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Antitumor effects of tyropeptin-boronic acid derivatives: New proteasome inhibitors

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Key words

Antitumor effect, boronic acid, multiple myeloma, proteasome inhibitor, tyropeptin

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Funding Information

Scientific Research (23510270). The Ministry of Education, Culture, Sports, Science and Technology in Japan Scientific Research on Innovative Areas Scientific Support Programs for Cancer Research The Ministry of Education, Culture, Sports, Science and Technology, Japan

Received July 28, 2014; Revised September 13, 2014; Accepted September 19, 2014

Cancer Sci **105** (2014) 1609–1615

doi: 10.1111/cas.12542

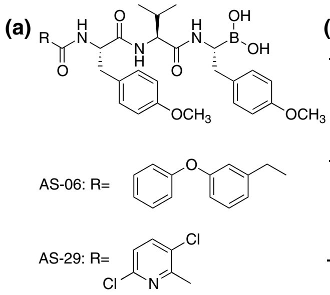
The proteasome degrades numerous regulatory proteins that are critical for tumor growth. Thus, proteasome inhibitors are promising antitumor agents. New proteasome inhibitors, such as tyropeptins and tyropeptin-boronic acid derivatives, have a potent inhibitory activity. Here we report the antitumor effects of two new tyropeptin-boronic acid derivatives, AS-06 and AS-29. AS-06 and AS-29 significantly suppress the degradation of the proteasome-sensitive fluorescent proteins in HEK293PS cells, and induce the accumulation of ubiquitinated proteins in human multiple myeloma cells. We show that these derivatives also suppress the degradation of the NF-κB inhibitor IκB-α and the nuclear translocation of NF-κB p65 in multiple myeloma cells, resulting in the inhibition of NF-κB activation. Furthermore, we demonstrate that AS-06 and AS-29 induce apoptosis through the caspase-8 and caspase-9 cascades. In a xenograft mouse model, i.v. administration of tyropeptin-boronic acid derivatives inhibits proteasome in tumors and clearly suppresses tumor growth in mice bearing human multiple myeloma. Our results indicate that tyropeptin-boronic acid derivatives could be lead therapeutic agents against human multiple myeloma.

The proteasome is a large multi-enzyme complex involved in the main degradation pathway for intracellular proteins in eukaryotic cells.[1–3] This multimeric protease possesses proteolytic activities that are classified into chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) activity. Regulatory proteins degraded by the proteasome include cyclins, cyclin-dependent kinase inhibitors (e.g. p21 and p27), tumor suppressors (e.g. p53) and NF-κB inhibitors (e.g. IκB-α), which are all critical for tumor growth.[4–7] Proteasome inhibitors can stabilize these regulatory proteins and induce cell cycle arrest, ER stress and apoptosis, resulting in a limitation of tumor development.[8,9] Thus, proteasome inhibitors are promising candidates for antitumor agents.[10,11] Both bortezomib[12,13] and carfilzomib[14,15] proteasome inhibitors have been approved and bortezomib is used as a frontline treatment of multiple myeloma.[16] In contrast, bortezomib induces many side effects, including painful peripheral neuropathy, orthostatic hypotension, pyrexia, cardiac and pulmonary disorders, adverse gastrointestinal events, myelosuppression and thrombocytopenia asthenia.[17–20] Furthermore, most multiple myeloma patients treated with bortezomib develop resistance in the short term.[21] The side effects and drug resistance justify the development of novel proteasome inhibitors.

Previously, we identified new proteasome inhibitors, tyropeptins, which are produced by *Kitasatospora* sp. MK993-dF2.[22,23] Tyropeptins specifically inhibit the CT-L activity of the 20S proteasome. With the aim of enhancing the inhibitory activities of these molecules, we constructed a structural model of tyropeptin A bound to the CT-L catalytic site of the mammalian 20S proteasome. We designed new tyropeptin derivatives[24,25] and conducted structure-activity relationship (SAR) studies of these derivatives. We found that tyropeptin-boronic acid derivatives display an enhanced inhibitory activity against CT-L activity of the human proteasome.[26] These results encouraged us to perform further SAR studies of tyropeptin-boronic acid derivatives to develop derivatives more potent than bortezomib.[27] In the present study, we report the antitumor effects of tyropeptin-boronic acid derivatives AS-06 and AS-29 (Fig. 1a).

Materials and Methods

Materials. Bortezomib was synthesized as described by Adams *et al.*[12] MG-132 was obtained from the Peptide Institute (Osaka, Japan). G418 was purchased from Promega (Madison, WI, USA). The antibodies used in western blotting were as follows: anti-IκB-α (FL), anti-nucleolin (H-250) and anti-NF-κB p65 (A), from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-IκB-α (Ser32/36) (5A5), anti-caspase-3 (3G2), anti-cleaved caspase-3 (Asp175), anti-caspase-8 (1C12), anti-caspase-9, anti-caspase-12 and anti-PARP (46D11)



(b)

Proteasome Inhibitory activity

Compounds	IC$_{50}$ (μM) 20S proteasome CT-L	C-L	T-L
AS-06	0.0022	0.57	0.032
AS-29	0.014	3.6	1.5
Bortezomib	0.0094	0.26	2.4

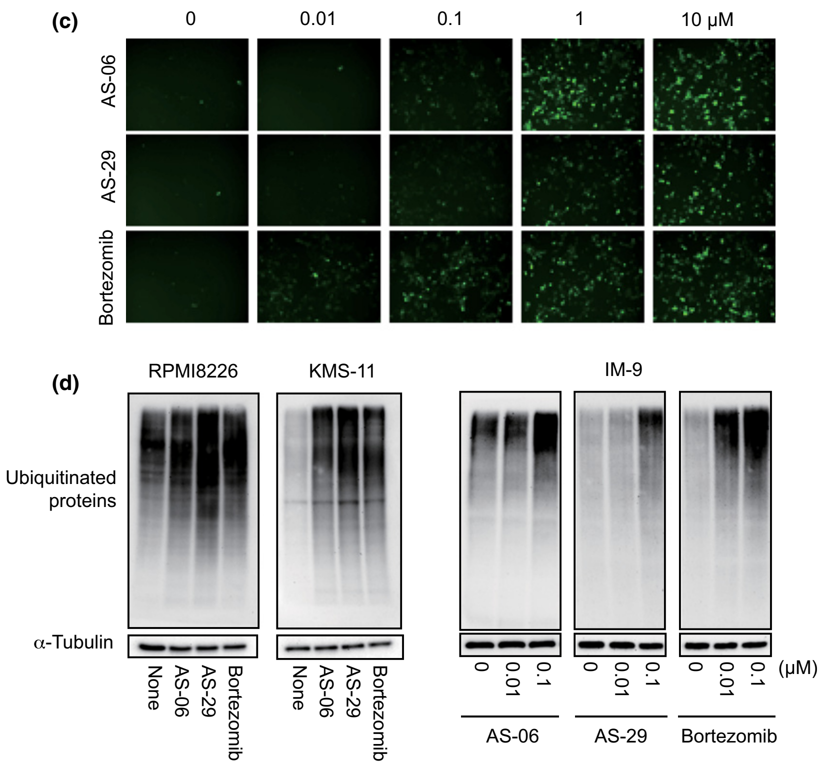


Fig. 1. Inhibition of the proteasome by tyropeptin-boronic acid derivatives. (a) Structures of tyropeptin-boronic acid derivatives. (b) Proteasome inhibitory activity *in vitro*. Proteasome activities were determined by the Proteasome-Glo Assay System using purified human erythrocyte-derived 20S proteasome. (c) Accumulation of proteasome-sensitive fluorescent proteins. HEK293PS cells were incubated with inhibitors for 18 h, and fluorescent protein levels were monitored by fluorescence microscopy. (d) Accumulation of ubiquitinated proteins in human multiple myeloma cells. RPMI8226, KMS-11 and IM-9 cells were incubated with inhibitors for 6 or 24 h, and ubiquitinated proteins were detected by western blotting.

from Cell Signaling Technology (Danvers, MA, USA); anti-mono- and polyubiquitinylated proteins (FK2) from Enzo Life Sciences (Farmingdale, NY, USA); and anti-α-tubulin (T5168) from Sigma-Aldrich (St Louis, MO, USA).

Cell lines and culture conditions. The human multiple myeloma RPMI8226 cells line was obtained from the American Type Culture Collection (Rockville, MD, USA). Human multiple myeloma IM-9 and KMS-11 cells were obtained from the Japan Health Sciences Foundation (Osaka, Japan). These cell lines were grown in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% FBS (Nichirei Biosciences, Tokyo, Japan), 100 U/mL of penicillin G, and 100 μg/mL of streptomycin at 37°C with 5% CO_2. The HEK293 ZsGreen Proteasome Sensor Cell Line (HEK293PS), a stably transfected human embryo kidney cell line expressing the ZsProSensor-1 fusion protein, was obtained from Takara Bio (Tokyo, Japan). HEK293PS was grown in DMEM (Nissui) supplemented with 10% FBS, 100 000 U/L penicillin G, and 100 mg/L streptomycin at 37°C with 5% CO_2. The G418 antibiotic reagent was added to the culture medium at a concentration of 0.2 mg/mL to select for stably transfected cells.

Proteasome activity. Proteasome activities were determined by the Proteasome-Glo Assay System (Promega) using purified human erythrocyte-derived 20S proteasome (Enzo Life Sciences).

Fluorescence microscopy. HEK293PS cells (1×10^5) grown for 24 h in 35-mm dishes were incubated for 18 h. Fluorescent proteins were monitored with a LEITZ-BMRM fluorescence microscope (Leica, Heidelberg, Germany) using the FITC filter.

Western blotting. Equal protein amounts were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies against ubiquitinated proteins, IκB-α, phospho-IκB-α, α-tubulin, p65, nucleolin, caspase-3, -8, -9, -12 and PARP for 1 h at room temperature. Primary antibodies were detected using either an anti-mouse or anti-rabbit HRP-linked sheep secondary antibody (GE Healthcare, Piscataway, NJ, USA). The blots were developed with ECL reagent according to the manufacturer's instructions (GE healthcare).

Accumulation of ubiquitinated proteins. RPMI8226, KMS-11 and IM-9 cells (5×10^5) were incubated for 6 or 24 h. The cells were washed twice with ice-cold PBS containing 100 μM Na_3VO_4 and then lysed in a lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM Na_3VO_4, and 25 μg/mL each of antipain, leupeptin and pepstatin. The lysates were centrifuged at 20 000 *g* for 10 min at 4°C. Ubiquitinated proteins in supernatants were detected by western blotting.

NF-κB activation. RPMI8226 cells (1×10^6) were preincubated with inhibitors for 2.5 h and further incubated with 10 ng/mL TNF-α (R&D Systems, Minneapolis, MN, USA) for 25 min. Cytosolic and nuclear fractions were prepared using the cytosol/nuclear fractionation kit (Biovision, Mountain View, CA, USA). Equal protein amounts of fractions were analyzed by western blotting. The DNA-binding activity of NF-κB p65 was measured using a TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

Flow cytometric analysis. RPMI8226 cells (5×10^5) were incubated with 1-μM inhibitors for 22 h. The cells were treated with annexin V-FITC and propidium iodide according to an annexin V-FITC apoptosis detection kit (Biovision) and analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Caspase activation. RPMI8226 cells (5×10^5) were incubated with 0.1 μM inhibitors, and caspase activation was detected by western blotting. To determine caspase-3 activity, RPMI8226 cells (1×10^4/well) were incubated in 96-well plates with inhibitors for 16 h. The caspase-3 activity was measured using the Caspase3/7-Glo Assay (Promega) according to the manufacturer's instructions.

Gene expression analysis. RPMI8226 cells (2×10^5) were incubated with 0.01, 0.1 and 1 μM inhibitors for 13 h. Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA). Fluorescent-labeled cRNA was generated using the Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) and hybridized to an oligonucleotide microarray (Human Whole Genome 4 × 44 K; Agilent Technologies). Fluorescent images of hybridized microarrays were obtained using an Agilent DNA Microarray Scanner (Agilent Technologies), which were then processed using Feature Extraction ver 9.5.3.1 software (Agilent Technologies). Gene expression data analysis was performed using the GeneSpring GX ver.12 software (Agilent Technologies).

***In vivo* imaging of proteasome inhibition.** Six-week-old, female BALB/c nude mice purchased from Charles River Japan (Yokohama, Japan) were inoculated with 1×10^7 HEK293PS cells in 50% Matrigel (BD Biosciences, San Jose, CA, USA) into the flank. Tyropeptin-boronic acid derrivatives AS-06 (8 mg/kg), AS-29 (8 mg/kg) and bortezomib (2 mg /kg) were administrated i.v. to mice bearing size-matched HEK293PS tumors. After 24 h, the tumors were monitored using the OV-110 *in vivo* imaging system (Olympus, Tokyo, Japan) using the GFP filter.

Intratumor proteasome activity. AS-06 (4 and 8 mg/kg), AS-29 (4 and 8 mg/kg) and bortezomib (1 and 2 mg/kg) were administrated i.v. to mice bearing size-matched RPMI8226 tumors, and the tumors were excised from mice at 24 h after administration. To measure proteasome activity in tumors, they were frozen and mechanically disrupted in a ShakeMaster Neo (Bio Medical Science, Tokyo, Japan) in lysis buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM DTT, 2 mM ATP and 20% glycerol. Tumor debris were removed by centrifugation at 90 000 *g* for 30 min. The supernatant (10 μL) was added to 96-well plates along with 90 μL of 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM DTT, 0.04% SDS and 100 μM Suc-LLVY-MCA. The reaction mixture was incubated for 30 min at 37°C. Proteasome activity was measured by monitoring the increase in fluorescence (excitation, 360 nm; emission, 460 nm) that accompanies the cleavage of 7-amino-4-methylcoumarin from Suc-LLVY-MCA using a fluorescence microplate reader (Powerscan HT; DS Pharma Biomedical, Osaka, Japan).

Mouse xenograft models. The mouse experiments were conducted in accordance with a code of practice established by the ethical committee of the Microbial Chemistry Research Foundation. Six-week-old female SCID mice were purchased from Charles River Japan and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. Tumor xenografts were established by subcutaneously injecting 2.0×10^7 RPMI8226 cells near the left lateral flank. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × width^2)/2. Tumors were allowed to grow to approximately 100 mm^3 before administration of inhibitors. AS-06 (4 mg/kg), AS-29 (4 mg/kg) and bortezomib (1 mg/kg) were administrated i.v. twice weekly for 4 weeks from day 11 or 13.

Statistical analysis. Representative examples are shown with similar results from several independent experiments. The data are expressed as the mean ± SD using descriptive statistics.

Results

Inhibition of the proteasome by tyropeptin-boronic acid derivatives. The inhibitory activities of tyropeptin-boronic acid derivatives AS-06 and AS-29 on human erythrocyte-derived 20S proteasome were examined by proteasome-Glo asssays. AS-06 inhibited the CT-L activity of the 20S proteasome with an IC_{50} of 0.0022 μM, while AS-29 inhibited this activity with an IC_{50} of 0.014 μM (Fig. 1b). To examine proteasome inhibition in living cells, we used stably transfected HEK293 cells (HEK293PS) that are continuously expressing the ZsProSensor-1 protein, a proteasome-sensitive fluorescent reporter.[28] The ZsProSensor-1 protein is a fusion of the green fluorescent protein ZsGreen and mouse ornithine decarboxylase, which can be degraded by the proteasome without being ubiquitinated.[29] In these cells, the fluorescent protein was undetectable by fluorescence microscopy because of rapid degradation by the proteasome under steady-state conditions, but detectable in the presence of proteasome inhibitors. AS-06 and AS-29 significantly induced the accumulation of fluorescent proteins in a dose-dependent manner. In addition, we examined the effect of AS-06 and AS-29 on ubiquitinated proteins, endogenous substrates of the proteasome (Fig. 1d). AS-06 and AS-29 clearly induced the accumulation of ubiquitinated proteins in human multiple myeloma RPMI8226, KMS-11 and IM-9 cells, respectively. The results show that tyropeptin-boronic acid derivatives inhibit the intracellular proteasome activities and increase the amount of ubiquitinated proteins in human multiple myeloma cells.

Inhibition of NF-κB activation by tyropeptin-boronic acid derivatives. The transcription factor NF-κB is involved in cell growth and confers a significant survival potential in a variety of tumors. Inhibition of NF-κB activation by proteasome inhibitors, such as bortezomib, is considered to be the major mechanism of action of antitumor activity. To evaluate whether tyropeptin-boronic acid derivatives inhibit NF-κB activation, we examined the effect of these derivatives on the degradation of the NF-κB inhibitor IκB-α and the nuclear translocation of NF-κB p65 subunits in RPMI8226 cells (Fig. 2a). The stimulation by TNF-α drastically decreases IκB-α levels and promotes the nuclear translocation of NF-

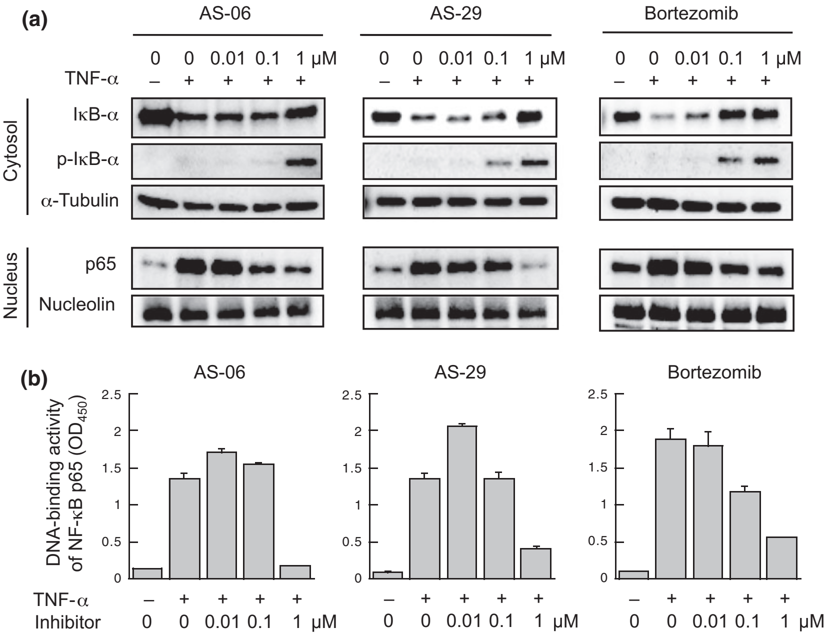


Fig. 2. Inhibition of NF-κB activation. (a) Inhibition of the degradation of IκB-α and nuclear translocation of NF-κB p65. RPMI8226 cells were preincubated with inhibitors for 2.5 h, and the cells were further incubated with 20 ng/mL TNF-α for 30 min. (b) Suppression of the DNA-binding activity of NF-κB. Columns, mean of triplicate determinations; bars, SD.

κB p65. AS-06 and AS-29 suppressed the decrease of IκB-α and increased IκB-α phosphorylation. Furthermore, AS-06 and AS-29 blocked the nuclear translocation of NF-κB p65 after TNF-α stimulation. To confirm the inhibition of NF-κB activation by the treatment of tyropeptin-boronic acid derivatives, we further examined whether AS-06 and AS-29 inhibit the DNA-binding activity of NF-κB p65 (Fig. 2b). The DNA-binding activity of NF-κB p65 was enhanced by TNF-α, but AS-06 and AS-29 repressed the DNA-binding activity of NF-κ B p65. Taken together, these results indicate that tyropeptin-boronic acid derivatives inhibit NF-κB activation by stabilizing IκB-α.

Induction of apoptosis by tyropeptin-boronic acid derivatives. We investigated whether tyropeptin-boronic acid derivatives induce apoptosis in RPMI8226 cells. Using annexin V and propidium iodide double staining and a flow cytometer (Fig. 3a), AS-06 and AS-29 significantly increased the number of late-apoptotic cells (annexin V-positive/propidium iodide-positive). We examined the effect of tyropeptin-boronic acid derivatives on the activation of caspases, a family of cysteine proteases playing a central role in apoptosis (Fig. 3b). AS-06 and AS-29 induced the degradation of full-length caspase-8 and full-length caspase-9, and cleaved fragments are detected. In addition, bortezomib was reported to activate ER-resident caspase-12 in multiple myeloma cells.[30] Although bortezomib induced the degradation of full-length caspase-12, neither AS-06 nor AS-29 induced the decrease of full-length caspase-12 (Fig. 3b). Caspase-3, a critical executioner of apoptosis, interacts with caspase-8 and caspase-9. AS-06 and AS-29 also stimulated the degradation of full-length caspase-3 and the appearance of cleaved forms, resulting in the cleavage of poly (ADP-ribose) polymerase (PARP), one of the main cleavage targets of caspase-3. In RPMI8226 cells, we found that AS-06 and AS-29 enhance caspase-3 activity in a dose-dependent manner (Fig. 3c). Furthermore, we performed gene expression analysis to compare the effects of tyropeptin-boronic acid derivatives and bortezomib on global gene transcription in RPMI8226 cells (Fig. 3d). We used whole human genome microarrays covering over 41 000 genes and transcripts. The transcription of 757, 744 and 2707 genes was altered over threefold in response to AS-06, AS-29 and bortezomib, respectively. The hierarchical clustering analysis for a total 2803 genes showed that the global gene expression signatures of AS-06 and AS-29 highly correlated with that of bortezomib. In summary, our data demonstrate that tyropeptin-boronic acid derivatives induce apoptosis through the caspase-8 and caspase-9 cascades, and that these derivatives and bortezomib have a similar effect on genome-wide transcriptional expression.

Antitumor activity of tyropeptin-boronic acid derivatives. To examine whether tyropeptin-boronic acid derivatives inhibit the proteasome in tumors, we used HEK293PS cells that are continuously expressing a proteasome-sensitive fluorescent protein. AS-06 and AS-29 were administrated at doses that were fourfold higher than bortezomib, because AS-06 and AS-29 had fourfold lower acute toxicity on mice than bortezomib. Intravenous administration of AS-29 to mice bearing HEK293PS tumors significantly induced strong fluorescence in tumors at 24 h after administration (Fig. 4a). Compared with AS-06, AS-29 inhibited more potently the proteasome activity in tumors (Fig. 4b). Furthermore, we assessed the *in vivo* antitumor activity of tyropeptin-boronic acid derivatives using xenograft models of human multiple myeloma RPMI8226 cells (Fig.4c). When administered i.v. twice weekly for 4 weeks, AS-06 moderately suppresses the growth of subcutaneous tumor of RPMI8226 xenograft, while AS-29 potently suppresses tumor growth. These results clearly show that tyropeptin-boronic acid derivatives inhibit the proteasome activity in tumors, and, in particular, AS-29 has potent antitumor activity.

Discussion

The successful development of bortezomib therapy for the treatment of multiple myeloma proves that proteasome inhibition is an attractive therapeutic strategy, but the prolonged treatment with bortezomib is associated with toxicity and development of drug resistance.[21] Recent studies have focused on the development of other proteasome inhibitors as therapeutics in cancer treatment. In 2012, carfilzomib, a novel

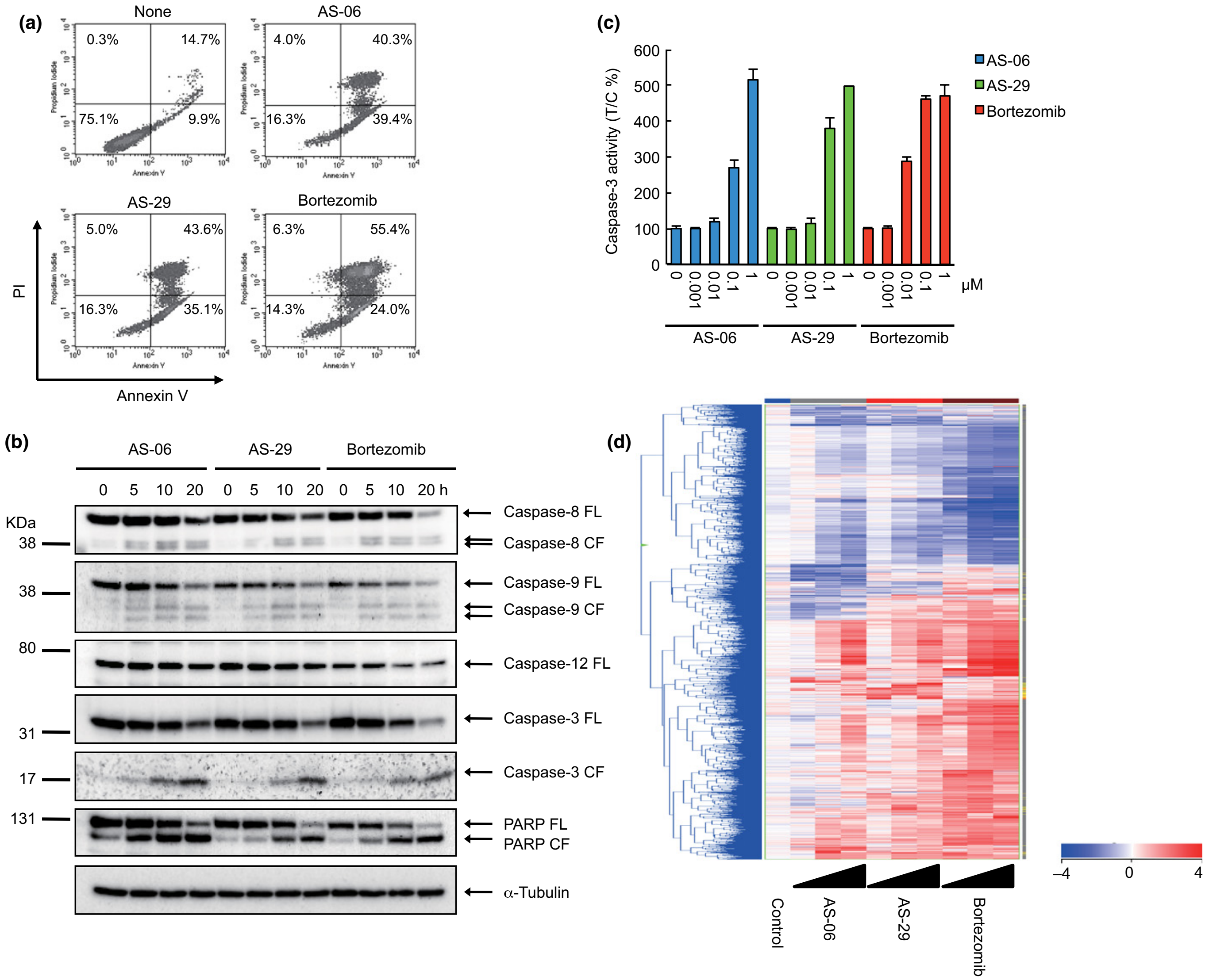


Fig. 3. Induction of apoptosis. (a) Detection of apoptotic cells. RPMI8226 cells were incubated with 1 μM inhibitors for 22 h. The cells were stained with an annexin V-FITC and propidium iodide and analyzed using a flow cytometer. (b) Induction of caspase activation. RPMI8226 cells were incubated with 0.1 μM inhibitors and caspase activation was detected by western blotting. (c) Induction of caspase-3 activity. RPMI8226 cells were incubated with inhibitors for 16 h and caspase-3 activity was measured using the caspase3/7-Glo assay. Columns, mean of triplicate determinations; bars, SD. (d) Gene expression analysis. RPMI8226 cells were incubated with 0.01, 0.1 and 1 μM inhibitors for 13 h. Gene expression analyses were performed using the Agilent human whole genome microarray.

second generation proteasome inhibitor, was approved for use in patients with relapsed/refractory multiple myeloma. In addition, several other proteasome inhibitors, such as ixazomib, oprozomib, marizomib and delanzomib, are currently in clinical trials.[31–38] Previously, we reported the isolation of novel proteasome inhibitors, tyropeptins, produced by *Kitasatospora* sp. MK993-dF2, and showed that tyropeptin-boronic acid derivatives exhibit a potent inhibitory activity against the CT-L activity of the human erythrocyte-derived 20S proteasome. In this study, we compare the potency to inhibit CT-L activity of two tyropeptin-boronic acid derivatives, AS-06 and AS-29, with bortezomib. Our results show that AS-29 and bortezomib have a similar potency, while AS-06 inhibited the CT-L activity more potently than bortezomib (Fig. 1b). Furthermore, we investigated the antitumor effect of these derivatives in detail. First, we examined the effects of these derivatives on the proteasome in cells. Both exogenous substrates (proteasome-sensitive reporter proteins) and endogenous substrates (ubiquitinated proteins) of the proteasome were markedly accumulated in cells treated with tyropeptin-boronic acid derivatives (Fig. 1c, d). These data show that the derivatives inhibit intracellular proteasome functions. Second, we identified the NF-κB pathway as a target of the inhibition. The proteasome degrades numerous regulatory proteins that are associated with tumor growth. For example, the degradation of IκB by the proteasome induces NF-κB activation, which is essential for the survival of cancer. In multiple myeloma, blocking of NF-κB activation by proteasome inhibitors such as bortezomib is considered to mediate therapeutic effects of bortezomib.[39,40] Both AS-06 and AS-29 inhibit the degradation of IκB-α and cause phosphorylated-IκB-α accumulation in multiple myeloma cells (Fig. 2a). In addition, these derivatives suppress the nuclear translocation of NF-κB p65 and the DNA-binding activity of NF-κB (Fig. 2a,b). These results indicate that tyropeptin-boronic acid derivatives inhibit NF-κB activation by blocking IκB-α degradation.

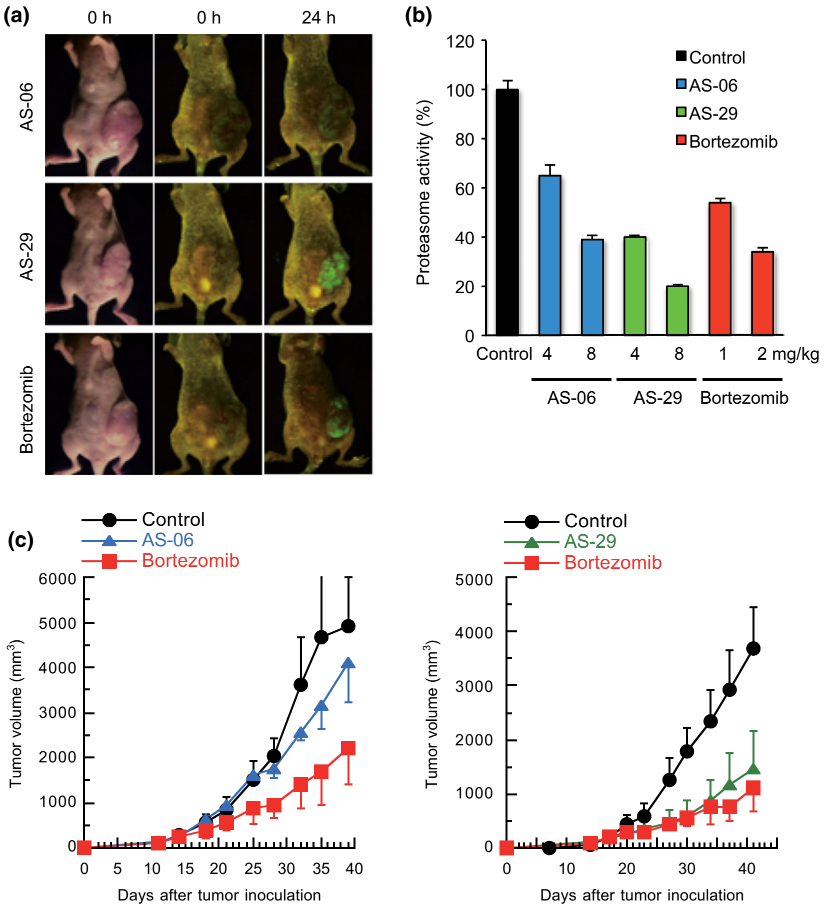


Fig. 4. Antitumor effect of tyropeptin-boronic acid derivatives. (a) *In vivo* imaging of proteasome inhibition. Inhibitors were administrated i.v. to mice bearing HEK293PS tumors. After 24 h, the tumors were monitored using the *in vivo* imaging system. (b) Inhibition of proteasome activity. Inhibitors were administered i.v. to mice bearing RPMI8226 tumors. Proteasome activity in tumor lysates was determined at 24 h after administration. (c) Antitumor activity on RPMI8226 xenografts. RPMI8226 cells were subcutaneously inoculated into SCID mice on day 0. Inhibitors were administrated i.v. twice weekly for 4 weeks from day 11 or 13.

Bortezomib-induced apoptosis is associated with activation of both extrinsic (caspase-8-mediated) and intrinsic (caspase-9-mediated) cell death signaling pathways.[30,41] AS-06 and AS-29, like bortezomib, induce apoptosis in multiple myeloma cells and activate both cell death signaling pathways (Fig. 3a–c). Thus, our data show that tyropeptin-boronic acid derivatives trigger both mitochondria-dependent and mitochondria-independent signaling pathways.

Bioactive compounds that interfere with cellular biological process influence specific signaling pathways and modulate the expression of individual subsets of signature genes. Compounds with similar mechanisms of action may induce similar gene expression profiles. Thus, genome-wide transcriptional expression analysis is a powerful strategy for characterizing the biological activity of bioactive compounds.[42–44] Genome-wide transcriptional expression analysis using hierarchical clustering showed a strong correlation in genome-wide gene expression signatures for AS-06 and AS-29, and bortezomib. These data suggest that tyropeptin-boronic acid derivatives and bortezomib have similar activities in multiple myeloma cells. However, the activities of tyropeptin-boronic acid derivatives were not identical to that of bortezomib, which were supported by the COMPARE analysis of de-dichloro-AS-29 derivative (AS-15) and bortezomib using a human cancer cell line panel JFCR39 (Peason correlation coefficient, 0.706) (Fig. S1).[45] In particular, AS-15 had a more potent cytotoxicity (LC50 value) against SF-539 cells than bortezomib. Tyropeptin-boronic acid derivatives might have possibilities of new antitumor agents against glioma cells like SF-539 cells. Therefore, tyropeptin-boronic acid derivatives are unique proteasome inhibitors that possess different mechanism to bortezomib.

Regarding the *in vivo* anticancer activities of tyropeptin-boronic acid derivatives, AS-29 was more potent than AS-06 in inhibiting the proteasome activity in tumors and more potently suppressed the tumor growth in xenograft models of human multiple myeloma RPMI8226 cells. Although AS-06 has a more potent inhibitory activity against the proteasome than bortezomib *in vitro*, the antitumor activity of AS-06 *in vivo* is relatively weak. This discrepancy between a potent inhibitory activity *in vitro* and a relatively weak antitumor effect may be ascribed to the low penetration of AS-06 in tumors. In contrast, AS-29 strongly inhibits the tumor growth and intratumor proteasome activity. Therefore, AS-29 could be a lead compound for the development of novel next generation antimultiple myeloma agents.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C, 23510270) from The Ministry of Education, Culture, Sports, Science and Technology in Japan. We thank Ms S. Kakuda for technical assistance and Dr T. Someno and Dr M. Kawada for helpful advice. We are also grateful to the Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan for supplying the measurement of growth inhibitory activities on 39 human cancer cell lines.

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. Growth inhibitory activity of AS-15 and bortezomib against 39 human cancer cell lines in the JFCR39 panel.