A New Proteasome Inhibitor, TP-110, Induces Apoptosis in Human Prostate Cancer PC-3 Cells

Isao Momose,¹ Masatomi Iijima, Manabu Kawada, and Daishiro Ikeda

Numazu Bio-Medical Research Institute, Microbial Chemistry Research Center, 18-24 Miyamoto, Numazu, Shizuoka 410-0301, Japan

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Proteasome inhibitors are useful in the treatment of cancer. Recently, we found a new proteasome inhibitor, TP-110, derived from tyropeptin A produced by Kitasatospora sp. Here we report that TP-110 induces apoptosis in human prostate cancer PC-3 cells. TP-110 showed strong cytotoxicity to PC-3 cells (IC₅₀ = 0.05 μM). It increased the number of cells in the G₂-M phase and increased the accumulated amounts of the p21 and p27 proteins, which are negative regulators of cell cycle progression. Furthermore, it induced apoptosis along with chromatin condensation and DNA fragmentation in PC-3 cells, and TP-110-induced apoptosis appeared to be associated with caspase activation. Additionally, TP-110 inhibited not only the degradation of IκB and the nuclear translocation of nuclear factor-κB (NF-κB), but also the DNA binding activity of NF-κB. These results indicate that TP-110 shows a strong growth inhibition and apoptosis in PC-3 cells.

Key words: proteasome inhibitor; apoptosis; TP-110

Materials and Methods

Antibodies. The antibodies used in Western blots were as follows: anti-p21 (sc-397), anti-p27 (sc-528), anti-Bax (sc-493), anti-Bcl-2 (sc-492), anti-C23 (sc-13057), anti-NF-κB p65 (sc-109) and anti-NF-κB p50 (sc-8414), from Santa Cruz Biotechnology (Santa Cruz, CA); anti-α-tubulin (T5168) from Sigma-Aldrich (St. Louis, MO); anti-XIAP (AF822) from R&D Systems (Minneapolis, MN); anti-Bcl-X (B22630) from Transduction Laboratories (Lexington, KY); and anti-poly (ADP-ribose) polymerase (PARP) (611038) from BD Biosciences (San Jose, CA).

Cells. Human prostate cancer PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA), 100 U/ml of penicillin G, and 100 μg/ml of streptomycin at 37°C with 5% CO₂.

Cell growth. The PC-3 cells were cultured in 96-well plates at 5,000 cells/well with a test sample for various times. Cell growth was determined by the 3-(4,5-
dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method.\textsuperscript{15)}

**Preparation of cell lystate and Western blotting.** PC-3 cells (2 × 10\(^5\)) were cultured in 35-mm dishes with or without TP-110 or MG132 for various durations. The cells were washed twice with ice-cold phosphate-buffered saline containing 100µM Na\(_2\)VO\(_3\) and then lysed in a lysis buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM Na\(_2\)VO\(_3\), pH 7.5, and 25µg/ml each of antipain, leupeptin, and pepstatin). Equal protein extracts were separated by SDS–polyacrylamide gel electrophoresis, transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) and Western-blotted with anti-p21, anti-bcl-x\(_l\), anti-XIAP, anti-Bax, anti-Bcl-2, anti-Bcl-X\(_l\), anti-PIPA, anti-p22, or anti-tubulin antibodies. Horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG antibodies were used as the secondary antibodies (GE Healthcare, Philadelphia, PA) and detected by ECL reagent according to the manufacturer’s instructions (GE Healthcare).

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis.** The PC-3 cells (2 × 10\(^5\)) were cultured in 35-mm dishes with or without the indicated concentrations of TP-110 for 24 h, and then total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using AMV reverse transcriptase (Promega, Madison, WI) with an equal quantity of total RNA, and amplified using Taq DNA polymerase (Promega). Specific primers of p21, p27, anti-p22, anti-p23, anti-Bax, anti-Bcl-2, anti-Bcl-X\(_l\), anti-PIPA, anti-p27, anti-PIPA, or anti-tubulin antibodies. Horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG antibodies were used as the secondary antibodies (GE Healthcare, Piscataway, NJ). The blots were developed with ECL reagent according to the manufacturer’s instructions (GE Healthcare).

**DNA fragmentation.** PC-3 cells (5 × 10\(^5\)) were cultured in 10-cm dishes with TP-110 for 14 or 24 h, and then the cells were washed with phosphate-buffered saline and lysed in lysis buffer containing 0.5% Triton X-100, 10 mM Tris–HCl (pH 7.4), and 10 mM EDTA at room temperature for 10 min. The supernatant fractions, collected by centrifugation at 15,000 rpm for 10 min, were treated with RNase A at 37°C for 1 h and then treated with Proteinase K (Invitrogen, Carlsbad, CA). The DNA in these fractions was precipitated overnight with NaCl and 2-isopropanol at −20°C. The DNA was dissolved in 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA buffer, and then separated on a 1.2% agarose gel.

**Cell cycle analysis.** PC-3 cells (5 × 10\(^5\)) were cultured in 10-cm dishes with or without 0.16µM TP-110 for 24 h. The harvested cells were fixed with ice-cold 70% ethanol. The fixed cells were then treated with 0.1% RNase A (Sigma-Aldrich) at 37°C for 15 min, and resuspended in phosphate-buffered saline containing 50µg/ml propidium iodide (Sigma-Aldrich). DNA fluorescence was measured using a flow cytometer (FACSCalibur, BD Biosciences).

**Hoechst 33342 staining.** The PC-3 cells (1 × 10\(^5\)) were cultured in 35-mm dishes with or without 0.16µM TP-110 for 24 h and then stained with 5µg/ml Hoechst 33342 for 90 min. The nuclear morphology of the cells was visualized using a fluorescence microscope (Leica DM, IRB, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

**NF-κB activation.** The PC-3 cells (2 × 10\(^5\)) were cultured in 35-mm dishes with 1.6µM TP-110 for 2.5 h before stimulation with 20 ng/ml tumor necrosis factor-α (TNF-α) (R&D Systems). Cytosolic and nuclear fractions were prepared using the cytosol/nuclear fractionation kit (BioVision, Mountain View, CA). Equal protein extracts were analyzed by Western blotting. The DNA-binding activity of NF-κB was quantified using the TransAM NF-κB p65 transcription factor assay kit (Active Motif North America, Carlsbad, CA) according to the manufacturer’s instructions.

**Statistical analysis.** All data are representative of three independent experiments with similar results. The statistical data are expressed as mean ± SD using descriptive statistics.

**Results**

**TP-110 inhibits PC-3 cell growth**

TP-110 is a new proteasome inhibitor derived from tyropeptin A isolated from actinomycete (Fig. 1A). In particular, TP-110 selectively inhibited the chymotrypsin-like activity of 20S proteasome and prevented the growth of various cancer cell lines (13). The chymotrypsin-like activity of proteasome in human prostate cancer PC-3 was inhibited 56% by 0.1 µg/ml of TP-110, but it is unclear whether TP-110 induces apoptosis in the cancer cells. Hence we examined the effect of TP-110 on the growth of human prostate cancer PC-3 cells (Fig. 1B). The PC-3 cells were grown with different concentrations of TP-110 for 1 d after TP-110 treatment. On day 2, high concentrations of TP-110 (0.1 and 0.4 µM) decreased the cell density. A medium concentration of 0.16µM TP-110 inhibited 56% by 0.1 µg/ml of TP-110, but it is unclear whether TP-110 induces apoptosis in the cancer cells. Hence we examined the effect of TP-110 on the growth of human prostate cancer PC-3 cells (Fig. 1B). The PC-3 cells were grown with different concentrations of TP-110 for 1 d after TP-110 treatment. On day 2, high concentrations of TP-110 (0.1 and 0.4 µM) decreased the cell density. A medium concentration of 0.16µM TP-110 maintained the starting density of the cells and a low concentration of 0.0063µM did not affect cell growth. The effect of TP-110 on the growth of PC-3 cells was similar to that of MG-132. Next we evaluated the effect of TP-110 on cell viability cultured for 72 h using MTT methods (Fig. 1C). TP-110 strongly inhibi-
edited the growth of the PC-3 cells, and the 50% growth inhibitory concentration (IC$_{50}$) was determined to be 0.05 µM.

Numerous proteins, including the cyclin-dependent kinase inhibitors p21 and p27, control the cell cycle progression. To examine the potent mechanism of TP-110-induced cell growth inhibition, we evaluated the effect of TP-110 on the protein levels of p21 and p27 in PC-3 cells (Fig. 2A). Accumulation of the p21 and p27 proteins were detected at 8 h after 0.16 µM TP-110 treatment. Next we examined the effect of the various concentrations of TP-110 (Fig. 2B). Although only a trace of p21 protein was detected in the untreated cells, treatment with 0.16 µM TP-110 significantly increased p21 protein at 12 h. p27 protein also increased with 0.16 µM TP-110. Expression of p21 and p27 on the mRNA levels was detected by RT-PCR (Fig. 2C). The expression of p21 was noticeably increased with 0.16 µM TP-110, but p27 did not increase even with 1.6 µM TP-110. Next we evaluated the effect of TP-110 on the cell cycle progression (Fig. 2D). The ability of TP-110 to inhibit cell cycle progression was determined by a combination of propidium iodide staining and the flow cytometer analysis. Treatment of the PC-3 cells with 0.16 µM TP-110 for 12 h resulted in an accumulation of cells in the G$_{2}$-M phase along with a decrease in the number of cells in the G$_{1}$ phase. Taken together, these results clearly indicate that TP-110 affects proteins that control cell cycle progression, and inhibits cell cycle progression and cell growth.

**TP-110 induces apoptosis in PC-3 cells**

To investigate whether TP-110 induces apoptosis in PC-3 cells, we examined the apoptotic morphological changes in the PC-3 cells with TP-110. The PC-3 cells were cultured with 0.16 µM TP-110 for 24 h and then stained with Hoechst 33342 (Fig. 3A). TP-110 induced chromatin condensation, which is characteristic of apoptotic cells. To confirm this result, we performed agarose gel electrophoresis of the genomic DNA purified from PC-3 cells cultured with the indicated concentrations of TP-110 for 14 and 24 h (Fig. 3B). TP-110 caused remarkable DNA fragmentation at 0.16 µM TP-110 during a 24 h treatment. To investigate the possible involvement of caspase activation in TP-110-induced apoptosis, we examined the effect of the pan-caspase inhibitor Z-VAD-FMK on apoptosis induction by TP-110 (Fig. 3C). The TP-110-induced DNA frag-
mentations were dose-dependently inhibited by Z-VAD-FMK. Next we evaluated the effect of TP-110 on the protein levels of several apoptosis-related molecules (Fig. 3D). TP-110 decreased the anti-apoptotic proteins Bcl-2 and XIAP, but the anti-apoptotic protein Bcl-XL did not change. In contrast, the pro-apoptotic protein Bax and the cleaved form of PARP increased. Taken together, these results indicate that TP-110 induces apoptosis in PC-3 cells, and that TP-110-induced apoptosis is dependent on the caspase activation.

**TP-110 inhibits TNF-α-stimulated activation of NF-κB in PC-3 cells**

The transcription factor NF-κB is involved in cell growth and confers a significant survival potential in a variety of tumors. Proteasome inhibitors, such as bortezomib, are known to inhibit NF-κB activity as one of their diverse actions. To evaluate whether TP-110 inhibits the TNF-α-stimulated activation of NF-κB, we examined the effect of TP-110 on the degradation of the NF-κB inhibitor IκB-α in TNF-α-treated PC-3 cells (Fig. 4A). IκB-α decreased drastically after stimulation of TNF-α in PC-3 cells, but not in TP-110-treated PC-3 cells. Next we examined the effect of TP-110 on the nuclear translocation of the NF-κB p50 and p65 subunits (Fig. 4A). Although TNF-α induced nuclear translocation of the NF-κB p50 and p65 subunits, TP-110 blocked these responses. To confirm the inhibition of NF-κB activation by TP-110 treatment, we further examined whether TP-110 inhibits the DNA-binding activity of NF-κB in PC-3 cells (Fig. 4B). The DNA-binding activity of NF-κB was enhanced by TNF-α, but TP-110 inhibited the DNA-binding activity of NF-κB induced by TNF-α. Taken together, these results indicate that TP-110 inhibits NF-κB activation in PC-3 cells by stabilizing IκB-α.

**Discussion**

The successful development of bortezomib therapy for the treatment of relapsed/refractory multiple myeloma has proved that proteasome inhibition is an
**Fig. 3.** Induction of Apoptosis by TP-110 in PC-3 Cells.

A. Chromatin condensation by TP-110 treatment. PC-3 cells were cultured with 0.16 μM TP-110 for 24 h, and then the cells were stained with Hoechst 33342. The arrows indicate condensed chromatin. B. DNA fragmentation due to TP-110 treatment. PC-3 cells were cultured with the indicated concentrations of TP-110 or MG132 for 14 or 24 h. Fragmented DNA was isolated and electrophoresed. C. The pan-caspase inhibitor inhibited TP-110-induced DNA fragmentation. PC-3 cells were cultured with 0.16 μM TP-110 and/or the indicated concentrations of the pan-caspase inhibitor Z-VAD-FMK for 24 h. D. The effect of TP-110 on apoptosis-related molecules. PC-3 cells were cultured with 0.16 μM TP-110 for the indicated times. The protein extracts were applied to Western blot analysis.

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**Fig. 4.** TP-110 Inhibited TNF-α-Activated NF-κB in PC-3 Cells.

A. TP-110 inhibited TNF-α-stimulated degradation of IκB and nuclear translocation of the NF-κB p50 and p65 subunits. PC-3 cells were precultured with and without 1.6 μM TP-110 for 2.5 h. The cells were further cultured with 20 ng/ml TNF-α for the indicated times. The cytosolic and nuclear fractions were prepared using a Cytosol/Nuclear Fractionation kit. The protein extracts were applied to Western blot analysis. B. TP-110 inhibited the TNF-α-stimulated DNA-binding activity of NF-κB. The DNA-binding activity of NF-κB was quantified using a TransAM NF-κB p65 Transcription Factor Assay kit. Columns, mean of triplicate determinations; bars, SD.
attractive therapeutic strategy, but the prolonged treatment with bortezomib is associated with toxicity and the development of drug resistance. Recent studies have focused on the development of other proteasome inhibitors as therapeutics in cancer treatment. We synthesized a new proteasome inhibitor, TP-110, derived from tyropeptin A produced by Kitasatospora sp. MK993-dF2.

First we investigated the effects of TP-110 on the growth of human prostate cancer PC-3 cells (Fig. 1). The PC-3 cells grow with any concentration of TP-110 until 1 d after TP-110 treatment. On day 2, high concentrations of TP-110 were found to decrease cell density. In other words, the cells might make the decision of life or death after the treatment with TP-110 within 1 d. This is supported by the data that 8 h or more were needed to accumulate p21 and p27 proteins under treatment with TP-110 (Fig. 2).

It is known that proteasome degrades p21 and p27. The proteasome inhibitor TP-110 was found to inhibit the degradation of p21 and p27 in a time- and dose-dependent manner (Fig. 2). When de novo synthesis of the p21 and p27 proteins was inhibited by the protein synthesis inhibitor cycloheximide, the p21 and p27 proteins gradually decreased, but TP-110 suppressed this decrease in the p21 and p27 proteins under cycloheximide treatment (data not shown). Thus it is conceivable that the increase in the p21 and p27 proteins was due to inhibition of the proteasomal degradation of the p21 and p27 proteins by TP-110. But the expression of p21 mRNA was increased by TP-110. Therefore, the increase in p21 protein was due to both inhibition of the proteasomal degradation of p21 protein and to an increase in p21 expression.

Next we evaluated the effect of TP-110 on the cell cycle progression, and found that TP-110 induced an accumulation of cells in the G2-M phase (Fig. 2). This effect was supported by increased p21 and p27 protein levels, because increased p21 and p27 proteins, in addition to blocking the G1-S transition, also block the G2-M transition leading to an accumulation of cells in the G2-M phase. These results clearly indicate that TP-110 affects the proteins that control the cell cycle progression. The expression of p21 mRNA was generally regulated by the p53 protein. However, p53 is null in PC-3 cells, and hence p53 is not required for the TP-110-induced increase in p21 expression in PC-3 cells. Although the mechanism of TP-110-induced p21 expression is not clear, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor lovastatin can induce p53-independent transcriptional up-regulation of p21 in human prostate cancer PC-3-M cells. TP-110 might induce p53-independent regulation of p21 as lovastatin does.

Inhibiting the degradation of the key proteins in cell-cycle regulation causes a disparity in the proliferative signals and eventually leads to apoptosis. Bortezomib and MG132 can arrest cell growth and induce apoptosis. Hence we investigated whether TP-110 can induce apoptosis. As expected, TP-110 induced apoptotic morphological changes with chromatin condensation and caused DNA fragmentation (Fig. 3). Since the TP-110-induced DNA fragmentations were inhibited by the pan-caspase inhibitor, TP-110-induced apoptosis was assumed to be dependent on the caspase pathway. Moreover, TP-110 evoked the cleaved form of the caspase-3 substrate PARP, suggesting caspase-3 activation by TP-110 treatment.

Proteasome inhibitors can overcome NF-κB activation by inhibiting the degradation of IκB. A major rationale for the therapeutic use of bortezomib is its ability to inhibit NF-κB activation. Hence we investigated whether TP-110 inhibits NF-κB activation (Fig. 4). TP-110 inhibited not only the degradation of IκB and the nuclear translocation of the p50 and p65 subunits of NF-κB, but also the DNA-binding activity of NF-κB after TNF-α stimulation, but inhibition of the DNA binding activity of NF-κB by TP-110 was less effective than that of nuclear translocation of NF-κB by TP-110. Phosphorylation of NF-κB p65 stimulates transcriptional activity by promoting an interaction with the coactivator CBP/p300. The discrepancy between the degrees of inhibition of nuclear translocation and DNA binding activity of NF-κB can be explained by phosphorylation of NF-κB p65 and co-activator CBP/p300. These results indicate that TP-110 inhibits NF-κB activation in PC-3 cells by stabilizing IκB-α.

Recently, a novel proteasome inhibitor, NPI-0052, was isolated from a new marine actinomycete. It is distinct from bortezomib in its chemical structure and mode of action. NPI-0052 was found to inhibit multiple myeloma cell growth in vivo and to prolong survival in a murine model. Thus proteasome inhibitors are promising in the therapeutics of cancer. In this study, we found that the proteasome inhibitor TP-110 shows not only cell-growth inhibition, but also induction of apoptosis in PC-3 cells. TP-110 is distinct from bortezomib and MG132 in its chemical structure, and hence it is an attractive lead compound in the treatment of cancer. However, TP-110 unfortunately had only a weak anti-tumor activity in vivo (data not shown). The blood concentration of TP-110 rapidly decreased, to half of the initial concentration, within 45 min of intravenous injection. Therefore, TP-110 has poor metabolic stability, and this might limit its anti-tumor activity in vivo. We intend to explore tumor cells significantly more sensitive to TP-110 and synthesize more effective and stable TP-110 derivatives.

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References


