The HIV-1 protease inhibitor nelfinavir impairs proteasome activity and inhibits the multiple myeloma cells proliferation in vitro and in vivo

by Camille Bono, Lionel Karlin, Stephanie Harel, Enguerran Moully, Sylvaine Labaume, Lionel Galicier, Sebastien Apcher, Helene Sauvageon, Jean-Paul Fermand, Jean-Christophe Bories, and Bertrand Arnulf

Haematologica 2012 [Epub ahead of print]


Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.

Haematologica (pISSN: 0390-6078, eISSN: 1592-8721, NLM ID: 0417435, www.haematologica.org) publishes peer-reviewed papers across all areas of experimental and clinical hematology. The journal is owned by the Ferrata Storti Foundation, a non-profit organization, and serves the scientific community with strict adherence to the principles of open access publishing (www.doaj.org). In addition, the journal makes every paper published immediately available in PubMed Central (PMC), the US National Institutes of Health (NIH) free digital archive of biomedical and life sciences journal literature.

Support Haematologica and Open Access Publishing by becoming a member of the European Hematology Association (EHA) and enjoying the benefits of this membership, which include participation in the online CME program.
The HIV-1 protease inhibitor nelfinavir impairs proteasome activity and inhibits the multiple myeloma cells proliferation in vitro and in vivo

Running title: Effect of HIV protease inhibitors in multiple myeloma

Camille Bono¹, Lionel Karlin¹-², Stephanie Harel¹, Enguerran Mouly¹, Sylvaine Labaume¹,
Lionel Galicier³, Sébastien Apcher⁴, Hélène Sauvageon⁵, Jean-Paul Fermand¹-³, Jean-Christophe Bories¹
and Bertrand Arnulf¹-³

¹EA 3963, Univ Paris Diderot, Sorbonne Paris Cité, Institut Universitaire d’Hématologie, Paris, France,
²Département d’Immuno-clinique, Lyon, France, ³Département d’Immuno-hématologie, Hôpital St Louis, Paris, France, ⁴Inserm U 716 Paris, France, and ⁵Département de pharmacie, Hôpital St Louis, Paris, France

Key words: HIV protease inhibitor; nelfinavir; proteasome; multiple myeloma; apoptosis; PI3K/AKT

Correspondence

Bertrand Arnulf, EA 3963, Paris VII University, Paris, France. Phone: international +33.1.57276753.
Fax: international +33.1.57276747. E-mail: bertrand.arnulf@sls.aphp.fr
Abstract

Background

Multiple Myeloma is characterized by the accumulation of tumor plasma cells in the bone marrow. Despite improvement brought by proteasome inhibitors such as bortezomib, myeloma remains an incurable disease. In a variety of human cancers, human immunodeficiency virus protease inhibitors (nelfinavir) effectively inhibit tumor progression, but their impact on myeloma is unknown. We assessed in vitro and in vivo effects of the nelfinavir, on myeloma.

Design and methods

The effect of nelfinavir (1-10µM) on proteasome activity, proliferation and viability of myeloma cell line and plasma cells from patients was assessed by measuring PERK, AKT, STAT3 and ERK1/2 phosphorylation and CHOP expression with immunoblotting or flow cytometry. The in vivo effect was assessed in NOD/SCID mice injected with luciferase expressing human myeloma cell lines and treated with nelfinavir at 75mg/kg/day. Tumor progression was evaluated using a bioluminescent system.

Results

Nelfinavir inhibited the 26S chymotrypsin-like proteasome activity, impaired proliferation and triggered apoptosis of the myeloma cell lines and fresh plasma cells. It activated the proapoptotic unfolded protein response pathway by inducing PERK phosphorylation and CHOP expression. Cell death triggered by nelfinavir treatment correlated with decreased phosphorylation of AKT, STAT3 and ERK1/2. Nelfinavir enhanced the anti-proliferative activity of bortezomib, dexamethasone and histone deacetylase inhibitors and delayed tumor growth in a myeloma mouse model.

Discussion

These results suggest that nelfinavir used at pharmacological dosage, alone or in combination, may be useful in the treatment of myeloma. Our data provide a preclinical basis for clinical trials using nelfinavir in patients with myeloma.
Introduction

Multiple Myeloma (MM) is characterized by the proliferation of clonal plasma cells in the bone marrow and accounts for approximately 10% of all hematological cancers \(^1\). Despite recent therapeutic advances including the use of “novel agents” such as immunomodulatory drugs and proteasome inhibitors (PI), MM remains an incurable disease with a median survival of approximately 5 to 6 years \(^2\)–\(^5\).

Bortezomib (Velcade®) is a selective inhibitor of the 26S proteasome that has led to improved patient survival \(^6\)–\(^8\). The three enzymatic activities of the 26S proteasome – chymotrypsin (CT), trypsin (T) and caspase-like (C) – are located on the \(\beta5\), \(\beta2\) and \(\beta1\) subunits, respectively \(^9\). This complex is constitutively activated in plasma cells and is involved in multiple biological functions, including degradation of damaged protein, regulation of cell cycle, transformation or apoptosis \(^7\),\(^8\). Thus, the 26S proteasome represents an interesting therapeutic target in cancer \(^10\)–\(^12\).

Bortezomib-mediated proteasome inhibition entails accumulation of unfolded protein leading to induction of pro-apoptotic genes of the unfolded protein response (UPR). The UPR is a multi-branch system located in the endoplasmic reticulum (ER) that regulates the production and secretion of immunoglobulin, allowing either the survival or apoptosis of the cell during ER stress \(^2\),\(^13\),\(^14\). Excessive accumulation of misfolded proteins leads to their preferential binding to BiP and its dissociation from the ER membrane, thereby rendering active the three pathways via three transmembrane protein (IRE-1\(\alpha\), ATF6 and PERK). During this ER stress, IRE1-\(\alpha\) and ATF6 induction leads to XBP1 activation and synthesis of the molecular chaperone proteins including BiP/GRP78 and Hsp90. In contrast, the PERK pathway is thought to decrease protein synthesis via eIF2\(\alpha\) phosphorylation as well as generate a pro-apoptotic signal mediated by the up-regulation of ATF4 (activating factor transcription 4) transcription factor \(^15\),\(^16\). ATF4 induces the expression C/EBP homologous protein (CHOP/GADD153) transcription factor, known to activate apoptosis in the case of overwhelming ER stress. Both the effect on
proteasome and on the UPR system may explain how proteasome inhibition induces apoptosis in tumor plasma cells (17).

In clinical practice, the use of bortezomib is limited by the occurrence of severe side effects (including peripheral neuropathy) and resistance (3). In this regard, a new generation of proteasome inhibitors is needed to further improve the outcome of patients with MM (15, 16).

HIV protease inhibitors (HIV PIs) were developed in the early 1990s to treat chronically HIV infected patients. In combination with nucleoside analogs as part of highly active antiretroviral therapy, they have led to a reduction in HIV-related morbidity and mortality (18, 19). HIV PIs are peptidomimetic analogues of the peptide bond between phenylalanine 167 and proline 168 of the gag-pol polyprotein, which is the target of the HIV aspartyl-protease. The cleavage sites of HIV-I protease were initially thought to be unique and distinct from those of mammalian proteases (20). However, it has been suggested that the 26S proteasome is able to cleave the same sites and therefore may be a target of HIV PIs (21). In addition, HIV PIs have been successfully used for treating HIV-related Kaposi’s sarcoma indicating anti-tumor properties (22). Recent studies have shown that these drugs are able to inhibit cell proliferation and induce apoptosis in numerous cancer cells including malignant glioma, melanoma, prostate tumor and carcinomas (20, 23, 24). Various mechanisms of action, depending on the HIV PI and the cell type have been hypothesized. For example, it has been suggested that HIV PIs saquinavir and ritonavir could induce apoptosis of fibroblast and prostate cancer cell lines through inhibition of the proteasome activity (25, 26).

In this study, we investigated the effect of the HIV PI nelfinavir on the 26S proteasome activity and on the proliferation and viability of MM plasma cells both in vitro and in vivo. We found that, at concentrations achievable in patients, nelfinavir inhibited the CT activity of the 26S proteasome, decreased the proliferation and induced apoptosis of MM cell lines and plasma cells from patients. It was also found to induce the pro-apoptotic pathway of the UPR system, including PERK phosphorylation and up-regulation of CHOP. Nelfinavir-induced apoptosis of plasma cells was associated with decrease phosphorylation of AKT, STAT3 and ERK1/2. In addition, we show that nelfinavir enhanced the anti-proliferative effect of bortezomib, dexamethasone or histone deacetylase-like (HDAC) inhibitor valproic acid. This effect was validated in vivo in a xenograft mouse model of
MM in which nelfinavir significantly delayed tumor growth. Together, these results suggest that nelfinavir, alone or in combination with conventional cytotoxic therapy, may be effective in treating patients with MM even in the absence of HIV infection.
Design and Methods

Cell lines

U266, MM1S, RPMI, OPM2 and LP1 human myeloma cells lines and 293T cells were obtained from ATCC (Manassas, VA) and were respectively grown in RPMI 1640, IMDM and GIBCO® Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Invitrogen, Paris, France) supplemented with 2mM l-Glutamine (l-Glu), 100U/ml penicillin, 100g/ml streptomycin and 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco BRL, Invitrogen, Paris, France) and maintained routinely in a humidified chamber at 37°C and 5% carbon dioxide.

Human MM plasma cells

Bone marrow aspirates or peripheral blood samples were harvested and mononuclear cells were isolated by Ficoll-Hypaque centrifugation (lymphocyte separation medium, Eurobio, Les Ulis, France) and washed twice with phosphate-buffered saline containing 1% bovine serum albumin (BSA). CD138⁺ plasma cells were purified using the Direct CD138 Progenitor Isolation kit using immunomagnetic beads conjugated to monoclonal mouse anti-human CD138 antibody (Miltenyi Biotech, Paris, France). The purity of sorted cells analyzed by flow cytometry (with anti-CD138-FITC) was found to be up to 90%. All patients gave written informed consent.

Drug treatment

Nelfinavir (Viracept® Roche) and the synthetic PI MG132 (SIGMA Z-Leu-Leu-Leu-al) were solubilized in DMSO (dimethyl sulfoxide SIGMA). Bortezomib (Velcade®, Janssen and Janssen) was solubilized in saline buffer (NaCl 9%). The Fibroblast Growth Factor (rh-FGFacidic) and Insulin Growth Factor 1 (rh-IGF-1) were from R&D system. The IL-6 (recombinant human IL-6) was from PeproTech. The HDAC inhibitor, valproic acid, was provided by Sanofi Aventis and dexamethasone from Mylan.

26S Proteasome activity
MM cells were lysed in RIPA buffer (Santa Cruz sc.24948) supplemented with protease inhibitors (protein inhibitor cocktail) and sodium orthovanadate (100 mM). 30µg of protein per cell line was collected in a Tris buffer (Tris 50mM (pH:7.5), DTT (1mM), MgCl2 (10mM), ATP (2mM)) and incubated with nelfinavir (5µM), bortezomib (10nM) or MG132 (1µM) for 2 hours at 37°C. Then 1mM of the fluorogenic substrate was added and the enzymatic activities measured by a FLUOstar OPTIMA (BMG Labtech) (λ_{exc} : 380nM and λ_{em} : 460nM). The fluorogenic substrates were Z-Leu-Leu-Val-Tyr-AMC (Calbiochem, San Diego, CA) for the chymotrypsin-like activity and Bz-Val-Gly-Arg-AMC (BIOMOL) for the trypsin-like activity. All experiments were performed in triplicate.

Cell Proliferation and Viability assay

The proliferation of plasma cell lines was measured by tritiated thymidine uptake ([6-³H] thymidine). 3.10^4 cells were plated in a 96-well flat bottomed plate and incubated 48 hours with the drugs. Then [6-³H] thymidine (Amersham Biosciences, UK) was added (1µCi/well) for a further 16 hours. The [6-³H] thymidine incorporation was analyzed using a liquid scintillation counter (Wallace, PerkinElmer).

The viability of the plasma cells from patients (5.10^4 cells per well) was measured using the WST-1 kit (Roche®) according to the manufacturer’s instructions. All experiments were performed in triplicate.

Flow Cytometry

Apoptosis was analyzed by flow cytometry (Becton Dickinson FACS Calibur / BD Bioscience) using rh-annexin V-FITC Staining (AbCys®, Paris, France). CellQuest Pro software (BD Bioscience) was applied for flow cytometry analyses. Data are presented as a dot plot of at least 50 000 counted events per sample. 1.10^6 cells were incubated with nelfinavir (17 hours) without FCS. The cells were then washed twice and dissolved in 1x binding buffer for annexin V-FITC before being incubated for 10 minutes at room temperature with rh-annexin V-FITC. Propidium iodure was added just before the FACS analysis.

For flow cytometry analysis of AKT phosphorylation, 10^6 cells were serum starved for 4 hours, incubated with IGF-1 (100ng/mL) for 30 minutes then cultured for 2 hours with nelfinavir (5µM). For analysis of STAT-3 and ERK1/2 phosphorylation, 10^6 cells were serum starved for 4 hours, incubated with nelfinavir (5µM and 20µM) for 2 hours before IL-6 stimulation (100ng/mL) during 30 minutes.
Cells were fixated with PFA-4% for 10 minutes at room temperature and permeabilized for 30 minutes in methanol 50% at 4°C. Cells were then wash in PBS and incubated with specific antibodies for 1 hour at room temperature. Antibodies recognizing P-AKT, P-STAT3 and P-ERK1/2 were purchased respectively from Cell signaling and BD Bioscience.

**Western-Blot and Antibodies**

Cells were lysed with RIPA buffer, samples (30µg of protein) were boiled, sheared and clarified by centrifugation, before being separated on an 8-10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. Membranes were blocked in PBS containing 0.1% Tween 20 and 5% BSA before adding the primary antibody. Antibody binding was detected using the ECL chemiluminescence kit (Amerham, Arlington Heights, IL), and digitized using an Arcus II scanner. The antibodies for CHOP, ubiquitine, AKT, P-AKT(ser473) and caspase-3 were from Cell Signaling, for ATF4 (H-290), and actin from Santa Cruz. The rabbit polyclonal antibody for P-PERK was from Biolegend. The HRP antibodies anti-mouse, goat and rabbit were from Santa Cruz.

**Generation of U226-luc cells**

Infectious non-replicative retroviral vector supernatants were produced in 293T cells by co-transfection of three plasmids using the liposomal method according to the manufacturer’s instructions (Effectene® Transfection Reagent – Qiagen). 293T cells were seeded in a 10 cm tissue culture dish 24 hours prior to transfection in DMEM medium to obtain semi-confluent cells the day of transfection. For the production of retroviral particles, cotransfection of the pMSCV/Luc plasmid driving the luciferase gene and neomycin resistance (G418 at 1mg/ml) was achieved with the pMD.G envelope plasmid containing the VSV-g envelope protein and a plasmid expressing the gag, and pol genes (27). The U266 cells were incubated for 48 hours with the supernatant of the retroviral producer with FCS (10%) and polybrene (2µg/mL). Then the U266-luc cells were selected on the basis of their neomycin resistance.

**Human MM xenograft model**

Immunodeficient non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (10-12 weeks old) were obtained from the Institut André Lwoff- Villejuif, France. The mice were maintained in a specific pathogen-free (SPF) breeding facility and, in line with the recommendations of the **IUH**
animal care and user committee, were sacrificed 25 days after inoculation of the U266-luc cells. The mice were handled and housed in compliance with the IUH animal care and user committee guidelines. Fourteen NOD/SCID mice received whole-body irradiation 2.5Gy the day of the subcutaneous injection of $8 \times 10^6$ U266-luc cells in 0.2mL in PBS into the flank. Treatments started 24 hours after cell inoculation. The mice were separated into two groups of seven. One group was treated daily with an intraperitoneal infusion of nelfinavir (75mg/kg) dissolved in a solution of PBS containing 50% PEG-10% DMSO and Tween® 80% (SIGMA) in a final volume of 300 µL, for 20 days. The control group received an intraperitoneal infusion of the same solution without nelfinavir (vehicle).

On designated days post-inoculation, U266-luc cells were detected using the IVIS imaging system (Xenogen Corporation, Alameda, CA). For imaging, mice were injected intraperitoneally with 150 µg/g luciferin (Xenogen) followed by anesthetization with isoflurane (Baxter). Ten minutes after the injection of luciferin, the mice were imaged using a charge coupled device camera in the IVIS imaging system to evaluate the bioluminescence of the cancer cells in the animal. A gray-scale image of the mice was captured followed by an overlay of a bioluminescence map representing the spatial distribution of photons detected from the cleaved luciferin in the cancer cells expressing luciferase. The signal intensity was quantified using a version of the IGOR Pro version 4.06A Software (WaveMetrics, Inc., Lake Oswego, OR) called Living Image Version 2.50.2 (Xenogen).

Statistics

Experimental values were expressed as ± SEM. In all experiments, statistical comparison of means values was done using the Student’s t-test.

The study was approved by the local Institut Universitaire d'Hématologie Ethics Committee. A written informed consent was obtained from each subject.
Results

Nelfinavir inhibits the 26S proteasome activity

To investigate the effect of nelfinavir on 26S proteasome in MM cells, we measured the enzymatic proteasomal activity in the presence or absence of nelfinavir, bortezomib or MG 132. As expected, bortezomib and MG132 readily inhibited CT-like activity in LP1, U266 and MM1S cells. Similarly, the 26S CT-like proteasome activity was reduced by 60% when using a pharmacological concentration of nelfinavir (5µM) (Figure 1A). As previously described for bortezomib, this inhibitory effect of nelfinavir was not restricted to MM cells and was also observed on peripheral blood lymphocytes from healthy donors (data not shown). Neither nelfinavir nor bortezomib had an effect on the T-like activity. Nelfinavir inhibited the CT-like activity of 26S proteasome of CD138 selected tumor plasma cells from MM patients as well as bortezomib (Figure 1B). As shown in figure 1C, nelfinavir induced a time-dependent accumulation of ubiquitinylated protein after 6 hours of treatment in MM cell lines. Next, we investigated the activities of saquinavir and tipranavir and found that both proteases inhibitors reduced the CT-like activity in MM cell lines (Online supplementary figure S1A). Thus, nelfinavir (as saquinavir and tipranavir) is an effective inhibitor of the 26S proteasome CT-like activity in MM cell lines and fresh plasma cells from patients.

Nelfinavir inhibits proliferation of MM cells in vitro even in the presence of pro-survival cytokines

To investigate the impact of nelfinavir induced proteasome inhibition on the proliferation of MM cells, RPMI, LP1, U266, OPM2 and MM1S cell lines were grown in the presence or absence of increasing concentration of nelfinavir. Nelfinavir inhibited the proliferation of RPMI, LP1, U266, OPM2 and MM1S cell lines in a dose-dependent manner with an IC 50 of 1 - 5µM. Similar results were observed with saquinavir and tipranavir (Online supplementary figure S1B). Interestingly, in U266 cells treated with nelfinavir, the kinetics of CT-like inhibition correlated with the inhibition of cell proliferation (Online supplementary figure S2). In addition, nelfinavir (1 to 5µM) also decreased the viability of CD138+ plasma cells purified from 3 patients (Figure 2A).
The effect of nelfinavir was then tested on the survival of U266 cells and plasma cells from an MM patient in the presence of the pro-survival cytokine known to be secreted *in vivo* in the plasma cell microenvironment. U266 cells treated with or without nelfinavir were grown in the presence of IGF-1 or FGF. As illustrated in Figure 2B, while FGF and IGF-1 markedly increased cell proliferation, neither of these cytokines could counteract the inhibitory effect of nelfinavir. The survival of CD138+ purified plasma cells from an MM patient was doubled in the presence of IGF-1 whereas the inhibitory effect of nelfinavir (5µM) was not counteracted by the addition of either of these cytokines. Similar results were obtained with interleukin-6 (IL-6) treated MM cells (*Online supplementary figure S3*). These data show that nelfinavir inhibits the proliferation not only of MM cell lines, but also of plasma cells from patients. Moreover, this activity is not reversed by pro-survival signals delivered by several soluble factors such as IGF-1 or FGF.

**Nelfinavir enhances the anti-proliferative activity of anti-myeloma agents**

Next we investigated whether nelfinavir could enhance anti-proliferative activities on MM cells in combination with other anti-myeloma drugs such as HDAC inhibitor valproic acid (VA) or dexamethasone (Dex). In this experiment, the drugs were used at the optimal concentration in order to unmask potential cooperative effects. Used separately, Dex (5µM) and nelfinavir (2µM) each induced a 40% inhibition of U266 cell proliferation. Combined in the culture medium, they induced an 80% reduction in the proliferation of U266 (Figure 3A). The cooperative effect was also observed on the LP1 cell proliferation using Dex (10µM) and nelfinavir (1µM). Similarly, an enhanced effect was found between VA (0.5mM) and nelfinavir (2µM) with a 90% inhibition of U266 cell proliferation when used together (Figure 3B) as compared to 50 to 60% when used separately. This combination also inhibited the proliferation of LP1 cells although, in this cell line, the cooperative effect of nelfinavir was achieved with VA, 1mM. Moreover, an enhanced anti-proliferative effect was found between the two proteasome inhibitors bortezomib and nelfinavir on U266 and LP1 cell lines (Figure 3C). These data demonstrate cooperative cytotoxic effects between nelfinavir and Dex, bortezomib or VA on MM cell lines.
**Nelfinavir induces apoptosis of MM cell lines and activates the cleavage of caspase-3**

To obtain further insights into the mechanism of the nelfinavir-induced anti-proliferative effect, we investigated the impact of HIV IP on apoptosis induction of LP1 and U266 cells (Figure 4A). After 17h hours of incubation with increasing concentrations of nelfinavir (1-10µM), the percentage of annexin V+/IP+ apoptotic cells was analyzed by flow cytometry. Nelfinavir induced a dose-dependent increase in the percentage of annexin V+/IP+ cells. The effect of nelfinavir on the activation of caspase-3 was then studied. Nelfinavir induced the pro-caspase-3 cleavage in a time-dependent manner (Figure 4B) on U266 cell line. These results suggest that nelfinavir inhibits proliferation of MM cells lines by inducing apoptosis.

**Nelfinavir decreases the phosphorylation of AKT, STAT-3, ERK1/2 and activates the pro-apoptotic pathway of the UPR system**

Knowing the importance of the PI3K/AKT pathway on cell survival, we studied the impact of nelfinavir on AKT phosphorylation. Western blot analysis revealed that the level of AKT phosphorylation in U266 cells decreased after 6 hours following nelfinavir (5µM) treatment (Figure 5A. left panel). In addition, intracellular FACS analysis revealed that, at this concentration, nelfinavir also reduced the mean intensity of fluorescence (MIF) corresponding to IGF1-mediated AKT phosphorylation (Figure 5A. right panel).

We next used FACS analysis to investigate the impact of nelfinavir on the phosphorylation of STAT3 and ERK1/2 following IL-6 stimulation. As previously described 29, our results show that, upon IL-6 treatment, high concentrations of nelfinavir (20µM) dramatically decreased the MIF corresponding to phosphorylated STAT3 and ERK1/2 in U266 cells (Figure 5B right panel). At 5µM, nelfinavir induced lower but significant reductions of phosphorylated STAT3 and ERK1/2 (Figure 5B left panel).

Next we investigated the impact of nelfinavir on the UPR system. No effect was observed on the level of chaperone protein BiP and Hsp90 (data not shown). We then studied the activation of the pro-apoptotic pathway of the UPR system. Nelfinavir induced a transient phosphorylation of PERK after 3 hours of treatment (Figure 5C). The expression of CHOP protein was up-regulated after 6 hours of
treatment but no variation of ATF4 protein level was observed. These results suggest that nelfinavir inhibits the phosphorylation of AKT, STAT3 and ERK1/2 and induces the pro-apoptotic pathway of the UPR system.

**Nelfinavir decreases MM cell growth in NOD/SCID mice**

To establish the anti-proliferative effect of HIV PI on MM cells *in vivo*, a xenograft model was set up by injecting U266-luc cells into NOD/SCID mice. No toxicity or drug related death was observed in the mice treated with nelfinavir (75mg/kg) administered intraperitoneally 5 days a week for 21 days. To serially monitor MM growth *in vivo*, we examined whole-body photon emission from the inoculated mice once a week starting 7 days after injection. Luciferase activity was detected in all mice at day 7 following cell inoculations (Figure 6A) indicating the presence of similar amounts of tumor cells in both nelfinavir and vehicle treated cohorts. At day 14, low levels of luciferase activity were detected in some nelfinavir treated animals even if the average light emission remained similar in both groups (Figure 6B). In contrast, at day 21 after inoculation, there were significantly lower photon emissions in mice treated with nelfinavir compared to vehicle treated mice (82 vs. 33 mean photon emission, p=0.026). This data indicates that nelfinavir delays MM cell growth *in vivo*. After day 21 the vehicle mice were killed due to tumor burden. Thus, nelfinavir was shown to significantly delay growth of MM cells *in vivo*. 

DOI: 10.3324/haematol.2011.049981
**Discussion**

Despite recent therapeutic advances, MM remains an incurable disease. A growing number of reports suggest that HIV PIs may act as anti-cancer agents as they are able to inhibit growth of solid tumors in part through the impairment of the proteasome activity \(^{(20, 28)}\). However, to date, most of the anti-neoplastic effects of HIV PIs have been tested on solid tumors and very little is known about their effect in MM plasma cells.

In this study we show that nelfinavir induces a dose-dependent inhibition on proliferation and induces cell death through apoptosis of MM cells lines and tumor plasma cell derived from patients. It has been suggested that ritonavir, saquinavir and nelfinavir induce growth arrest but that high concentrations (50µM) are needed to trigger a significant inhibitory effect \(^{(29)}\). Our results show that nelfinavir is effective at relatively low doses (between 1 and 10µM) that are achievable in patients. *In vivo*, it could either triggers apoptosis of MM cells at high concentrations or inhibits their growth at lower dosage. Furthermore, we found that the addition IL-6, IGF-1 and FGF secreted in the bone marrow microenvironment do not reverse the inhibitory effect of nelfinavir suggesting that human growth factors may not counteract HIV PI activity *in vivo*. Furthermore, our data show that nelfinavir was able to inhibit IL-6 induced phosphorylation of STAT3 and ERK1/2. Considering the critical role of both pathways *in vitro* and *in vivo*, their inhibition might contribute to repress the growth of MM cells.

Indeed, using a human MM xenograft model in NOD/SCID mice, we show that nelfinavir (75mg/kg 5d/week) is able to delay the growth of engrafted MM cell lines. Given the short half-life and the well characterized pharmacokinetics of nelfinavir in mice, the efficacy could well be improved by more frequent dosing \(^{(24, 30)}\).

Proteasome activity is crucial for the survival of MM cells \(^{(31)}\). Our results show that both nelfinavir and bortezomib selectively inhibit the CT-like activity of the 26S proteasome in MM cells. The *in vitro* inhibition of proteasome activity by the HIV PIs saquinavir and ritonavir has been suggested in several types of cancer including human leukemic cells and lymphomas \(^{(21, 23)}\). However, again, in all these studies, the inhibition of cell proliferation and the induction of apoptosis were only shown for
concentrations over 50µM, a dosage which is not achievable in patients. Here, we show that, 5µM of nelfinavir reduces the CT-like proteasome activity by 40% in MM cells. This level of inhibition is achieved with bortezomib at 10nM. Altogether, our pre-clinical findings show that nelfinavir, used at pharmacological dosage exhibits anti-myeloma activity.

Bortezomib-induced proteasome inhibition is thought to entail the accumulation of misfolded proteins thereby leading to ER stress, induction of terminal UPR activation and apoptosis of MM plasma cells. During ER stress, induction of IRE1-α and ATF6 trigger XBP1 activation and the synthesis of molecular chaperone proteins including GRP78/BIP and Hsp90. In addition, the PERK/ATF4/CHOP pathway reduces the level of protein synthesis and may induce apoptosis through an unclear mechanism (32). Bortezomib is thought to induce the UPR pro-apoptotic pathway by triggering PERK autophosphorylation and subsequent activation of the PERK/ATF4/CHOP cascade. We show here that nelfinavir activates the UPR pro-apoptotic pathway as revealed by PERK phosphorylation and increased CHOP expression. Although an intricate link between ER stress and apoptosis has been suggested (32-35), the precise mechanisms inducting apoptosis through ER stress in MM cells are still unclear (36). Whatever the mechanism, we show that apoptosis induced by nelfinavir, as well as by bortezomib, correlates with the activation of the PERK/ATF4/CHOP pro-apoptotic pathway of the UPR.

The PI3K/AKT pathway has been shown to play a major role in malignant growth and survival of MM cells and is involved in cell-cycle and apoptosis regulation in MM cell lines and primary tumor samples (37-39). In addition, AKT has been shown to be frequently activated in primary MM plasma cells and its pharmacological inhibition has been associated with cell death induction (40). We found that nelfinavir also inhibits growth factor induced AKT phosphorylation in MM cells. Interestingly, nelfinavir-induced inhibition of proteasome activity in carcinoma cell lines, may prevent the degradation of the phosphatase PP1, thereby leading to an increase in AKT dephosphorylation (41). This inhibition of the PI3K/AKT pathway may represent a mechanism of nelfinavir-induced apoptosis. Indeed, AKT is in part involved in maintaining mitochondria integrity by phosphorylating BAD (Bcl2 Antagonist of Death) and inhibiting its binding to the Bcl2 protein. AKT also decreases the expression of FoxO transcription factors that regulate the transcription of pro-apoptotic genes (42). Furthermore, a proteasome independent AKT inactivation may induce CHOP expression (43). During the UPR stress triggered by bortezomib
treatment, up-regulation of CHOP is mainly due to the rapid induction of ATF4 as a consequence of PERK activation. Although our results show that PERK is phosphorylated in response to nelfinavir treatment and that CHOP is up-regulated, we were unable to monitor ATF4 modulation. This could indicate that CHOP is up-regulated in a PERK independent manner. This hypothesis is strengthened by the kinetics of PERK phosphorylation and CHOP accumulation that seems to be much slower on nelfinavir than bortezomib treatment \(^{(14)}\). These results suggest that, nelfinavir induced AKT inhibition and subsequent CHOP up-regulation may represent a UPR independent mechanism of cell death in MM cells.

In our study, the time course of the nelfinavir-induced PERK/ATF4/CHOP pathway is different to that reported for bortezomib \(^{(14)}\). Bortezomib has been shown to induce more rapid PERK activation (as early as 30 minutes) and subsequent CHOP up-regulation after as little as 4 hours of treatment. These results suggest a potential additive effect between nelfinavir and bortezomib which we indeed observed in MM cells. Of note, it has been reported that the HIV PI ritonavir, which is ineffective when used alone, can sensitize sarcoma cells to bortezomib \(^{(44)}\). In patients with MM, combinations including HIV PIs could also be particularly useful in case of peripheral neuropathy which frequently complicate bortezomib or thalidomide therapies. Indeed, the well-known side effects of most HIV PIs do not include neurotoxicity.

Importantly, all in vivo and in vitro results in our study were obtained at pharmacological concentrations that are usually measured in HIV PI treated patients with chronic HIV infection \(^{(18)}\). We also showed that nelfinavir significantly enhanced the anti-proliferative effect of drugs currently used or recently developed in the treatment of MM including dexamethasone and the HDAC inhibitor valproic acid. These combinations could also benefit from the low hematological toxicity of HIV PIs promoting their combination with potentially synergistic conventional chemotherapeutic drugs or novel agents. Indeed, numerous clinical trials currently evaluating the safety and efficacy of nelfinavir in combination with other drugs in patients with solid cancer indicate an acceptable toxicity with promising anti-tumor activity (http://clinicaltrials.gov).

In conclusion, our results show for the first time the effect of nelfinavir on MM tumor growth both in vitro and in vivo. They provide a rational for the therapeutic evaluation of the HIV PIs, alone or in
combination, in patients with relapse/refractory MM, especially with bortezomib-induced severe side effects such as peripheral neuropathy.
Abbreviations


Funding

This work was supported by grants from the Association pour la Recherche Contre le Cancer N° 3539 and 3750 and a grant from the Fondation de France N° 2004004116. C. Bono is a recipient of the Convention Industrielle de Formation par la REcherche (CIFRE) fellowship and from the Association pour la Recherche Contre le Cancer. L. Karlin was supported by the Association pour la Recherche Contre le Cancer.

Acknowledgments

We thank M. Bargis for helpful technical assistance, M. Chopin for assistance with the animal studies.

Authorship and Disclosures

C Bono prepared the manuscript and conducted the bulk of the experimental work. L. Karlin, S. Harel, E. Mouly and S. Labaume have also contributed to experiments. S. Apcher helped for the proteasome experiments. L. Galicier and H. Sauvageon have contributed to the start of the study and have provided sample of patients and drugs. JP. Fermand, JC. Bories and B. Arnulf have supervised the work and have jointly prepared the manuscript. The authors declare that they do not have commercial or other associations that might cause conflict of interest.
References


Figure legends

Figure 1. Nelfinavir inhibits the 26S proteasome activity.

Histograms show the chymotrypsine-like (CT-like) and trypsin-like (T-like) activities of the 26S proteasome (A) Cellular lysates of LP1, U266 or MM1S cell lines were incubated with 5µM nelfinavir (Nelf), 10nM bortezomib (Bort) or 1µM MG132 (MG) or cultured in medium alone (Med). (B) Cellular lysates of plasma cells from patients were incubated with nelfinavir (Nelf) 10µM, bortezomib (Bort) 10nM and MG132 (MG) 1µM or cultured in medium alone (Med). (C) U266 cells were incubated with nelfinavir (5µM) during the indicated times and the level of the ubiquitinylated proteins was determined by western-blot. Error bars correspond to the standard of deviation. The p value was calculated with the Student’s t-test, (*p≤0.05, **p≤0.01).

Figure 2. Nelfinavir inhibits proliferation of MM cell lines in vitro even in the presence of pro-survival cytokines.

(A. Right). Histograms show the proliferation of RPMI, LP1, U266, OPM2 and MM1S cell lines treated with nelfinavir (to 1µM to 10µM). (A. Left). Shown are the viabilities of plasma cells from three patients having received bortezomib (“P” and “M” were non-responsive to bortezomib) and that were cultured with the indicated concentrations of nelfinavir. (B. Right). Histograms show the proliferation of U266 cells treated (filed) or not (open) with 5µM nelfinavir in the presence of IGF-1 (100ng/mL) or FGF (100ng/mL) + heparin (100µg/mL). (B. Left). Histograms show the viability of the plasma cells from a patient treated (filed) or not (open) with 10µM of nelfinavir in the presence of IGF-1 (100ng/mL) or FGF (100ng/mL) + heparin (100µg/mL). Error bars correspond to the standard of deviation. The p value was calculated with Student’s t-test, (*p≤0.05, **p≤0.01).
Figure 3. Nelfinavir cooperates with anti-myeloma agents to inhibit the proliferation of multiple myeloma cells.

Bar graphs show the proliferation of U266 and LP1 cells cultured in the presence of nelfinavir and (A) dexamethasone (Dex), (B) valproic acid (VA), (C) bortezomib. The concentrations at which each drug was used are indicated. Error bars correspond to the standard of deviation. The p value was calculated with Student’s t-test, (*p≤0.05, **p≤0.01).

Figure 4. Nelfinavir induces the cleavage of pro-caspase 3 and apoptosis in MM cell lines.

(A) Dot plots show apoptosis of LP1 and U266 cells cultured with nelfinavir at the indicated concentrations. The percentage of dead cells measured as Annexin V and propidium iodide staining are indicated in each quadrant. (B). Western-blot analysis of the kinetic of pro-caspase-3 cleavage in U266 cells cultured with nelfinavir (5µM). Cleaved and native forms of the Caspase-3 are indicated as the Actin control.

Figure 5. Nelfinavir decreases the phosphorylation of AKT, STAT3, ERK, and activates the pro-apoptotic pathway of the UPR system

(A) Right. U266 cell were starved for 4 hours and incubated at the indicated times with 5µM nelfinavir. The levels of AKT, phosphorylated AKT (P-AKT) and the control Actin were detected by western-blot. 

Left. Histograms show the level of phosphorylated AKT in IGF1 stimulated U266 cells in the presence of nelfinavir (5µM). (B) Histogram show the intracellular FACS analysis of phosphorylated STAT3 (P-STAT3) and ERK1/2 (P-ERK1/2) in IL-6 stimulated U266 cells. Nelfinavir treated cells are indicated by the dotted lines (5µM) or dashed line (20µM), non treated cells correspond to the bold line and the isotype control is represented in filled grey. The mean intensity of fluorescence (MIF) of each treatment are indicated on the histograms. (C) Western blot analysis of the kinetic of P-PERK, ATF4 and CHOP expression in U266 cells treated with nelfinavir (5µM). Actin is used as a loading control.
Figure 6. Nelfinavir decreases MM cell growth in NOD/SCID mice.

8.10^6 U266-luc cells were injected subcutaneously into the flank of NOD/SCID mice. Intraperitoneal administration of nelfinavir (75mg/kg 5 days/week) was initiated 24 hours after cell inoculation and continued all through the end of the experiment. The tumor burden was measured based on photon emission at the indicated day. (A) Representative bioluminescence images of mice at days 7, 14 and 21 are shown (days after the inoculation). (B) The mean photon emission readout indicated for each group (n=7).
Figure 2

A

B

U266

Plasma Cells

% Cell Proliferation

% Cell Viability

Neof. (µM) 0 1 2 5 10

RPMI
LP1
U266
OPM2
MM1S

# L
# P
# M

Medium
IGF1
FGF

- Nelfinavir
+ Nelfinavir

200
180
160
140
120
100
80
60
40
20
0

200
180
160
140
120
100
80
60
40
20
0

* *
Figure 4

A

Medium  1 µM  5 µM  10 µM  
LP1  
11%  16%  25%  48%  
ANNEXIN V  
IP  
13%  13%  16%  26%  
U266  

B

U266 - Nelfinavir 5 µM  
0  3  6  12  24 (h)  
Caspase 3  
17 kDa  
Cleaved Caspase 3  
actin
Supplementary Appendix

The HIV-1 protease inhibitor nelfinavir impairs proteasome activity and inhibits the multiple myeloma cells proliferation in vitro and in vivo

Camille Bono¹, Lionel Karlin¹², Stephanie Harel¹³, Enguerran Mouly¹, Sylvaine Labaume¹, Lionel Galicier³, Sébastien Apcher⁴, Hélène Sauvageon⁵, Jean-Paul Fermand¹³, Jean-Christophe Bories¹ and Bertrand Arnulf¹³

¹EA 3963, Univ Paris Diderot, Sorbonne Paris Cité, Institut Universitaire d’Hématologie, Paris, France, ²Département d’Immuno-clinique, Lyon, France, ³Département d’Immuno-hématologie, Hôpital St Louis, Paris, France, and ⁴Inserm U 716 Paris, France, ⁵Département de pharmacie, Hôpital St Louis, Paris, France

Online supplementary Figure S1. (A) Bar graphs show the effects of HIV protease inhibitors nelfinavir, tipranavir and saquinavir on the CT-like activity of RPMI, U266, LP1 and OPM2 cells. The inhibitory activity of the bortezomib is also indicated. (B). Proliferation of LP1 (Left) and U266 (Right) cells treated with increasing concentrations of HIV PIs nelfinavir, saquinavir and tipranavir.

Online supplementary Figure S2. (A). Histograms show the kinetic of bortezomib (10nM) inhibition of U266 cell proliferation. (B). Shown are the corresponding kinetics of the CT-like activity in bortezomib treated U266 cells as in A.

Online supplementary Figure S3. (A). Histograms show the proliferation of LP1 cells treated (filled) or not (open) with 5μM nelfinavir in the presence of IGF-1 (100ng/mL), IL-6 (100ng/mL) or FGF (100ng/mL) + heparin (100µg/mL). (B). Histograms show the viability of the plasma cells from a patient treated (filled) or not (open) with 10μM of nelfinavir in the presence of IGF-1 (100ng/mL), IL-6 (100ng/mL) or FGF (100ng/mL) + heparin (100µg/mL). Error bars correspond to the standard of deviation.
Online supplementary data S1

A

% CT-like activity

120
100
80
60
40
20
0

10% Nelfinavir Tipranavir Saquinavir Bortezomib

B

% cell proliferation

120
100
80
60
40
20
0

LP1 U266

Nelfinavir Saquinavir Tipranavir

Nelf (µM) 0 1 2 5 10

Nelf (µM) 0 1 2 5 10