HIV-1 protease inhibitor induces growth arrest and apoptosis of human multiple myeloma cells via inactivation of signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2

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Abstract
We previously showed that HIV-1 protease inhibitors slowed the proliferation of human myeloid leukemia cells and enhanced their differentiation in the presence of all-trans retinoic acid (ATRA). In this study, we found that protease inhibitors, including ritonavir, saquinavir, and nelfinavir, but not indinavir, induced growth arrest and apoptosis of U266, RPMI8226, and ARH77 human multiple myeloma (MM) cells in association with down-regulation of anti-apoptotic protein Mcl-1. Also, protease inhibitors inhibited the survival of freshly isolated MM cells from patients. In contrast, these protease inhibitors did not affect survival of normal B cells and colony formation of myeloid committed stem cells (CFU-GM) from healthy volunteers. In addition, we found that all of the protease inhibitors, except for indinavir, blocked interleukin-6 (IL-6)-stimulated phosphorylation of both signal transducer and activator of transcription 3 (STAT 3) and extracellular signal-regulated kinase 1/2 in U266 and RPMI8226 MM cells. Moreover, the protease inhibitors inhibited both the basal and IL-6-stimulated STAT 3/DNA binding activity in U266 cells as measured by an ELISA-based assay. Furthermore, ritonavir inhibited production of vascular endothelial growth factor, one of the targets of STAT 3, in U266 and RPMI8226 cells as measured by ELISA. Taken together, protease inhibitors might be useful for treatment of individuals with MM. [Mol Cancer Ther. 2004;3(4):473–479]

Introduction
Multiple myeloma (MM) is characterized by the clonal proliferation of malignant plasma cells in the bone marrow associated with bone loss, renal disease, and immunodeficiency (1). Despite recent new insights into the pathogenesis of MM, it remains incurable with a median survival of approximately 3–4 years with conventional chemotherapies and 4–5 years with high-dose chemotherapy followed by autologous stem-cell transplantation which is limited to selected non-elderly patients (1). Therefore, novel treatment strategies for MM are urgently needed.

Interleukin-6 (IL-6) is a pleiotropic cytokine that stimulates the proliferation of MM cells in either an autocrine or paracrine fashion and acts as an anti-apoptotic factor in these cells (2). Its intracellular signaling is mediated by two pathways. One is through the IL-6 receptor and gp-130, which together signal through the Janus family tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) proteins. The second is through the Ras/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. MM cells often have enhanced expression of IL-6 resulting in activation of both their JAK/STAT and their Ras signal pathways (3). Thus, these pathways might be potential therapeutic targets in MM.

Bcl-2 family members, including Bcl-2, Bcl-xL, and Mcl-1, contribute to the survival and drug resistance of MM cells (4). Recent studies showed that among Bcl-2 family members, Mcl-1 plays a critical role in survival of MM cells; Mcl-1, but not Bcl-2 and Bcl-xL, antisense oligonucleotides induced apoptosis of MM cells (5, 6). In addition, the cyclin-dependent kinase inhibitor flavopiridol induces apoptosis of MM cells in conjunction with down-regulation of Mcl-1 (7). Flavopiridol did not modulate levels of other Bcl-2 family members in these cells (7). More recently, Mcl-1 has been shown to be regulated by both STAT 3-dependent and -independent manners in MM cells (8). These observations suggest that Mcl-1 might be a promising molecular target in MM.

HIV-1 protease inhibitors have become important tools in the treatment of HIV infection; these include saquinavir mesylate, ritonavir, nelfinavir mesylate, and indinavir sulfate. Recent studies showed that protease inhibitors possess antitumor activity which is independent from their ability to inhibit HIV protease: we found that saquinavir, ritonavir, and indinavir induced growth arrest and differentiation of NB4 and HL-60 human myeloid leukemia cells, and enhanced the ability of all-trans retinoic acid (ATRA) to decrease proliferation and increase differentiation of these cells (9). Other investigators have shown that protease inhibitors can decrease proliferation of Kaposi sarcoma as well as prostate cancer cells via inhibition of nuclear factor-κB (NF-κB) activity (10–12).

In this study, we found that ritonavir, nelfinavir, and saquinavir, but not indinavir, induced growth arrest and
apoptosis of human MM cells in association with down-regulation of antiapoptotic protein Mcl-1 in these cells. These protease inhibitors, except for indinavir, blocked IL-6-induced activation of STAT 3 and ERK signaling in these myeloma cells.

Materials and Methods

Cell Lines

U266, RPMI8226, and ARH77 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies).

Isolation of Fresh MM and CD-19-Positive B Cells

Bone marrow samples were obtained from nine MM patients after their informed consent, according to institutional guidelines. Mononuclear cells were purified by Ficoll-Hypaque (Sigma, St. Louis, MO) density-gradient centrifugation. Myeloma cells were purified (>95% CD138+) by positive selection with anti-CD138 magnetic-activated cell separation microbeads (Miltenyi, San Diego, CA). CD138+ myeloma cells were cultured in RPMI 1640 medium, supplemented with 20% FCS and 20 ng/ml IL-6 (Kirin, Tokyo, Japan). CD-19 positive B cells from healthy volunteers were isolated by immunomagnetic selection technique according to the manufacturer’s instructions (Dynal Inc., Lake Success, NY).

Chemicals

Saquinavir mesylate (Roche, Branchburg, NJ), ritonavir (Abbott Labs, North Chicago, IL), indinavir sulfate (Merck, West Point, PA), and nelfinavir mesylate (Japan Tobacco Specification, Tokyo, Japan) were dissolved in 50% DMSO (Burdick & Jackson, Muskegon, MI) to a stock concentration of 10^{-2} M and stored at -80°C.

Trypan Blue Exclusion Test

MM cells (2 \times 10^5/ml) were incubated with various concentrations of protease inhibitors (1 \times 10^{-6} to 5 \times 10^{-5} M) for 4 days in 96-well plates (Flow Laboratories, Irvine, CA). Freshly isolated MM cells from patients and CD-19 positive B cells from healthy volunteers were cultured with either ritonavir (2 \times 10^{-5} M) or control diluent (0.5% DMSO) for 2 days. After culture, cell number and viability were evaluated by staining with trypan blue and counting using light microscopy.

Colony-Forming Assay

Resuspended mononuclear bone marrow cells from healthy volunteers (5 \times 10^5 cells/ml) were added 1:10 to methylcellulose medium H4534 (StemCell Technologies Inc., Vancouver, British Columbia, Canada) to yield a final concentration of 1% methylcellulose, 30% FCS, 1% BSA, 10^{-4} M mercaptoethanol, 2 \times 10^{-3} M l-glutamine, 50 ng/ml stem cell factor, 10 ng/ml GM-CSF, and 10 ng/ml IL-3. Cells were plated in six-well plates in a volume of 1 ml. Before this step, 2 \times 10^{-5} M or 5 \times 10^{-5} M protease inhibitors were pipetted into the wells. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 10 days later, colonies were counted. All experiments were done twice using duplicate plates per experimental point.

Assessment of Apoptosis

Apoptotic cell death was examined by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) method using the In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instruction. For quantification, three different fields were counted under the microscope and at least 300 cells were enumerated in each field. All experiments were performed twice.

Cell Cycle Analysis by Flow Cytometry

Cell cycle analysis was performed on U266 and RPMI8226 cells incubated for 24 h with ritonavir (2 \times 10^{-5} M). The cells were fixed in chilled methanol overnight before staining with 50 μg/ml propidium iodide, 1 mg/ml RNase (100 units/ml; Sigma), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using a FACScan (Becton Dickinson, Mountain View, CA).

Western Blot Analysis

Lysates were made by standard methods as previously described (13). Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 4–15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL), and probed sequentially with antibodies. Anti-poly-adenyl ribonucleic polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (Santa Cruz Biotechnology), anti-Mcl-1 (Santa Cruz Biotechnology), anti-STAT 3 (Santa Cruz Biotechnology), anti-ERK (Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology) antibodies were used. The band intensities were measured using densitometry.

Evaluation of DNA Binding Activity of STAT3 by ELISA

The DNA binding activity of STAT 3 was quantified by ELISA using the Trans-AM STAT Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA), according to the instructions of the manufacturer. Briefly, nuclear extracts were prepared as previously described and incubated in 96-well plates coated with immobilized oligonucleotides containing a consensus (5’-TTCCCGGAA-3’) binding site for STAT 3. STAT 3 binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of STAT 3 (Active Motif North America), visualized by anti-IgG horseradish peroxidase conjugate and Developing Solution, and quantified at 450 nm with a reference wavelength of 655 nm. Background binding was subtracted from the value obtained for binding to the consensus DNA sequence.

Measurement of Vascular Endothelial Growth Factor

Cells (5 \times 10^5/ml) were plated in six-well plates and cultured with either ritonavir (2 \times 10^{-5} M or 5 \times 10^{-5} M) or control diluent. After 24 h, culture medium was collected and the concentration of vascular endothelial growth factor (VEGF) was measured by ELISA kit (PharMingen, San Diego, CA).
Results

Effect of Protease Inhibitors on the Proliferation of MM Cells

Protease inhibitors were examined for their effects on proliferation of U266, RPMI8226, and ARH77 cells in a liquid culture system (Fig. 1, A–C). Ritonavir, nelfinavir, and saquinavir effectively inhibited the proliferation of U266 cells with an ED50 of approximately $2 \times 10^{-5}$ M as measured by trypan blue exclusion test on the 4th day of culture (Fig. 1A). On the other hand, indinavir did not affect the growth of U266 cells (Fig. 1A). Similarly, ritonavir, nelfinavir, and saquinavir, but not indinavir, effectively inhibited the growth of ARH77 and RPMI8226 cells with an ED50 of approximately $2 \times 10^{-5}$ and $1 \times 10^{-5}$ M, respectively (Fig. 1, B and C). In addition, we explored anti-MM effect of protease inhibitors on freshly isolated MM cells from patients (Table 1). In seven of nine cases of MM (78%), ritonavir ($2 \times 10^{-5}$ M, 2 days) reduced the survival of MM cells by more than 30%. Of note, all cases except for patient #1 and 2 relapsed after conventional chemotherapy, and MM cells from five cases (Pt. # 3–7) showed sensitivity to ritonavir. Moreover, conventional chemotherapy, and MM cells from five cases of MM (78%), ritonavir ($2 \times 10^{-5}$ M, 24 h) down-regulated levels of Mcl-1 in U266 and RPMI8226 cells (Fig. 2C); however, ritonavir did not modulate level of Bcl-2 protein in these cells (data not shown). Furthermore, exposure of U266 cells to ritonavir (2 or $5 \times 10^{-5}$ M, 24 h) cleaved PARP which is a target of caspases in a dose-dependent manner (Fig. 2D).

Effect of Protease Inhibitors on Cell Cycle and Apoptosis of MM Cells

The analysis of cell cycle of U266 and RPMI8226 cells after exposure to ritonavir ($2 \times 10^{-5}$ M, 24 h) was determined (Table 2). The appearance of cells with a fractional DNA content, a feature characteristic of apoptosis, was prominent in these cells after their exposure to ritonavir (13.6 ± 2.6% in U266 cells, 16.0 ± 2.3% in RPMI8226 cells). The accumulation of cells in the G2-M phase of the cell cycle occurred with a concomitant decrease in the proportion of cells in S phase after culture with ritonavir (Table 2). Further experiments employing TUNEL showed that ritonavir, nelfinavir, and saquinavir caused apoptosis of U266 and RPMI8226 cells in a time-dependent manner (Fig. 2, A and B). For example, after 24 h culture, $2 \times 10^{-5}$ M of ritonavir, saquinavir, and nelfinavir induced a mean 22 ± 3%, 25 ± 2%, and 21 ± 1% of U266 cells to become apoptotic, respectively, which increased to a mean of 33 ± 4%, 29 ± 3%, and 31 ± 3%, respectively, after another 24 h culture (Fig. 2A). The proapoptotic effect of ritonavir was explored by examining the level of Mcl-1 in MM cells after exposure to ritonavir (Fig. 2C). Ritonavir $5 \times 10^{-5}$ M, 24 h) down-regulated levels of Mcl-1 in U266 and RPMI8226 cells (Fig. 2C); however, ritonavir did not modulate level of Bcl-2 protein in these cells (data not shown). Further experiments employing TUNEL showed that ritonavir, nelfinavir, and saquinavir caused apoptosis of U266 and RPMI8226 cells in a time-dependent manner (Fig. 2, A and B). For example, after 24 h culture, $2 \times 10^{-5}$ M of ritonavir, saquinavir, and nelfinavir induced a mean 22 ± 3%, 25 ± 2%, and 21 ± 1% of U266 cells to become apoptotic, respectively, which increased to a mean of 33 ± 4%, 29 ± 3%, and 31 ± 3%, respectively, after another 24 h culture (Fig. 2A). The proapoptotic effect of ritonavir was explored by examining the level of Mcl-1 in MM cells after exposure to ritonavir (Fig. 2C). Ritonavir $5 \times 10^{-5}$ M, 24 h) down-regulated levels of Mcl-1 in U266 and RPMI8226 cells (Fig. 2C); however, ritonavir did not modulate level of Bcl-2 protein in these cells (data not shown). Furthermore, exposure of U266 cells to ritonavir (2 or $5 \times 10^{-5}$ M, 24 h) cleaved PARP which is a target of caspases in a dose-dependent manner (Fig. 2D).

Effect of Protease Inhibitors on Basal and IL-6-Stimulated STAT 3 in MM Cells

U266 cells were serum-starved for 24 h. These cells constitutively expressed the phosphorylated form of STAT 3 (Fig. 3A). Exposure of these cells to IL-6 (50 ng/ml, 30 min) increased the level of the phosphorylated form of STAT 3 by 10-fold, and pretreatment (3 h) of these cells with $2 \times 10^{-5}$ M of either ritonavir, saquinavir, or nelfinavir almost completely blocked the IL-6-induced phosphorylation of STAT 3 (Fig. 3A). In addition, ritonavir, saquinavir, and nelfinavir decreased basal level of the phosphorylated form of STAT 3 in U266 cells (Fig. 3A). On the other hand, indinavir, which did not inhibit the growth of the MM cells, failed to inhibit the IL-6-induced phosphorylation of STAT 3 in these cells. None of protease inhibitors modulated basal and IL-6-stimulated levels of total STAT 3 (Fig. 3A).

The effect of protease inhibitors on STAT 3 activity was further investigated by an ELISA-based assay. Control serum-starved U266 cells possessed strong STAT 3 DNA binding activity, and $2 \times 10^{-5}$ M of either ritonavir, saquinavir, or nelfinavir inhibited this activity by about 50%, 40%, and 50%, respectively (Fig. 3B). Addition of IL-6 (50 ng/ml, 30 min) to these cells further increased DNA binding activity (2.5-fold); and preincubation of these cells with $2 \times 10^{-5}$ M of ritonavir, saquinavir, and nelfinavir for

Figure 1. Effect of HIV-1 protease inhibitors (PIs) on the growth of multiple myeloma cells. U266 (A), ARH77 (B), and RPMI8226 (C) cells (10^5/ml) were cultured in the presence of various concentrations (1 × 10^{-6} to 5 × 10^{-5} M) of ritonavir, saquinavir, nelfinavir, and indinavir. On the fourth day of culture, cell numbers were counted by the trypan blue exclusion test. Results are expressed as a mean percentage of cell counts in control plates. Points, mean of three independent experiments done in triplicate plates; bars, SD.
3 h almost completely blocked IL-6-induced STAT 3 DNA binding activity (Fig. 3B). In contrast, indinavir did not affect either basal or IL-6-induced levels of STAT 3/DNA binding activity (Fig. 3B).

The effect of protease inhibitors on STAT 3 was also examined in other MM cells. Pretreatment of RPMI8226 cells with ritonavir (2 x 10^{-5} M, 3 h) completely blocked IL-6-induced phosphorylation of STAT 3 in these cells (Fig. 3C).

**Effect of Protease Inhibitors on Phosphorylation of ERK Mediated by IL-6 in U266 Myeloma Cells**

Levels of the phosphorylated form of ERK1/2 were negligible in control U266 cells which were serum-starved for 24 h. Addition of IL-6 (50 ng/ml, 30 min) to these cells induced phosphorylation of ERK; pretreatment of these cells with 2 x 10^{-5} M of either ritonavir, saquinavir, or nelfinavir for 3 h decreased expression of the IL-6-induced phosphorylated form of ERK1/2 by approximately 80%, 70%, and 10%, respectively (Fig. 4A). Again, indinavir did not affect the IL-6-induced levels of the phosphorylated form of ERK1/2 in these cells (Fig. 4A). The phosphorylated form of ERK1/2 was also induced by IL-6 in RPMI8226 cells; ritonavir completely blocked IL-6-induced phosphorylation of ERK in these cells (Fig. 4B).

**Effect of Protease Inhibitors on Production of VEGF by U266 and RPMI8226 Cells**

Previous studies showed that expression of VEGF is closely associated with marrow neoangiogenesis and correlates with tumor progression and poor prognosis in individuals with MM (14). In addition, recent studies have shown that STAT 3 regulates expression of VEGF (15). These observations prompted us to explore whether protease inhibitors decreased the production of VEGF by U266 and RPMI8226 cells; ritonavir completely blocked IL-6-induced phosphorylation of ERK in these cells (Fig. 4B).
MM cells. We measured the level of VEGF in culture medium of U266 cells treated with either ritonavir or control diluent. Control diluent cultured U266 cells secreted $2583 \pm 175$ pg/ml VEGF/$1 \times 10^5$ cells during a 24-h culture period (Fig. 5A). Exposure of these cells to ritonavir ($5 \times 10^{-5}$ M, 24 h) decreased the mean level of VEGF to $920 \pm 48$ pg/ml/$1 \times 10^5$ cells. Addition of IL-6 (50 ng/ml, 24 h) further increased the level of VEGF to $3541 \pm 69$ pg/ml/$1 \times 10^5$ cells, and ritonavir (2 or $5 \times 10^{-5}$ M) decreased the expression of VEGF in these cells by 45% and 70%, respectively (Fig. 5A). Also, ritonavir (2 or $5 \times 10^{-5}$ M) decreased the production of VEGF by RPMI8226 cells in a dose-dependent manner (Fig. 5B).

**Discussion**

This study found that protease inhibitors, including ritonavir, saquinavir, and nelfinavir induced growth arrest and apoptosis of MM cells and their anti-MM cell activity was almost identical. Protease inhibitors inhibited both activated STAT 3 and ERK1/2 signaling mediated by IL-6. Interestingly, among protease inhibitors, only indinavir failed to inhibit the growth of MM cells and did not inhibit STAT 3 and ERK1/2. Nelfinavir effectively blocked IL-6.
induced phosphorylation of STAT 3; however, its inhibition of EKR was only 10%. Further studies to identify the chemical differences between the protease inhibitors may lead to development of specific inhibitors of STAT 3 and/or ERK1/2.

The results presented here suggest that anti-MM cell activity of protease inhibitors may be mediated in part via inhibition of signaling by STAT 3 and ERK1/2. In a wide variety of types of cancer, including those from breast, lung, prostate, and head and neck, as well as malignant lymphoma and leukemia, STAT 3 and/or ERK1/2 signaling are constitutively activated and thought to contribute to carcinogenesis and progression of disease (16 – 25). Protease inhibitors may be useful for the treatment of individuals with these cancers.

Recent studies have found that saquinavir could inhibit the ability of the 20S and 26S proteasomes; these proteasomes can control the level of IκBα, a regulator of NF-κB (12). In addition, other investigators showed that ritonavir and saquinavir induced apoptosis of Kaposi sarcoma and prostate cancer cells via inhibition of NF-κB activity (10, 11). NF-κB was shown to play an important role in development and progression of MM (1). We, therefore, investigated the effect of protease inhibitors on NF-κB in MM cells using reporter assay. U266 cells had a measurable constitutive NF-κB transcriptional activity; none of the protease inhibitors (5 x 10^{-10} M, 24 h) significantly inhibited basal activity of NF-κB in these cells, suggesting that growth inhibition mediated by protease inhibitors is probably independent of inhibition of NF-κB in MM cells. MM cells initially respond to conventional chemotherapy; however, they eventually acquire drug resistance and no longer respond to anticancer drugs. The mechanisms of drug resistance include overexpression of P-glycoprotein (P-gp) which pumps drugs out of the cells, resulting in decreased intracellular concentrations of drugs (26). Another explanation of drug resistance relates to cytochrome P450 3A4 (CYP 3A4) which is associated with metabolism of chemotherapeutic agents. Xenobiotics, including anticancer drugs such as doxorubicin, promote nuclear translocation of pregnancy X receptor that binds to the promoter of CYP 3A4 and activates its expression, resulting in detoxification of drugs (27). Previous studies showed that ritonavir inhibited activities of both P-gp and CYP 3A4 (28 – 30). Co-administration of docetaxel and ritonavir increased plasma levels of docetaxel by 50-fold in mice as compared with those who received docetaxel alone (30). In addition, we recently found that ritonavir inhibited docetaxel-induced expression of CYP 3A4 in androgen-independent prostate cancer DU145 cells, which resulted in enhancement of antitumor activity of docetaxel in vitro and in vivo.3 Future studies will explore whether protease inhibitors can overcome drug resistance of MM cells.

In summary, we have found that protease inhibitors induced growth arrest and apoptosis of MM cells in conjunction with down-regulation of Mcl-1. protease inhibitors may be useful as adjunctive therapeutic agents for individuals with MM as well as other types of cancer in which activated STAT 3 and/or ERK1/2 play a role.

References

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