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TM-233, a novel analog of 1′-acetoxychavicol acetate, induces cell death in myeloma cells by inhibiting both JAK/STAT and proteasome activities

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Key words

1′-acetoxychavicol acetate, apoptosis, bortezomib, multiple myeloma, NF-κB

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Although the introduction of bortezomib and immunomodulatory drugs has led to improved outcomes in patients with multiple myeloma, the disease remains incurable. In an effort to identify more potent and well-tolerated agents for myeloma, we have previously reported that 1′-acetoxychavicol acetate (ACA), a natural condiment from South-East Asia, induces apoptotic cell death of myeloma cells *in vitro* and *in vivo* through inhibition of NF-κB-related functions. Searching for more potent NF-κB inhibitors, we developed several ACA analogs based on quantitative structure–activity relationship analysis. TM-233, one of these ACA analogs, inhibited cellular proliferation and induced cell death in various myeloma cell lines with a lower IC_{50} than ACA. Treatment with TM-233 inhibited constitutive activation of JAK2 and STAT3, and then downregulated the expression of anti-apoptotic Mcl-1 protein, but not Bcl-2 and Bcl-xL proteins. In addition, TM-233 rapidly decreased the nuclear expression of NF-κB and also decreased the accumulation of cytosolic NF-κB. We also examined the effects of TM-233 on bortezomib-resistant myeloma cells that we recently established, KMS-11/BTZ and OPM-2/BTZ. TM-233, but not bortezomib, inhibited cellular proliferation and induced cell death in KMS-11/BTZ and OPM-2/BTZ cells. Interestingly, the combination of TM-233 and bortezomib significantly induced cell death in these bortezomib-resistant myeloma cells through inhibition of NF-κB activity. These results indicate that TM-233 could overcome bortezomib resistance in myeloma cells mediated through different mechanisms, possibly inhibiting the JAK/STAT pathway. In conclusion, TM-233 might be a more potent NF-κB inhibitor than ACA, and could overcome bortezomib resistance in myeloma cells.

Multiple myeloma is a plasma cell malignancy, which still remains incurable despite the use of conventional high-dose chemotherapy with stem cell transplantation.[1] Since 2000, novel agents such as thalidomide, lenalidomide and bortezomib have been introduced in clinical settings and have remarkably improved patients' outcomes.[2,3] Subsequently, many clinical trials of second generations of these agents, such as pomalidomide, carfilzomib and ixazomib, have been conducted with better outcomes even in drug-resistant cases.[4–8] However, it is still difficult to cure patients with multiple myeloma; because most patients are elderly, resistance to novel drugs often appears, and severe side effects, such as peripheral neuropathy and serious infections, occur in many patients. Therefore, the identification and validation of novel targeted agents with less toxicity are necessary to overcome drug resistance and to improve clinical outcomes of multiple myeloma.

1′-Acetoxychavicol acetate (ACA) is obtained from the rhizomes of *Languas galanga* (Zingiberaceae), a traditional condiment in South-East Asia and in Thailand in particular.[9] Recent studies have revealed that ACA has potent chemo-preventive effects against rat oral carcinomas and inhibits the chemically-induced tumor formation and cellular growth of various cancer cells.[10,11] Furthermore, we have previously reported that ACA has an inhibitory effect on NF-κB and induces cell death in myeloma cells both *in vitro* and *in vivo*.[12,13]

With the aim of discovering more potent NF-κB inhibitors, we subsequently developed several ACA analogs based on quantitative structure–activity relationship (QSAR) analysis. We and other groups have reported QSAR studies of ACA for apoptotic activity towards human leukemia HL-60 cells, showing that the two acetyl groups and the unsaturated double bond between the Cβ and Cγ positions of ACA are essential for its activity, and synthesized novel constructs that differ at the Cβ and Cγ positions of ACA.[11,14] TM-233 is a novel benzhydrol-type analog of ACA that exhibits greater growth inhibition of HL-60 leukemia cells. In the present study, we examined the effects of TM-233 on various myeloma cells, including those resistant to bortezomib, and we investigated the molecular mechanism of TM-233-induced death in these cells.

Material and Methods

Cells and cultures. Human myeloma cell lines (U266, RPMI-8226, KMS-11, OPM2 and MM-1S) were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Bortezomib-resistant myeloma cell lines (KMS-11/BTZ and

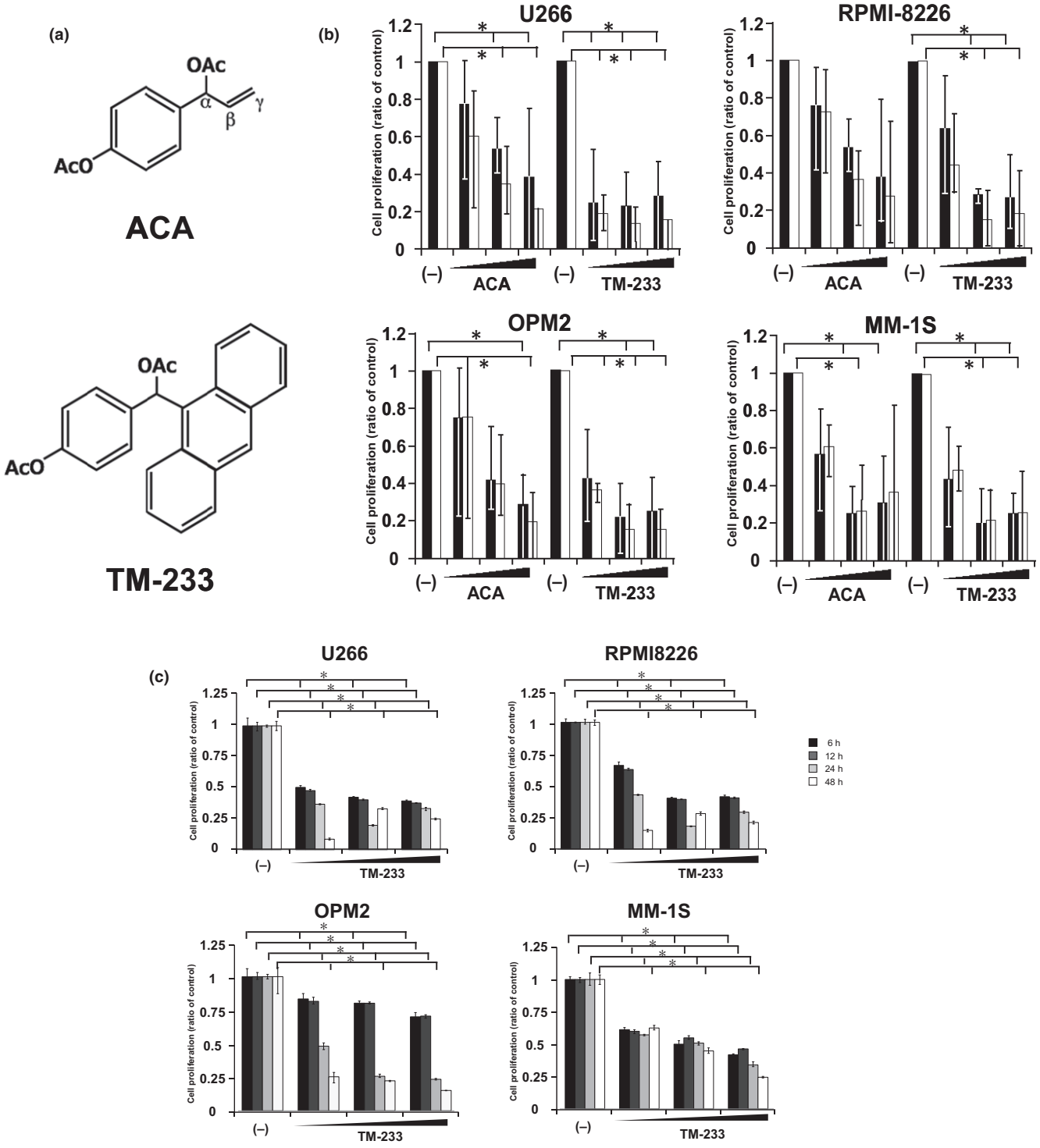

Fig. 1. Effects of TM-233 treatment on myeloma cells, fresh samples with patients and normal peripheral blood mononuclear cell (PBMC). (a) Chemical structures of parental 1′-acetoxychavicol acetate (ACA) (upper panel) and its derivative TM-233 (lower panel). (b) Detection of growth inhibition of parental ACA, and TM-233 by MTS assay at various doses (1, 2.5, 5 μM) and times (24 h, black; 48 h, white) in four myeloma cell lines (U266, RPMI-8226, OPM2, MM-1S). (c) Detection of growth inhibition of TM-233 by MTS assay at various doses (1, 2.5, 5 μM) and times (6 h, black; 12 h dark gray; 24 h, light gray; 48 h, white) in myeloma cell lines. (d) U266 and RPMI8226 cells were pre-treated with 25 ng/mL of interleukin-6 (IL-6) or vehicle for 30 min prior to treatment with various doses (0, 2.5, 5 μM) of TM-233 and cell proliferation was detected by MTS assay. (e) Bone marrow samples from two myeloma patients (Pt 1 and Pt 2) were sorted with CD138-beads and were treated with either vehicle or 2.5 μM of TM-233 for 24 h. Cell viability was measured by using trypan blue exclusion. (f) Normal human peripheral blood mononuclear cells (PBMC) were treated with low dose (2.5 μM) and high dose (10 μM) of TM-233 for 24 to 72 h. Viable cells were counted by using trypan blue exclusion. Asterisks (*) indicate $P < 0.05$ versus control.

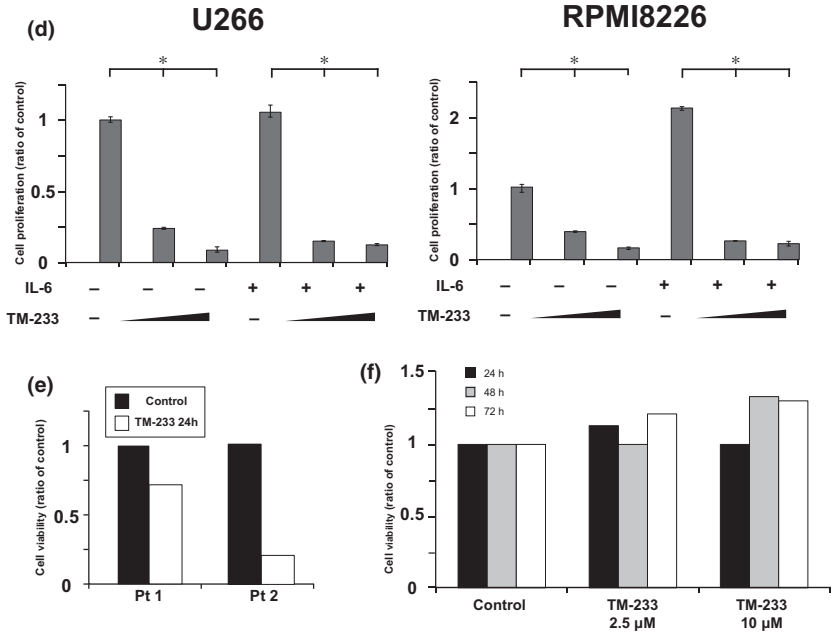

Fig. 1. (Continued).

Table 1. IC_{50} values of ACA and TM-233 against various human myeloma cell lines

Cell line	ACA (μM)	TM-233 (μM)
OPM2*	1.99	0.82
U266*	2.83	0.67
PRMI-8226*	2.99	1.44
MM-IS	1.19	0.94

*$P < 0.05$. The concentration of 1′-acetoxychavicol acetate (ACA) and TM-233 that inhibits 50% viability (IC_{50}) as compared with control after 24 h incubation of each agent.

OPM2/BTZ) were previously reported by our group.[15] Bone marrow samples from two Japanese patients with multiple myeloma were obtained according to appropriate Human Protection Committee validation at Saitama Medical University with written informed consent. Mononuclear cells were separated by Lymphoprep (Nycomed Pharma, Oslo, Norway). CD138-positive plasma cells were sorted using MACS MicroBeads (Miltenyi Biotec, Tokyo, Japan). Normal human peripheral blood mononuclear cell (PBMC) were purchased from Precision Bioservices (Frederick, MD, USA). Cells were maintained in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich), 100 units/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2. Morphology was examined on cytospin slides stained with Giemsa.

Reagents. TM-233 (Fig. 1a, lower panel) is a novel benzhydrol-type analog of ACA (1′-acetoxychavicol acetate) (Fig. 1a, upper panel), which we had previously developed[14] and which was dissolved in DMSO at a stock concentration of 10 mM. Interleukin-6 (IL-6) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Assays for cellular viability and proliferation. Cellular viability was examined by counting the viable cells using trypan blue dye exclusion, and cellular proliferation was measured using an MTS proliferation assay kit (Promega, Madison, WI, USA). For the MTS assay, cells were plated on 96-well tissue culture plates at 5×10^4/mL in a total volume of 100 μL with the indicated agents and assayed according to the manufacturer's instructions. The absorbance at 490 nm was expressed as a relative value of the control culture.

Assays for apoptotic cell death. Apoptotic cell death was determined by morphologic change as well as staining with Annexin V-FITC and propidium iodide (PI) labeling by using a staining kit purchased from BD Bioscience (San Jose, CA, USA). BD FACSVerse was used for flowcytometric analysis. In addition, the induction of apoptotic cell death was detected by a Cytotoxicity Detection Kit^{PLUS} [LDH] purchased from Roche Diagnostics (Mannheim, Germany). Each experiment was performed according to manufacturers' instructions.

Cell cycle analysis. Cells were suspended in hypotonic solution (0.1% Triton X-100, 1 mM Tris-HCl [pH 8.0], 3.4 mM sodium citrate, 0.1 mM EDTA) and stained with 50 μg/mL of PI. BD FACSVerse was used for flowcytometric analysis and the population of cells in each cell cycle phase was determined using ModiFIT (Verity Software House, Topsham, Maine, USA) software.

Western blot analysis. Cells were collected by centrifugation at 500 *g* for 5 min, and the pellets were resuspended in a lysis buffer (1% NP40, 1 mM phenylmethylsulfonyl fluoride, 40 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM NaOV) at 4°C for 15 min. Cell lysates (20 μg protein per lane) were fractionated on 12.5% SDS-polyacrylamide gels before being transferred to the membrane (Immobilon-P membranes [Merck Millipore, Billerica, MA, USA]) according to the standard protocol. Antibody binding was detected by using the enhanced chemiluminescence kit with hyper-ECL film (GE Healthcare Japan, Hino, Japan). Antibodies against caspase-3, carpase-8 and carpase-9, PARP, Bid, STAT3, pTyr705-STAT3, pTyr1007/1008-JAK2, Akt, p44/42 MAPK (Erk1/2) and NF-κB p65 were purchased from Cell Signaling Technology (Beverly, MA, USA), while those against Bcl-2, Bcl-xL,

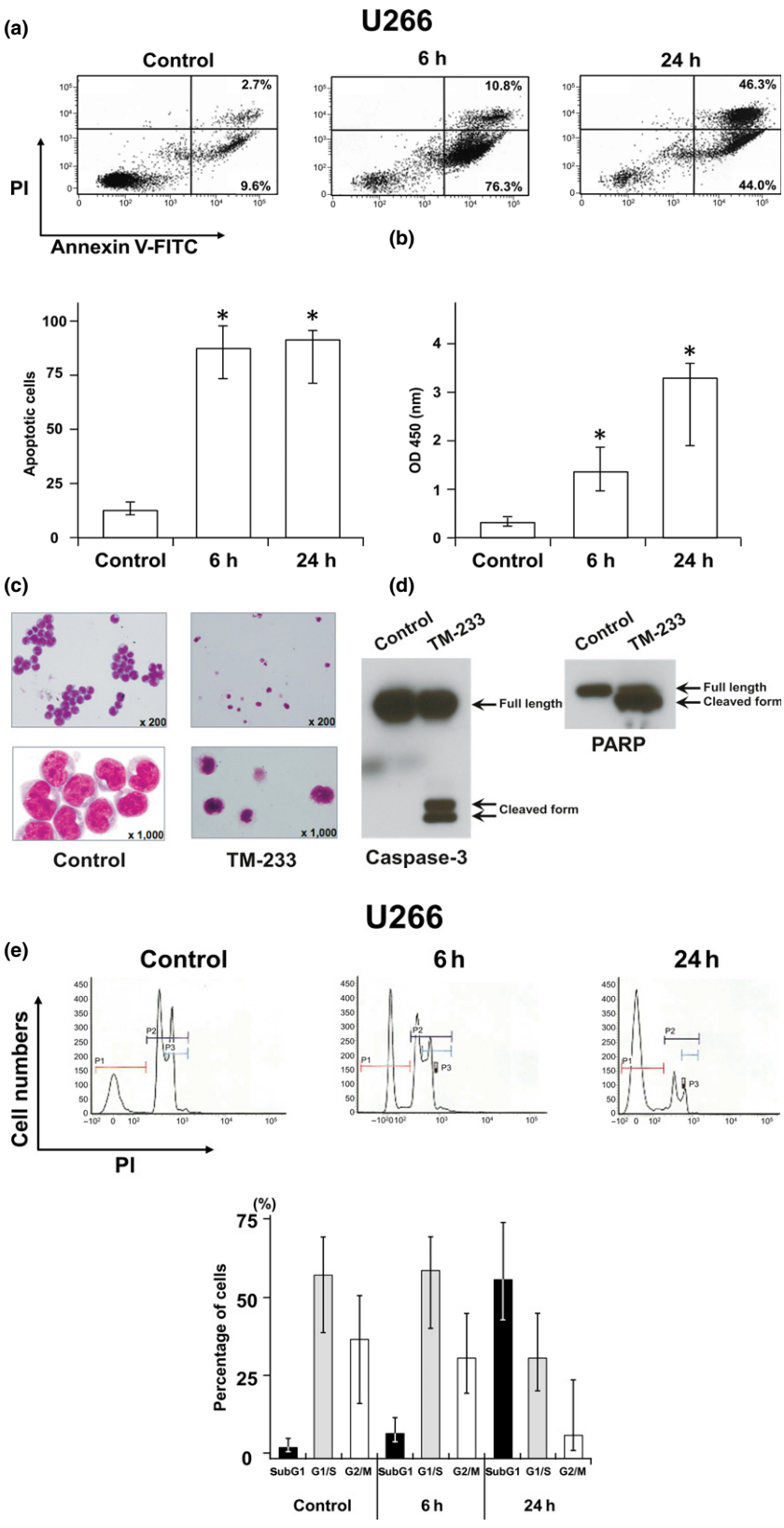

Fig. 2. Effects of TM-233 treatment on myeloma cell apoptotic cell death. (a) Detection of apoptotic cell death by Annexin V-PI assay and lactate dehydrogenase (LDH) immunofluorescence assay. U266 cells were cultured with 2.5 μM TM-233 for 0, 6 or 24 h, then stained with Annexin V-FITC and PI, then analyzed by flow cytometry. Asterisks (*) indicate $P < 0.05$ versus control. (b) In the same conditions using U266 cells, LDH activity was measured by immunofluorescence. Asterisks (*) indicate $P < 0.05$ versus control. (c) Morphological changes show characteristics of apoptotic cell death in U266 myeloma cells. Cells were treated with 2.5 μM TM-233 for 24 h, and then cytospin slides were prepared and stained with Giemsa. Original magnification ×1000. (d) Western blot analysis of caspase-3 and PARP proteins in TM-233-treated U266 cells. Protein levels were detected using antibodies against caspase-3 and PARP. TM-233 treatment-induced processing of caspase-3 and PARP is indicated by the appearance of cleaved active forms, respectively. (e) Cell cycle analysis. U266 cells were treated with 2.5 μM TM-233 for the indicated time, and then stained with PI. The DNA content was analyzed by flow cytometry. SubG1 content refers to the portion of apoptotic cells. Similar results were obtained in RPMI8226 cells (Suppl. Fig. S2). Three independent experiments were performed and all gave similar results. PI, propidium iodide.

Mcl-1, RelB, c-Rel and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Reverse transcription-polymerase chain reaction analysis. Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. Ten pmol of primers for Mcl-1 (forward, 5′-GCCAAG GACACAAAGCCAAT-3′; and reverse, 5′-AACTCCACAAA CCCATCC CA-3′), and NF-κB p 65 (forward, 5′-ACAAGTG GCCATTGTGTTCC-3′; and reverse, 5′-ACGTTTCTCCTCA ATCCGGT-3′) were used in the PCR reactions. Primer sets for

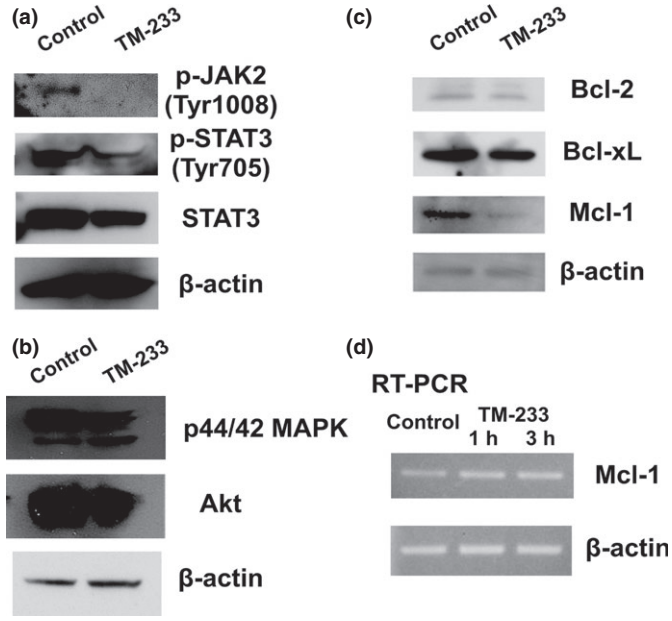

Fig. 3. JAK-STAT signaling pathway in TM-233-induced cell death. (a) U266 cells were cultured with 2.5 μM TM-233 for 3 h versus control. Western blot analyses were performed using whole cell lysates. Antibodies against phospho-JAK2 (Tyr1008), phospho-STAT3 (Tyr705) and STAT3 were used. Activation of JAK2 and STAT3 was confirmed. β-actin was used as an internal control. (b) Western blot analyses were performed by using antibodies against p44/42 MAPK (Erk1/2) and Akt. Either pathway was not activated in TM-233-treated U266 cells. β-actin was used as an internal control. (c) The expression of apoptosis-associated proteins (Bcl-2, Bcl-xL, Mcl-1) was detected. Only Bcl-2, but not Bcl-xL or Mcl-1 protein, was activated. β-actin was used as an internal control. (d) Mcl-1 transcription was analyzed by using semi-quantitative RT-PCR assay.

β-actin (forward, 5′-CAAGAGATGGCCACGGCTGCT-3′; and reverse, 5′-CAAGAG ATGGCCACGGCTGCT-3′) was used as the internal control. After an initial denaturation at 94°C for 2 min, 30 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, and final extension at 72°C for 7 min were performed using the Superscirpt III First-Strand Synthesis System for RT-PCR (Life Technologies Japan, Tokyo, Japan), The PCR products were electrophoresed in 2% agarose gels.

***In vitro* proteasome activity assays.** *In vitro* proteasome activity assays were performed using Proteasome-Glo Assay Systems (Promega KK, Tokyo, Japan) according to the manufacturer's instructions. Briefly, chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) activities of the 20S proteasome were detected using luminogenic substrates such as Suc-LLVY-Glo, Z-LRR-Glo and Z-nLPnLD-Glo, respectively. A TR717 Microplate Luminometer (Life Technologies Japan) was used to detect fluorescence.

Statistical analysis. Data are expressed as means ± SD. The unpaired Student's *t*-test was used to evaluate statistical significance. Differences with $P < 0.05$ were considered statistically significant.

Results

TM-233 inhibits cellular proliferation of various multiple myeloma cell lines and fresh samples from patients, but not normal peripheral blood mononuclear cells. We first examined the effects of TM-233 on several myeloma cell lines (U266, RPMI-8226, OPM2 and MM-1S) and found that TM-233 inhibited cell proliferation and induced cell death in various myeloma cell lines in a time (0–48 h)-dependent and dose (0–5 μM)-dependent manner (Fig. 1b,c). Notably, in each cell line, the dose to induce cell death was lower, and the time was earlier than those of its parental derivative, ACA. The IC_{50} values at 24 h for each myeloma cell line of TM-233 compared to ACA are shown in Table 1. IL-6 is one of the important growth factors inducing myeloma cell growth. IL-6 is produced by both autocrine from myeloma cells and paracrine from their microenvironment.[16] To make a similar condition of co-culture with myeloma cells and bone marrow stromal cells, we next investigated whether IL-6 could block TM-233-induced cell death in U266 and RPMI8226 myeloma cells, and found that TM-233 did not block cell death of myeloma cells even in the presence of IL-6 (Fig. 1d). Treatment of TM-233 (2.5 μM for 24 h) was also effective for bone marrow samples from two myeloma patients (Fig. 1e), but TM-233 had no effect on normal human PBMC even in higher doses (up to 10 μM) and with longer exposure (up to 72 h) (Fig. 1f).

TM-233 exerts G1 cell cycle arrest followed by apoptotic cell death in myeloma cells. We next examined whether the anti-proliferative effects of TM-233 on myeloma cells represented the induction of apoptotic cell death. The induction of apoptotic cell death of two myeloma cell lines (U266 and RPMI-8226) treated with 2.5 μM TM-233 using Annexin V-FITC and PI double staining was analyzed by flow cytometry, and we found that Annexin V-positive fractions were increased in a time-dependent manner in U266 and RPMI8226 cells (Fig. 2a and Suppl. Fig. S1). Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. The cytotoxicity Detection Kit^{PLUS} [LDH] can easily show damaged cells by measuring the LDH activity by immunofluorescence. Figure 2b shows that treatment with 2.5 μM TM-233 remarkably released LDH activity at 24 h. In addition, the exposure of myeloma cells to 2.5 μM of TM-233 resulted in the typical morphological appearance of apoptosis in U266 cells (Fig. 2c). Furthermore, TM-233 activated apoptosis-related caspase-3 and caspase-9 and PARP in U266 cells, suggesting that TM-233 activates an extrinsic pathway of caspase (Fig. 2d). We also performed cell cycle analysis by staining myeloma cells with PI and analyzed them by flow cytometry and found that TM-233 induced G1 cell cycle arrest followed by apoptotic cell death in U266 and RPMI8226 cells (Fig. 2e and Suppl. Fig. S2).

TM-233 induces cell death of myeloma via the JAK2/STAT3/Mcl-1 pathway, but not other kinase pathways. We then investigated the molecular mechanisms of TM-233-induced cell death via various signaling pathways in myeloma cells. Using western blot analysis, we found that treatment of myeloma cells with TM-233 (2.5 μM, 3 h) inhibited constitutive activation of JAK2 and STAT3 (Fig. 3a). Furthermore, we investigated other kinase pathways frequently detected in myeloma using western blot analysis, and found that expression of Akt and p44/42 MAPK was not changed after TM-233 treatment (Fig. 3b). TM-233 downregulated the expression of anti-apoptotic Mcl-1 protein, but not that of Bcl-2 or Bcl-xL proteins in myeloma cells (Fig. 3c). Next, we examined the transcription of Mcl-1 using semi-quantitative RT-PCR assay, and found that Mcl-1 expression was not changed during the time-course after TM-233 treatment (Fig. 3d). These results suggested that TM-233-induced Mcl-1 downregulation occurred at the post-transcription level.

TM-233 induces cell death of myeloma via the NF-κB pathway. The NF-κB pathway is crucial for the proliferation of

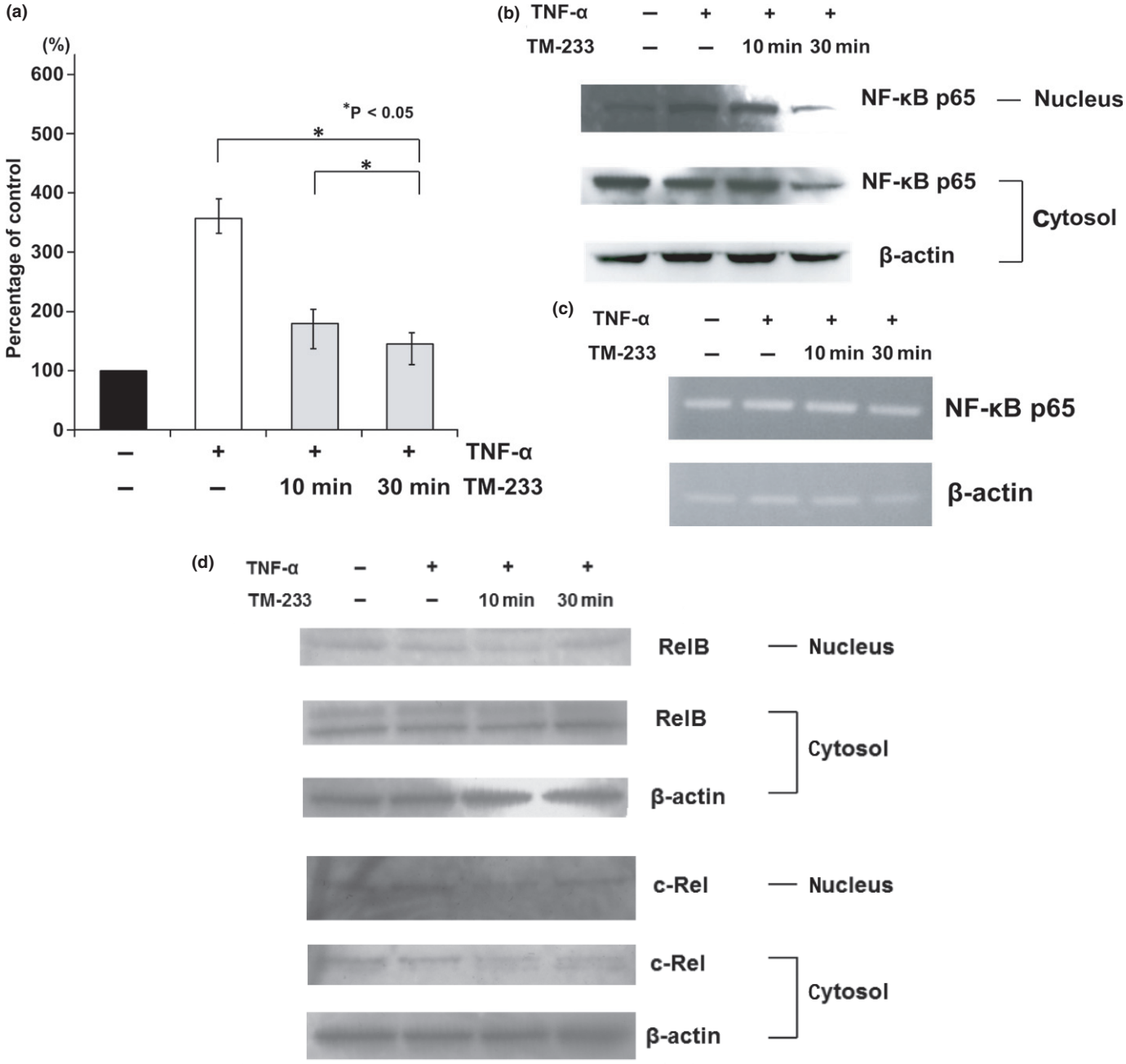

Fig. 4. NF-κB signaling pathway in TM-233-induced cell death. (a) NF-κB p65 activity (canonical pathway) was detected using by ELISA. U266 myeloma cells were first treated with TNFα. Fifteen minutes after stimulation, 2.5 μM of TM-233 were added for the indicated time. Nuclear protein was extracted and NF-κB p65 activity was detected by immunofluorescence. After TM-233 treatment, NF-κB p65 activity was downregulated in a time-dependent manner. (b) Under the same conditions, both nuclear and cytosolic proteins were extracted and analyzed with western blotting using antibodies against NF-κB p65. In both experiments, NF-κB p65 activation was confirmed. (c) Transcription of NF-κB p65 was detected using RT-PCR assay with the same conditions. At the transcription level, NF-κB p65 mRNA level was not changed, indicating that NF-κB p65were downregulated at the post-transcription level. (d) Other NF-κB pathways were detected using western blot analysis, under the same conditions. RelB, and c-Rel levels were not change indicating that TM-233 effected only at canonical pathway. β-actin was used as an internal control.

myeloma cells; therefore, we examined how the NF-κB pathway is influenced when treating myeloma cells with TM-233. First, we examined the DNA binding activity of NF-κB p65 (the canonical pathway) in TM-233-treated myeloma cells using ELISA, and found that NF-κB p65 activity was inhibited by treatment with TM-233 in a time-dependent manner (Fig. 4a). We next investigated the nuclear translocation of NF-κB p65 using western blot analysis, and found that TM-233 rapidly decreased both the nuclear and cytosolic expression of NF-κB, suggesting that TM-233 inhibited the activation of NF-κB p65 (Fig. 4b). In addition, we examined the transcription of NF-κB p65 after TM-233 treatment, and found that expression of NF-κB p65 was not changed during the same time course by using semi-quantitative RT-PCR assay (Fig. 4c), indicating that TM-233-induced NF-κB p65 was occurred at the post-transcription level. ACA inhibits the translocation of NF-κB from the cytosol

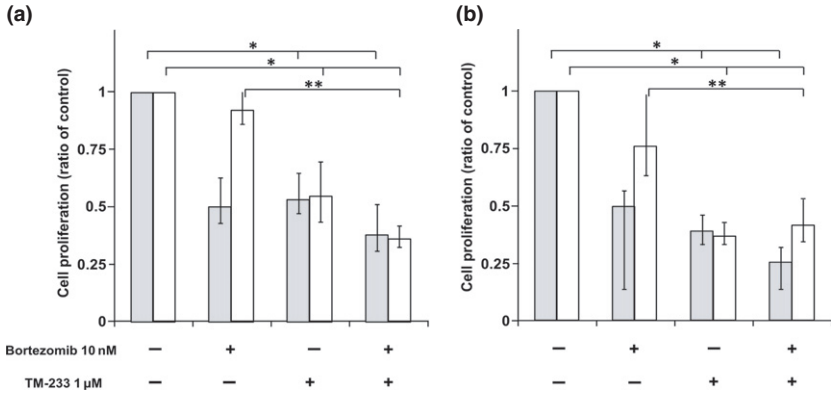

Fig. 5. TM-233 overcomes bortezomib resistance. Effect of TM-233 on KMS-11 (gray bar), KMS-11/BTZ (white bar) cells (a), and OPM-2 (gray bar) and OPM-2/BTZ (white bar) cells (b) were examined. In bortezomib-sensitive cell lines (KMS-11 and OPM2, gray bars), bortezomib and TM-233 alone induced cell death. In bortezomib-resistant cell lines (KMS-11/BTZ, OPM2/BTZ, white bars), bortezomib alone induced only mild cell death; however, TM-233 induced cell death at almost the same degree as in sensitive cells, and using both agents induced additive effects. Single asterisks (*) indicate $P < 0.05$ versus controls at the same time period, whereas double asterisks (**) indicate $P < 0.05$ versus controls and 10 nM bortezomib in the same period.

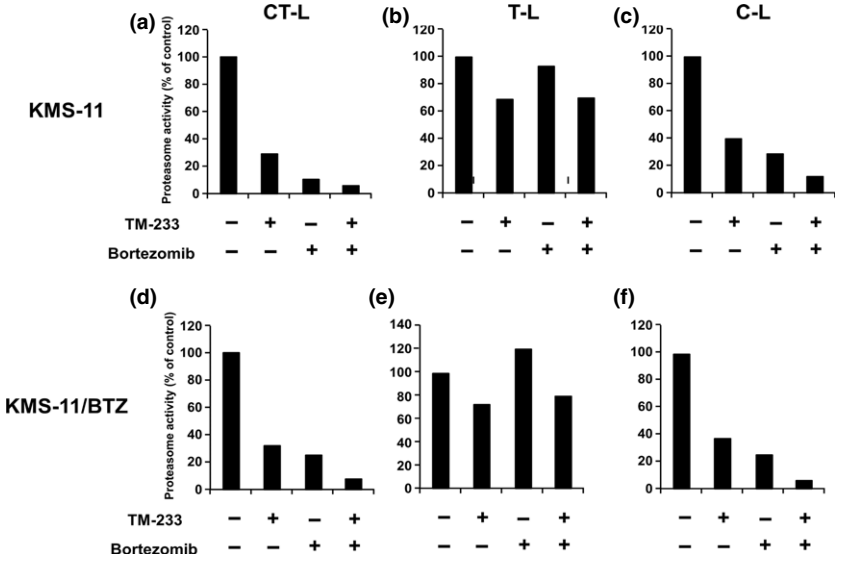

Fig. 6. *In vitro* proteasome assay. KMS-11 (a–c) and KMS-11/BTZ (d–f) cells were treated with low-dose bortezomib (10 nM) and TM-233 (1 μM) for 6 h, and *in vitro* proteasome assay was performed. Chymotrypsin-like (CT-L) (a,d), trypsin-like (T-L) (b–e) and caspase-like (C-L) (c,f) activities were detected using a luminometer. TM-233 as well as bortezomib inhibited both CT-L and C-L activities in KMS-11 myeloma cells, and a combination of bortezomib and TM-233 additively inhibited these activities. TM-233, but not bortezomib, slightly inhibited T-L activity. Interestingly, TM-233 and bortezomib inhibited both CT-L and C-L activities in bortezomib-resistant KMS-11/BTZ cells; however, bortezomib did not induce cell death in resistant KMS/BTZ myeloma cell lines.

to the nucleus;[13] therefore, the mechanism of NF-κB inhibition of TM-233 might be different from that of ACA. We also examined for other NF-κB pathways, such as non-canonical pathways. We investigated the nuclear translocation of RelB and c-Rel using western blot analysis, and found that RelB and c-Rel was not changed after TM-233 treatment, indicating that TM-233 did not inhibit activation of RelB and c-Rel (Fig. 4d).

TM-233 exerts cell death in bortezomib-resistant myeloma cells. We further examined the effects of TM-233 on bortezomib-resistant myeloma cells. We recently established bortezomib-resistant myeloma cell lines KMS-11/BTZ and OPM-2/BTZ.[15] We found that these cells have a unique point mutation, G322A, in the gene encoding the proteasome β5 subunit, resulting in bortezomib-resistance mediated through the prevention of the accumulation of unfolded proteins and fatal ER stress.[15] TM-233 inhibited cellular proliferation and induced cell death in KMS-11/BTZ and OPM-2/BTZ cells in a time-dependent and dose-dependent manner, whereas bortezomib alone only slightly inhibited cellular proliferation and induced cell death in KMS-11/BTZ and OPM-2/BTZ (Fig. 5a,b). Interestingly, the combination of TM-233 and bortezomib significantly induced cell death in these bortezomib-resistant myeloma cells. These results indicate that TM-233 can overcome bortezomib resistance in myeloma cells via a different mechanism, probably inhibition of the JAK/STAT pathway.

TM-233 inhibits proteasome activity similar to bortezomib in myeloma cells. The 20S proteolytic core region of 26S proteasome, which has proteolytic active sites, consists of four highly homologous rings (α-β-β-α). Two central β-rings contain multiple proteolytic sites that function together in protein degrada-

tion,[17,18] and each of these two β-rings comprises three proteolytic sites: β1 (C-L), β2 (T-L) and β5 (CT-L).[19,20]. Chauhan *et al.*[21] report that bortezomib inhibits both proteasome CT-L and C-L activities in myeloma cells. Therefore, we examined the *in vitro* proteasome activity of TM-233 in myeloma cells to compare the effects with bortezomib. Figure 6 shows that TM-233 as well as bortezomib inhibited both CT-L and C-L activities in KMS-11 myeloma cells, and a combination of bortezomib and TM-233 additively inhibited these activities. TM-233, but not bortezomib, slightly inhibited T-L activity, although it was not statistically significant. Interestingly, TM-233 and bortezomib inhibited both CT-L and C-L activities in bortezomib-resistant KMS-11/BTZ cells; however, bortezomib did not induce cell death in resistant KMS/BTZ myeloma cell lines. Taken together, these results and our previous report show that TM-233 can inhibit not only NF-κB but also other proteasome activities, resulting in overcoming bortezomib resistance in myeloma cells.[15]

Discussion

Since novel drugs such as bortezomib, thalidomide and lenalidomide have been introduced into routine practice for the treatment of multiple myeloma, the clinical outcomes of both newly diagnosed and relapsed/refractory patients have improved.[3] Moreover, second generations of these agents, such as carfilzomib, pomalidomide and ixazomib, are now being used in clinical trials and have been reported to result in better clinical outcomes even in relapsed/refractory cases.[4–8] However, myeloma is still incurable and often the treatments are discontinued due to the serious side effects of these new agents. Therefore, there is great need to develop new agents with novel mechanisms of action and lower toxicity.

NF-κB is a dimeric transcription factor of the Rel homology domain-containing proteins, which include p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2), and which regulate many biological phenomena, including cell proliferation, immune responses, anti-apoptotic cell death and cytokine secretion.[22,23] NF-κB has emerged as a therapeutic target in a variety of cancers, such as breast cancer,[24] melanoma,[25] prostate cancer,[26] MLL-leukemia[27] and multiple myeloma.[28,29] It has been reported that proteasome inhibition is a critical pathway for the treatment of multiple myeloma. Bortezomib, which inhibits the β5 subunit of the proteasome (representing chymotrypsin-like activity), is the most widely used first generation proteasome inhibitor, and it inhibits growth, induces apoptotic cell death, and overcomes drug resistance in myeloma cells.[28] Novel second generation proteasome inhibitors, such as carfilzomib, ixazomib and marizomib, can work even in bortezomib-resistant situations according to preclinical and clinical studies.[5–8,21,30,31]

In a previous study, we investigated the effects of ACA and found that it inhibits NF-κB activity in multiple myeloma cells *in vitro* and *in vivo*.[12,13] ACA also sensitizes myeloma cells to TNF-α and has a synergistic, pro-apoptotic effect with the NF-κB inhibitors MG-132 and TLCK. In contrast, an NF-κB activator, PMA, dramatically abrogates ACA-induced apoptosis. These results provide the framework for targeting NF-κB inhibition by treatment with ACA in multiple myeloma therapy. However, the doses required to eradicate myeloma cells are too high for clinical settings. TM-233 is a newly developed ACA analog based on QSAR analysis.[14] Its IC_{50} against three out of four different myeloma cell lines is significantly lower than that of its parental ACA. Therefore, we assumed that TM-233 has a higher potential for anti-myeloma activity and is more likely to be developed into a novel medication.

In the present study, we found that TM-233 is more effective than the parental ACA because of a statistically lower IC_{50} against various myeloma cell lines (Table 1). The molecular mechanisms by which TM-233 acts against myeloma cells are similar to those of ACA in that both agents can induce caspase-dependent cytotoxicity and G1-S cell-cycle arrest followed by apoptotic cell death. However, there are two major differences between these two agents. First, the mechanism through which these agents inhibit NF-κB is different. ACA inhibits the translocation of NF-κB p65 into the nucleus from the cytosol,[13] whereas TM-233 inhibits the activation of NF-κB p65. Second, TM-233 inhibits the JAK2-STAT3-Mcl-1 pathway, whereas ACA does not.

The JAK-STAT signaling pathway is also important in the proliferation of myeloma cells. IL-6 promotes the survival and proliferation of myeloma cells through the phosphorylation of both JAK2 and STAT3.[32,33] The phosphorylation of STAT3 results in the upregulation of anti-apoptotic Bcl-2 family proteins, including Mcl-1, Bcl-xL and Bcl-2.[34] In this study, we clearly showed that TM-233 treatment suppressed the phosphorylation of JAK2 and STAT3, followed by suppression of the downstream molecule Mcl-1, but not of Bcl-xL or Bcl-2 (Fig. 3a), in contrast to ACA (data not shown).

Bortezomib is widely used for the treatment of multiple myeloma in both newly diagnosed and relapsed/refractory settings. The survival of these patients has dramatically improved with the introduction of this medication.[2–4] However, bortezomib resistance is now an important clinical issue. The mechanisms of bortezomib resistance have been widely studied, and include, for example, a point mutation in the proteasome β5 subunit gene (PSMB5),[15,35] upregulation of the insulin-like growth factor (IGF)-1 axis[36] and bone marrow stromal cell-derived exosomes.[37] In this study, we examined the effects of TM-233 on bortezomib-resistant myeloma cell lines having a point mutation in PSMB5, and showed that TM-233 could overcome bortezomib resistance, suggesting that the JAK-STAT pathway might be involved in the acquisition of bortezomib resistance in multiple myeloma. Further studies to investigate the mechanisms of bortezomib resistance in myeloma are warranted.

In conclusion, we report here for the first time that the ACA derivative, TM-233, induces apoptotic cell death in human multiple myeloma cells via NF-κB and the JAK-STAT dual pathway. TM-233 induced cell death even in bortezomib-resistant myeloma cells, mediated through the JAK-STAT pathway. TM-233 is a promising candidate therapeutic agent for the treatment of multiple myeloma.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Detection of apoptotic cell death by Annexin V-PI assay in RPMI 8226 cells. RPMI8226 cells were cultured with 2.5 μM TM-233 for 0, 6 or 24 h, then stained with Annexin V-FITC and PI, and then analyzed by flow cytometry. Asterisks (*) indicate $P < 0.05$ versus control. PI, propidium iodide.

Fig. S2. Cell cycle analysis in RPMI8226 cells. RPMI8226 cells were treated with 2.5 μM TM-233 for the indicated time, and then stained with propidium iodide (PI). The DNA content was analyzed by flow cytometry. SubG1 content refers to the portion of apoptotic cells. Three independent experiments were performed and all gave similar results.