (1% O₂), then harvested and probed with the indicated antibodies by Western blot analysis. B, qRT-PCR analysis of the expression p53 and its target genes in TH-302–treated OPM2 cells. OPM2 cells were cultured with vehicle (Veh) or 5 μmol/L TH-302 for 16 hours in hypoxic condition (1% O₂), then harvested for RNA extraction and cDNA synthesis and PCR analysis. For more details see Materials and Methods. C and D, bortezomib activates p53 in OPM2 cells. C, OPM2 cells were cultured with indicated doses of bortezomib for 16 hours in hypoxic condition (1% O₂), then harvested and probed with the indicated antibodies by Western blot analysis. D, qRT-PCR analysis of the expression p53 and its target genes in bortezomib-treated OPM2 cells. OPM2 cells were cultured with vehicle or 5 nmol/L bortezomib for 16 hours in hypoxic condition (1% O₂), then harvested for RNA extraction and cDNA synthesis and PCR analysis. E and F, TH-302 activates ER stress signaling in LP1 cells. E, LP1 cells were cultured with vehicle or 5 or 10 μmol/L TH-302 for 16 hours in hypoxic condition (1% O₂), then harvested and probed with the indicated antibodies by Western blot analysis. (Continued on the following page.)
protein PUMA and NOXA were increased by both TH-302 and bortezomib.

**p53-dependent and -independent pathways involved in the induction of PUMA and NOXA**

To further address the mechanisms underlying the induction of PUMA and NOXA, we investigated the roles of p53-dependent and -independent pathways in response to TH-302 and bortezomib treatment. When exposed to hypoxic conditions, TH-302 reduction at the nitroimidazole site of the prodrug by intracellular reduc-
tases leads to the release of the DNA alkylating agent bromo-isophosphoramidate mustard, which is genotoxic (24). In response to genotoxic stress, the activation of p53 plays a crucial role in governing cell apoptosis. Because PUMA and NOXA are direct targets in p53-mediated apoptosis (25), we then investigated whether p53 is involved in TH-302- and bortezomib-induced apoptosis. As shown in Fig. 4A, we confirmed that p53 was upregulated and activated by TH-302 in OPM2 cells, which express the wild-type p53. In parallel to the activation of p53, as monitored by the phosphorylation on Ser-15, the expression level of the target gene p21\(^{WAF1/CIP1}\) was also increased. Similar results were also observed in bortezomib-treated multiple myeloma cells in hypoxic condition (Fig. 4C), and the results confirmed the finding that the induction and activation of p53 is one of the important molecular mechanisms mediating antimyeloma activity of bortezomib (26, 27). Moreover, by quantitative measuring of the transcriptional expression levels of the p53-related DNA repair genes such as BRCA1, BRCA2, RAD51, and FANC D2, we found that TH-302 upregulates the transcription of these DNA repair genes, suggesting the activation of DNA repair machinery. However, when treated with bortezomib, the expression levels of DNA repair genes were decreased (Fig. 4B and D). Interestingly, in the p53-mutated multiple myeloma cell line LP1, we still observed the induction of PUMA and NOXA, implicating the role of p53-independent pathways in the regulation of PUMA and NOXA. We then investigated the contribution of endoplasmic reticulum (ER) stress to the induction of PUMA and NOXA. As expected, we observed upregulated expression levels of the ER stress markers GRP-78 and CHOP in response to treatment by both bortezomib and TH-302. Moreover, the expression of ATF4, one major regulator of ER stress signaling pathway, was also increased (Fig. 4E and F). Furthermore, by using qRT-PCR, we show that the expression of ER stress markers and PUMA as well as NOXA were all upregulated at transcription level (Fig. 4F and H).

**Combination of bortezomib and TH-302 suppresses multiple myeloma cell growth in vivo**

To test whether the enhanced myeloma cell apoptotic response is observed in vivo, we examined the efficacy of the combination of bortezomib and TH-302 in the 5T33MM mouse model. In the first series of experiments, C57BL/ KaLwRijHsd mice inoculated with 5T33MMv cells were either assigned to receive TH-302, bortezomib, or a combination of both starting at day 7 after inoculation (Fig. 5A). In mice treated with TH-302 alone, bortezomib alone, or the combination, a significant reduction in serum paraprotein concentrations and plasmacytosis in the bone marrow were observed compared with the vehicle-treated mice (Fig. 5B and C). Importantly, the combination-treated group showed more decreases in the serum paraprotein concentrations and plasmacytosis than in the single-drug–treated groups. Immunohistochemical staining for CD31 showed significantly decreased MVD in the treatment groups (Fig. 5D). Similarly, combination treatment led to more significant decrease of MVD than each single-drug group. No significant reduction in body weight was observed in treated mice compared with vehicle-treated controls (data not shown).

To examine whether the combination of TH-302 and bortezomib can prolong the survival of multiple myeloma–inoculated mice, a second series of experiments was carried out. As shown in Fig. 5E, in vivo treatment of 5T33MM mice with a combination of TH-302 and bortezomib resulted in a significantly prolonged survival when compared with the vehicle group \((P < 0.0001)\), the TH-302-alone–treated group \((P < 0.0001)\), and the bortezomib-alone–treated group \((P < 0.0001)\). The median survival time of each group was 25.5 (vehicle), 34 (bortezomib), 41.5 (TH-302), and 53.5 (bortezomib and TH-302) days, respectively. These data clearly show that the combination of TH-302 and bortezomib results in improvements in multiple disease parameters and increased overall survival of the mice.

**Discussion**

Despite advances in chemotherapy and stem-cell transplantation, multiple myeloma remains an incurable disease. As shown in our previous report, multiple myeloma cells localize at the hypoxic regions in the bone marrow (6, 10). This finding not only provides us new insights into the multiple myeloma–bone marrow microenvironment, but also provides us a new treatment target taking advantage of hypoxia in multiple myeloma. By using a HAP TH-302, we previously showed that targeting hypoxia is a potential new treatment option for multiple myeloma (6).
In the present study, we investigated whether the combination of hypoxia-activated treatment with bortezomib can achieve better therapeutic efficacy, based on a finding that bortezomib has preferential cytotoxicity toward hypoxic solid tumor cells (28). We showed here that the combinatorial use of TH-302 and bortezomib can mediate synergistic antitumor effects in multiple myeloma.

Induction of apoptosis in tumor cells represents a key strategy of cancer chemotherapy. It has been shown that Bcl-2 family members are central regulators of apoptosis (29). All Bcl-2 family members contain at least one of four conserved Bcl-2 homology (24) domains. According to their function and structure, the Bcl-2 family can be divided into two classes: the antiapoptotic and proapoptotic members. The antiapoptotic proteins have all four BH domains and include Bcl-2, Bcl-xl, Mcl-1, Bcl-w, and A1. The proapoptotic Bcl-2 proteins are further functionally divided into two subgroups: the effector molecules such as BAX and BAK, containing BH domains 1–3, and the BH3-only proteins such as BAD, BID, BIK, BIM, BMF, BNIP3, HRK, NOXA, and PUMA, which contain only the BH3 domain (30). In the current study, following the findings that the combination of bortezomib and TH-302 can synergistically induce apoptosis (Fig. 1 and Table 1), we further investigated the changes of some important Bcl-2 family members in multiple myeloma cells. The changes of Bcl-2 family members are shown in Fig. 3 and are summarized in Table 2 based on quantitative analysis.

We found that the protein levels of antiapoptotic Bcl-2, Bcl-xl, and Mcl-1 were decreased by TH-302 in hypoxic conditions in all tested multiple myeloma cell lines. Moreover, when treated with bortezomib alone, the expression...
of Bcl-2 was decreased, the expression of Bcl-xL was not disturbed, and the expression of Mcl-1 was increased. Nevertheless, when the multiple myeloma cells were treated with the combination of drugs, we observed clearly a decrease of all three antiapoptotic Bcl-2 family proteins. Among these three investigated antiapoptotic Bcl-2 family members, the changes of Mcl-1 are noticeable. Mcl-1 is overexpressed in multiple myeloma cells, and plays essential roles for the survival of multiple myeloma cells by its ability to oppose to a wide variety of proapoptotic stimuli (31). Downregulation or reduction of Mcl-1 has been proposed to play very important roles in response to drug-induced apoptotic stimuli (32). The expression of Mcl-1 can be regulated at both transcriptional and post-translational levels. The accumulation of Mcl-1 upon bortezomib treatment has been shown to induce multiple myeloma cell resistance to bortezomib-induced lethality, and was speculated to be the result of the inhibition of ubiquitination-mediated degradation (33). More recently, we showed that the accumulation of Mcl-1 by bortezomib was transcriptionally upregulated by the selective activation of the ATF4 signaling branch of the unfolded protein response (UPR; ref. 34). Moreover, Mcl-1 was also shown to be a substrate for caspases during induction of apoptosis (35–37). In this regard, the cleavage of Mcl-1 by caspases is related to the decrease of the full length of the Mcl-1 protein. More importantly, the cleaved Mcl-1 shows strong proapoptotic activity by enhancing its turnover and impairing its ability to interact with BH3-only proapoptotic proteins such as NOXA, PUMA, and BIM-induced apoptosis (38). In line with the finding of Mcl-1 cleavage, the cleavage of PARP and pro-caspase-3/8/9 shows similar changes when treated with TH-302 and bortezomib either alone or in combination, suggesting a tight correlation between caspase activation and Mcl-1 reduction (Figs. 2 and 3A). Similar to the changes of Mcl-1 antiapoptotic proteins, we also observed that TH-302 and bortezomib can result in the cleavage of several BH3-only proapoptotic proteins such as BAD and BID (Fig. 3B and Table 2). However, the cleavage of BAD and BID does not imply the decrease of proapoptotic function, because previous studies have shown that the cleavage of BAD and BID is a caspase-dependent process, and the cleaved BAD and BID fragments exhibit stronger mitochondrial localization and higher apoptotic capability than intact ones, thus promoting apoptosis (21, 22).

One of the most striking findings of the study was that both TH-302 and bortezomib can induce the expression of proapoptotic BH3-only proteins PUMA and NOXA. The induction of PUMA and NOXA was speculated to be another crucial event mediating the synergistic apoptosis. PUMA and NOXA have been shown to be the transcriptional targets of p53 and essential mediators in p53-induced apoptosis (39–42). In the current study, we found that both TH-302 and bortezomib can activate p53 and its downstream target gene p21\(^{\text{WAF1/CIP1}}\) in multiple myeloma cells expressing wild-type p53 (Fig. 4A and C). However, the mechanisms underlying p53 activation by TH-302 and bortezomib are different. TH-302 is a hypoxia-activated DNA cross-linking agent and under hypoxic condition will lead to genotoxic stress in multiple myeloma cells, inducing p53 signal transduction and apoptosis. In response to the genotoxic stress caused by TH-302, the activation of p53 may play a direct role in triggering the onset of DNA repair processes. Accordingly, we found that the transcription levels of DNA repair genes such as BRCA1, BRCA2, RAD51, and FANCD2 were significantly increased by TH-302 (Fig. 4B). However, in multiple myeloma cells treated with bortezomib, although p53 was activated, the DNA repair machinery induction was delayed by downregulating the transcription of these DNA repair genes (Fig. 4D). Growing evidence has shown that bortezomib is particularly toxic to multiple myeloma cells due to an increased susceptibility of multiple myeloma cells to ER stress-induced apoptosis (43). Treatment of multiple myeloma cells with bortezomib leads to induction of proapoptotic UPR components, including growth arrest and CHOP. A previous study has indicated that the major apoptotic regulator p53 was significantly increased in response to ER stress, and participates in ER stress-induced apoptosis (40, 44). In this regard, our findings are consistent with the previous studies that the antimyeloma activity of bortezomib was partly mediated by the induction and activation of p53, and downregulation of the expression of the DNA repair machinery (26, 27). Very recently, Lin and colleagues further showed that ER stress stimulates p53 expression through NF-κB.

### Table 2. The changes of Bcl-2 family proteins by TH-302 and bortezomib in multiple myeloma cells

<table>
<thead>
<tr>
<th>Member</th>
<th>Pro/antiapoptotic</th>
<th>Bortezomib</th>
<th>TH-302</th>
<th>Bortezomib–TH-302</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Antiapoptotic</td>
<td>Downregulation</td>
<td>Downregulation</td>
<td>Downregulation</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Antiapoptotic</td>
<td>No change</td>
<td>Downregulation</td>
<td>Downregulation</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Antiapoptotic</td>
<td>Upregulation, cleavage</td>
<td>Downregulation, cleavage</td>
<td>Downregulation, cleavage</td>
</tr>
<tr>
<td>BAX</td>
<td>Proapoptotic</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>BAD</td>
<td>Proapoptotic</td>
<td>Cleavage</td>
<td>Cleavage</td>
<td>Cleavage</td>
</tr>
<tr>
<td>BID</td>
<td>Proapoptotic</td>
<td>Cleavage</td>
<td>Cleavage</td>
<td>Cleavage</td>
</tr>
<tr>
<td>PUMA</td>
<td>Proapoptotic</td>
<td>Upregulation</td>
<td>Upregulation</td>
<td>Upregulation</td>
</tr>
<tr>
<td>NOXA</td>
<td>Proapoptotic</td>
<td>Upregulation</td>
<td>Upregulation</td>
<td>Upregulation</td>
</tr>
</tbody>
</table>
activation (45). Work by Hideshima and colleagues showed that bortezomib actually activates two upstream NF-κB-activating kinases (RI2 and IKKβ), promotes downregulation of NF-κB’s inhibitor (IkBα), and increases NF-κB DNA binding both in vitro and in vivo (46). Taken together, it suggests that the induction and activation of p53 by bortezomib was mediated by ER stress rather than genotoxic stress.

In multiple myeloma cell lines with p53 mutations, we still observed the induction of PUMA and NOXA by TH-302 and bortezomib, strongly implicating the existence of p53-independent mechanisms. We found that the activation of CHOP by TH-302 and bortezomib through ER stress may be another upstream regulator. As shown in Fig. 4E and G, both TH-302 and bortezomib can trigger ER stress signaling, confirmed by upregulation of ER stress markers GRP-78 and CHOP. As an important ER stress downstream effector, the transcription factor CHOP works as a critical initiator of ER stress-induced apoptosis through upregulating the expression of proapoptotic BH3-only proteins PUMA and NOXA (47, 48). The mechanism how bortezomib induces ER stress has been well shown; however, the reasons why TH-302 can trigger both ER and genotoxic stresses have not been fully clarified. TH-302 consists of two distinct parts: a bis-alkylating effector and an oxygen concentration-activating trigger. The hypoxia-selective release of the Br-IPM may lead to protein alkylation in addition to DNA cross-linking, disturb the homeostasis of the ER, and induce ER stress.

In summary, our findings in the present study show that the combination of TH-302 and bortezomib has synergistic cytotoxicity in multiple myeloma, and the synergy is tightly related to the changes in the balance between proapoptotic and antiapoptotic Bcl-2 proteins favoring induction of apoptosis. Importantly, the experiments carried out in the in vivo murine 5T33MM model showed impressive improvements in multiple disease parameters and significantly prolonged survival after treatment with the combination. Taken together, our data provide a novel insight into the potential application of bortezomib and TH-302 for multiple myeloma, and provide support for further clinical evaluation of the combination of bortezomib and TH-302 for patients with multiple myeloma.

Disclosure of Potential Conflicts of Interest

D. Handisides has ownership interest (including patents) in Threshold. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J. Hu, D. Handisides, C.P. Hart, K. Vanderkerken
Development of methodology: J. Hu, E. Van Valkenborgh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Hu, H. De Raeve, S. Xu, K. Vanderkerken
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Hu, D. Xu, E. Menu
Writing, review, and/or revision of the manuscript: J. Hu, E. Van Valkenborgh, E. Menu, H. De Raeve, E. De Bryune, B. Van Camp, C.P. Hart, K. Vanderkerken
Study supervision: K. Vanderkerken

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