

The Human Immunodeficiency Virus (HIV)-1 Protease Inhibitor Saquinavir Inhibits Proteasome Function and Causes Apoptosis and Radiosensitization in Non-HIV-associated Human Cancer Cells¹

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ABSTRACT

Cancer cells frequently show high constitutive activity of the antiapoptotic transcription factor nuclear factor κ B (NF- κ B), which results in their enhanced survival. Activation of NF- κ B classically depends on degradation of its inhibitor I κ B α by the 26S proteasome. Specific proteasome inhibitors induce apoptosis in cancer cells and, at nonlethal concentrations, sensitize cells to the cytotoxic effects of ionizing radiation and chemotherapeutic drugs. Recently, the protease coded by the HIV-1 virus has been shown to share cleavage activities with the proteasome. For this reason, we investigated whether the HIV-1 protease inhibitor saquinavir can inhibit NF- κ B activation, block 26S proteasome activity in prostate cancer cells, and promote their apoptosis. The effect of saquinavir on LPS/IFN- γ -induced activation of NF- κ B was assessed by gel-shift assays and by Western analysis of corresponding I κ B α -levels. Its effect on 20S and 26S proteasome activity was analyzed with a fluorogenic peptide assay using whole cell lysates from LnCaP, DU-145, and PC-3 prostate cancer cells pretreated with saquinavir for 9 h. Proteasome inhibition in living cells was assessed using ECV 304 cells stably transfected with an expression plasmid for an ubiquitin/green fluorescence protein fusion protein (ECV 304/10). Apoptosis was monitored morphologically and by flow cytometry. Saquinavir treatment prevented LPS/IFN- γ -induced activation of NF- κ B in RAW cells and stabilized expression of I κ B α . It inhibited 20S and 26S proteasome activity in lysates from LnCaP, DU-145, and PC-3 prostate cancer cells with an IC₅₀ of 10 μ M and caused the accumulation of an ubiquitin/green fluorescence protein fusion protein in living ECV 304/10 cells. Incubation of PC-3 and DU-145 prostate cancer, U373 glioblastoma, and K562 and Jurkat leukemia cells with saquinavir caused a concentration-dependent induction of apoptosis. In the case of PC-3 and DU-145, saquinavir sensitized the surviving cells to ionizing radiation. We conclude that saquinavir inhibits proteasome activity in mammalian cells as well as acting on the HIV-1 protease. Because saquinavir induced apoptosis in human cancer cells, HIV-1 protease inhibitors might become a new class of cytotoxic drugs, alone or in combination with radiation or chemotherapy.

INTRODUCTION

HIV-1 encodes for a protease required for the cleavage of the viral gag-pol polyprotein, and its inhibition leads to the release of noninfectious virus particles (1). The development of specific HIV-1 PIs³ has revolutionized HIV therapy. At present, five different HIV-1 PIs (ritonavir, saquinavir, nelfinavir, indinavir, and amprenavir) are clinically used (2). Bioavailability of at least ritonavir, saquinavir, and indinavir is limited by the fact that they are substrates, and in part

inhibitors (3), of the same multidrug resistance gene product (*mdr-1*), P-glycoprotein (4, 5), that is a common cause for failure of chemotherapy in cancer patients.

The cleavage sites of action for HIV-1 protease were once thought to be unique and distinct from those of mammalian proteases. However, recently, the 20S proteasome has been shown to cleave the same sites (1). This led us to investigate whether saquinavir is an inhibitor of the 20S proteasome, as was previously reported for ritonavir (6). Proteasome inhibition may contribute to some of the effects of PIs that seem to be independent of virus inhibition, such as its immunomodulatory properties in HIV patients and its antitumoral action on HIV-associated Kaposi-sarcoma (6). We also examined whether saquinavir exhibits antitumoral effects in non-HIV-associated cancer of the prostate.

MATERIALS AND METHODS

Cell Culture. Cultures of PC-3, LnCaP, and DU-145 human prostate carcinoma and U373 glioblastoma cells and K562 erythroleukemia and Jurkat T-cell leukemia cells (American Type Culture Collection, Rockville, MD), RAW 264.7 murine macrophages (a gift of Dr. G. Hildebrandt, Department of Radiation Oncology, University Clinic Leipzig, Leipzig, Germany), and ECV 304 human bladder carcinoma cells (DSMZ, Braunschweig) were grown in 75-cm² flasks (Greiner) at 37°C in a humidified atmosphere of 5% CO₂/95% air. DMEM (Cell Concepts, Freiburg, Germany) and RPMI 1640 (Cell Concepts) were supplemented with 10% FCS and 1% penicillin/streptomycin (Life Technologies, Inc.) before use. Saquinavir (a generous gift of Dr. Christiane Moecklinghoff, Hoffmann La-Roche, Grenzach, Germany) was solubilized in ethanol/H₂O at a concentration 10 mM and used at concentrations of 0, 20, 50, 60, 80, and 100 μ M. Controls received solvent only.

Irradiation. PC-3 and DU-145 cells were trypsinized, counted, and diluted to a concentration of 10⁶ cells/ml. The cell suspension was immediately irradiated at room temperature using a ¹³⁷Cs-laboratory irradiator (IBL 637; CIS bio international) at a dose rate of 0.78 Gy/min. Corresponding controls were sham irradiated.

Clonogenic Survival. Colony-forming assays were performed immediately after irradiation by plating an appropriate number of cells (2×10^3 to 2×10^4) into Petri dishes, in triplicate. After 14-days culture, cells were fixed, stained with crystal violet, and colonies consisting of more than 50 cells were counted. Resulting survival plots were fitted using a linear-quadratic model.

Transfection. ECV 304 cells were maintained in DMEM (10% FCS, 1% penicillin/streptomycin). Twelve h before transfection, cells were trypsinized and plated at a density of 250,000 cells/well into six-well plates. Cells were transfected with 5 μ g of a plasmid (pEGFP-N1; Clontech) coding for an Ub-R-GFP fusion protein under control of a cytomegalovirus promoter (7; a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10% FCS, 1% penicillin/streptomycin) supplemented with 500 μ g/ml G418 (Sigma), and clones were obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL1-HI FACSCalibur, Becton Dickinson) using CellQuest Software before and after treatment with the proteasome inhibitor MG-132 (50 μ M, Calbiochem) for 10 h at 37°C. Clone 10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

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³ The abbreviations used are: PI, protease inhibitor; NF- κ B, nuclear factor κ B; GFP, green fluorescence protein; PMSF, phenylmethylsulfonyl fluoride; AMC, 7-amido-4-methylcoumarin; Ub, ubiquitin; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

Cell Extracts and Electrophoretic Mobility Shift Assays. Cellular extracts were prepared from normal and saquinavir-treated cells by dislodging the cells mechanically, washing with ice-cold PBS, and lysing them in TOTEX buffer [20 mM HEPES (pH 7.9), 0.35 mM NaCl, 20% glycerol, 1% NP40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 50 μ M PMSF, and 90 trypsin inhibitor units/ml aprotinin] for 30 min on ice. The lysate was centrifuged at $12,000 \times g$ for 5 min. Protein concentration in the supernatant was determined by the Micro BCA method (Pierce). Fifteen μ g of protein were incubated for 25 min at room temperature with 2 μ l of BSA (10 μ g/ μ l), 2 μ l of dIdC (1 μ g/ μ l), 4 μ l of Ficolll buffer (20% Ficolll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, and 0.1 mM PMSF), 2 μ l of buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40, 2 mM DTT, and 0.1 mM PMSF), and 1 μ l of the [γ - 32 P]ATP-labeled oligonucleotide (Promega; NF- κ B, AGTTGAGGGGACTTCCAGG). A negative control was prepared for one sample by adding unlabeled oligonucleotide in 50-fold excess. Electrophoresis was carried out in native 4% polyacrylamide/0.5-fold Tris/boric acid/EDTA gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphorimager (IPR 1500; Fuji).

Proteasome Function Assays. Proteasome function was measured as described previously (8) with some minor modifications. Briefly, cells were washed with PBS, then with buffer I [50 mM Tris (pH 7.4), 2 mM DTT, 5 mM MgCl₂, and 2 mM ATP], and pelleted by centrifugation. Glass beads and homogenization buffer [50 mM Tris (pH 7.4), 1 mM DTT, 5 mM MgCl₂, 2 mM ATP, and 250 mM sucrose] were added and cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at $1,000 \times g$ for 5 min, and the supernatant was further clarified by spinning at $10,000 \times g$ for 20 min. Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. Twenty μ g of protein from each sample was diluted with buffer I to a final volume of 200 μ l. To assess 26s function, fluorogenic proteasome substrate SucLLVY-AMC (chymotrypsin-like; Sigma) was dissolved in DMSO and added in a final concentration of 80 μ M (in 0.8% DMSO). To assess 20s function, buffer I was replaced by buffer containing SDS [20 mM HEPES (pH 7.8), 0.5 mM EDTA, and 0.03% SDS (9)]. Proteolytic activity was monitored continuously using a fluorescence plate reader (Spectra Max Gemini XS; Molecular Devices; 37°C) at 380/460 nm by release of the fluorescent group AMC.

Immunoblotting. Cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP40, SDS, 10 mM PMSF, aprotinin, and sodium vanadate]. Protein concentrations were determined using the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. Ten μ g of protein were separated on SDS gel (0.1% SDS/12% polyacrylamide) and blotted to polyvinylidene difluoride membranes at 4°C. After blocking with Blotto-buffer (Tris-buffered saline, 0.1% Tween 20, and 5% skim milk) for 1 h at room temperature, the membranes were incubated with a polyclonal antibody against I κ B α (0.5 μ g/ml; BD PharMingen) for 1 h at room temperature. A secondary horseradish peroxidase-conjugated antibody and the ECLplus system (Amersham) were used for visualization.

Flow Cytometry. For assessment of Ub-R-GFP expression, cells were trypsinized, pelleted (5 min, $500 \times g$) and washed twice in PBS. GFP content was analyzed by flow cytometry using the CellQuest Software (FL1-H, FACSCalibur; Becton Dickinson). The DNA profiles of adherent and nonadherent cells in drug-treated populations were analyzed by flow cytometry using CellQuest Software (FL2-A, FACSCalibur; Becton Dickinson). Cells were washed in PBS and fixed overnight in ice-cold 75% ethanol. The next day, cells were washed in PBS. The cell pellet was incubated with 50 μ g/ml RNase A and 1 mg/ml propidium iodide for 10 min and washed in PBS before examination. TUNEL assay was performed using the FlowTACS *in situ* kit (R&D Systems GmbH, Wiesbaden, Germany) following the manufacturer's instructions.

RESULTS

Saquinavir Prevents Activation of Transcription Factor NF- κ B. NF- κ B signaling is normally dependent on proteasomal degradation of the binding inhibitor I κ B α and is prevented by treatment of cells with proteasome inhibitors (10). Murine RAW 264.7 macrophages respond strongly with NF- κ B activation to treatment with LPS (0.1 μ g/ml) and IFN- γ (100 units/ml) for 6 h. This model was,

therefore, used to determine whether simultaneous addition of different concentrations of saquinavir (0, 6.25, 12.5, 25, and 50 μ M) affected the activation process. When total cellular lysates were analyzed at 6 h, we found a concentration-dependent inhibitory effect of saquinavir on constitutive and LPS/IFN- γ -induced increase of NF- κ B DNA-binding activity (Fig. 1A). This inhibition coincided with an accumulation of I κ B α , which indicated involvement of the classical activation pathway of NF- κ B by proteasome-dependent degradation of I κ B (Fig. 1B). Similar results were observed for human PC-3 prostate cancer cells using 25- μ M concentrations of saquinavir (data not shown).

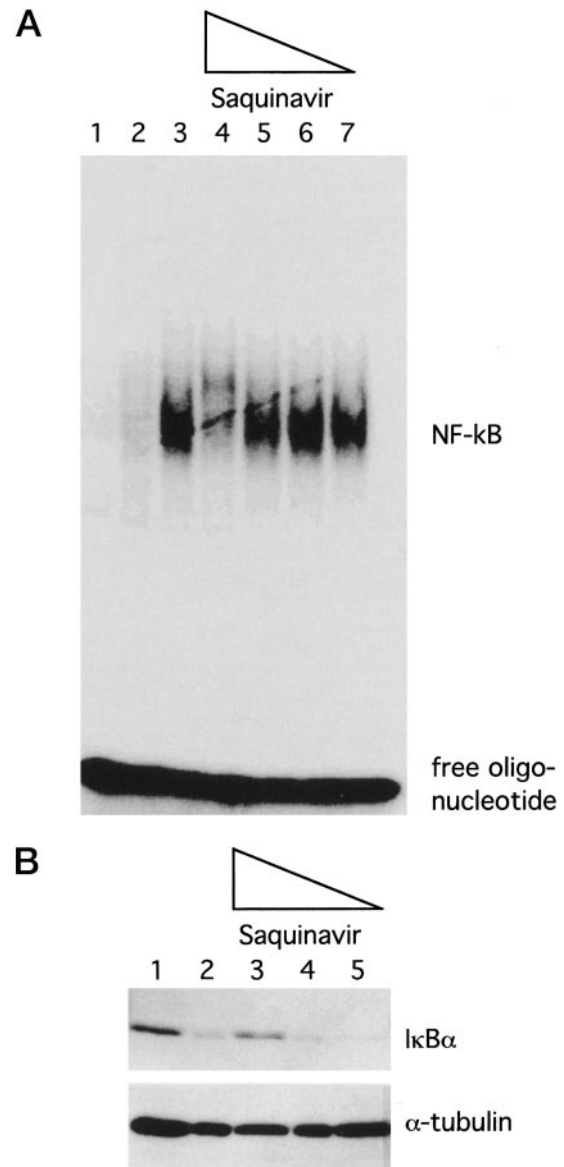


Fig. 1. Saquinavir inhibits activation of NF- κ B. In A, RAW 264.7 macrophages were stimulated with LPS (0.1 μ g/ml) and IFN- γ (100 units/ml) for 6 h in the presence or absence of different concentrations of saquinavir, and cell extracts were analyzed for NF- κ B expression by electrophoretic mobility shift assay. Lane 1, negative control with unlabeled oligonucleotide added in 50-fold molar excess; Lane 2, control nonstimulated cells; Lane 3, cells stimulated with LPS/IFN- γ in the absence of saquinavir; Lanes 4–7, cells stimulated with LPS/IFN- γ in the presence of saquinavir (Lane 4, 50 μ M; Lane 5, 25 μ M; Lane 6, 12.5 μ M; Lane 7, 6.25 μ M). B, Western blot analysis for I κ B α in cell extracts from RAW 264.7 macrophages stimulated with LPS (0.1 μ g/ml) and IFN- γ (100 units/ml) for 6 h in the presence or absence of different concentrations of saquinavir (Lane 1, untreated controls; Lane 2, LPS/IFN- γ , 0 μ M saquinavir; Lane 3, LPS/IFN- γ , 100 μ M saquinavir; Lane 4, LPS/IFN- γ , 50 μ M saquinavir; Lane 5, LPS/IFN- γ , 25 μ M saquinavir).

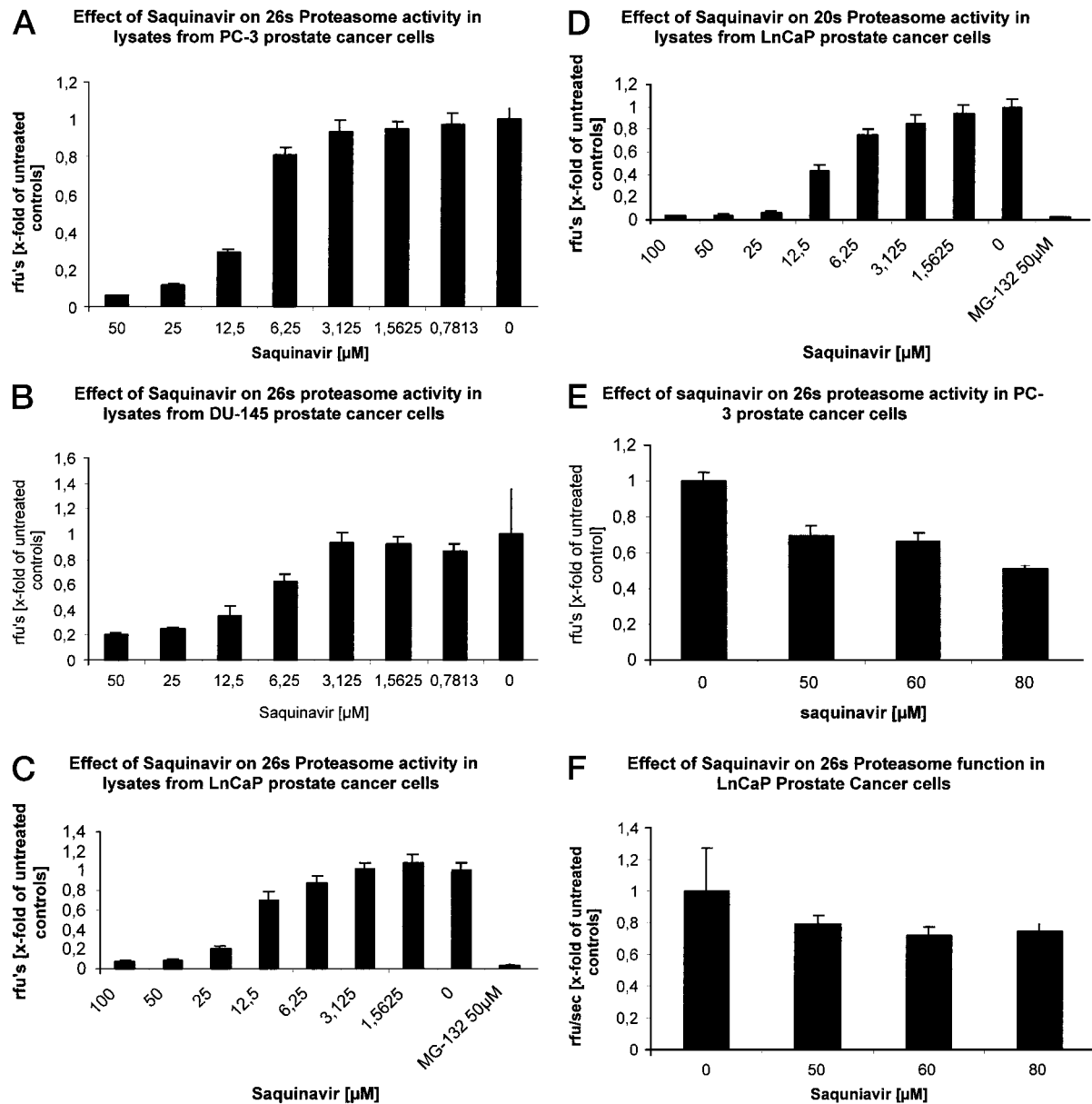


Fig. 2. Saquinavir is a 20s proteasome inhibitor. Chymotryptic 26s and 20s proteasome activity in lysates from PC-3, DU-145, and LnCaP human prostate cancer cells. Cleavage activity was monitored for 30 min and expressed as relative fluorescence units (rfu's) expressed as x -fold activity of untreated controls. Cellular extracts from PC-3 (A), DU-145 (B), and LnCaP (C) cells were incubated with different concentrations of saquinavir. Chymotryptic 26s proteasome activity was assessed using a fluorogenic peptide assay with the specific proteasome substrate SucLLVY-AMC. Release of the fluorogenic compound AMC was monitored continuously in a fluorescence plate reader (excitation, 380 nm; emission, 460 nm). The specificity of the cleavage reaction was demonstrated by the addition of the proteasome inhibitor MG-132 (C, 50 μ M). Saquinavir inhibited chymotryptic 26s proteasome activity in all three of the cell lines in a concentration-dependent manner. Saquinavir also inhibited chymotryptic 20s proteasome activity in LNCaP cells (D), indicating an effect on the 20s core unit rather than on the 19s regulatory unit. The inhibitory effect was also observed when cells, rather than extracts, were incubated in growth media supplemented with different concentrations of saquinavir for 45 min (E and F), which indicated that saquinavir enters the cells, although the concentration needed of the drug was higher.

Saquinavir Is an Inhibitor of the 26s Proteasome. We have previously shown that prostate cancer cells express high constitutive levels of NF- κ B that can be blocked by treatment with proteasome inhibitors (11). The ability of saquinavir to inhibit proteasome function was examined by incubating cellular extracts of PC-3, DU-145, and LnCaP prostate cancer cells with different concentrations of the drug (100, 50, 25, 12.5, 6.25, 3.125, 1.6, and 0 μ M). Chymotryptic, tryptic, and peptidyl-glytamyl 20s and 26s proteasome activities were continuously monitored for 30 min by the release of AMC from the fluorogenic proteasome substrates SucLLVY-AMC, Z-AAR-AMC, and Z-LLE-AMC. Saquinavir inhibited the function of both chymotryptic 26s (Fig. 2, A, B, and C) and 20s (Fig. 2D) and peptidyl-glutamyl (data not shown) proteasome activity in a concentration-

dependent fashion. The IC₅₀ for the chymotryptic 26s proteasome activity was 10 μ M, 1.8 μ M for the chymotryptic 20s proteasome activity, 5.2 μ M for the peptidyl-glutamyl 26s proteasome activity, and 1.6 μ M for the peptidyl-glutamyl 20s proteasome activity. We could not show any effect on tryptic 20s or 26s proteasome activity. In contrast, when PC-3 and LnCaP cells were treated with saquinavir supplemented media for 45 min and the extracts were then tested for proteasome activity, the IC₅₀ was about 80 μ M (Fig. 2, E and F). The difference in the ability of saquinavir to inhibit proteasome activity in whole cells and in extracts is probably attributable to its bioavailability, which may be adversely affected by saquinavir being a substrate of the *mdr-1* gene product P-glycoprotein, which is expressed at high levels in prostate cancer cells (12).

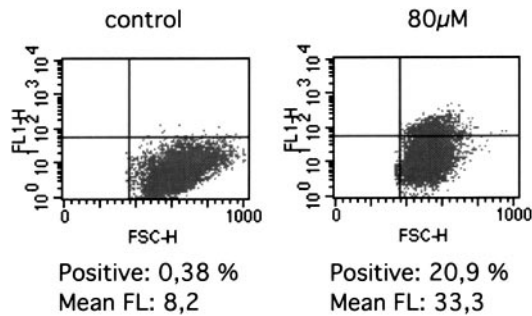


Fig. 3. Saquinavir causes accumulation of the Ub-R-GFP reporter of proteasomal function. Flow cytometric analysis of ECV 304 cells stably transfected with Ub-R-GFP reporter of proteasomal function. Incubation with saquinavir (80 μ M) for 9 h caused an accumulation of Ub-R-GFP in cells. Positive cells were detected in the upper right quadrant. *Positive*, the number of positive cells; *Mean FL*, their average fluorescence.

To confirm that saquinavir can inhibit proteasome activity in cells, ECV 304 cells, transfected with Ub-R-GFP fusion protein (clone 10), were treated for 12 h with media supplemented with different concentrations of saquinavir and incubated for an additional 9 h. Subsequent flow cytometric analysis revealed a concentration-dependent

increase in GFP-positive cells from, initially, 0.38% (0 μ M) to 20.9% (80 μ M; Fig. 3).

Saquinavir Induces Apoptosis in Non-HIV-associated Human Cancer Cells. One of the many consequences of proteasome inhibition is induction of apoptosis (13). The ability of saquinavir treatment to achieve this end point was, therefore, tested. In PC-3, cells, 100 μ M induced apoptosis starting within 60 min (Fig. 4A). Comparable results were obtained in DU-145 (data not shown), U373 (data not shown), and K562 and Jurkat cells (Fig. 4B). By 24 h, all of the cell lines showed typical morphological criteria of apoptosis. Most PC-3 cells tolerated up to 50 μ M for over 24 h, but 60 μ M induced considerable apoptosis by this time point. By 48 h, PC-3 cells showed an increase of the apoptotic (sub-G₁) fraction from 10.4% (0 μ M) to 48.2% (50 μ M), 55.7% (60 μ M) and 78.2% (80 μ M; Fig. 4C). The induction of apoptosis was confirmed by TUNEL-staining (Fig. 4D).

Saquinavir Sensitizes PC-3 and DU-145 Prostate Cancer Cells to Ionizing Radiation. Transient inhibition of proteasome function has been shown to sensitize tumor cells to ionizing radiation (14). To test a possible effect of saquinavir on the clonogenic survival of PC-3 cells after radiation therapy, clonogenic assays were performed. Two-h pretreatment of PC-3 or 3-h pretreatment of DU-145 cells with

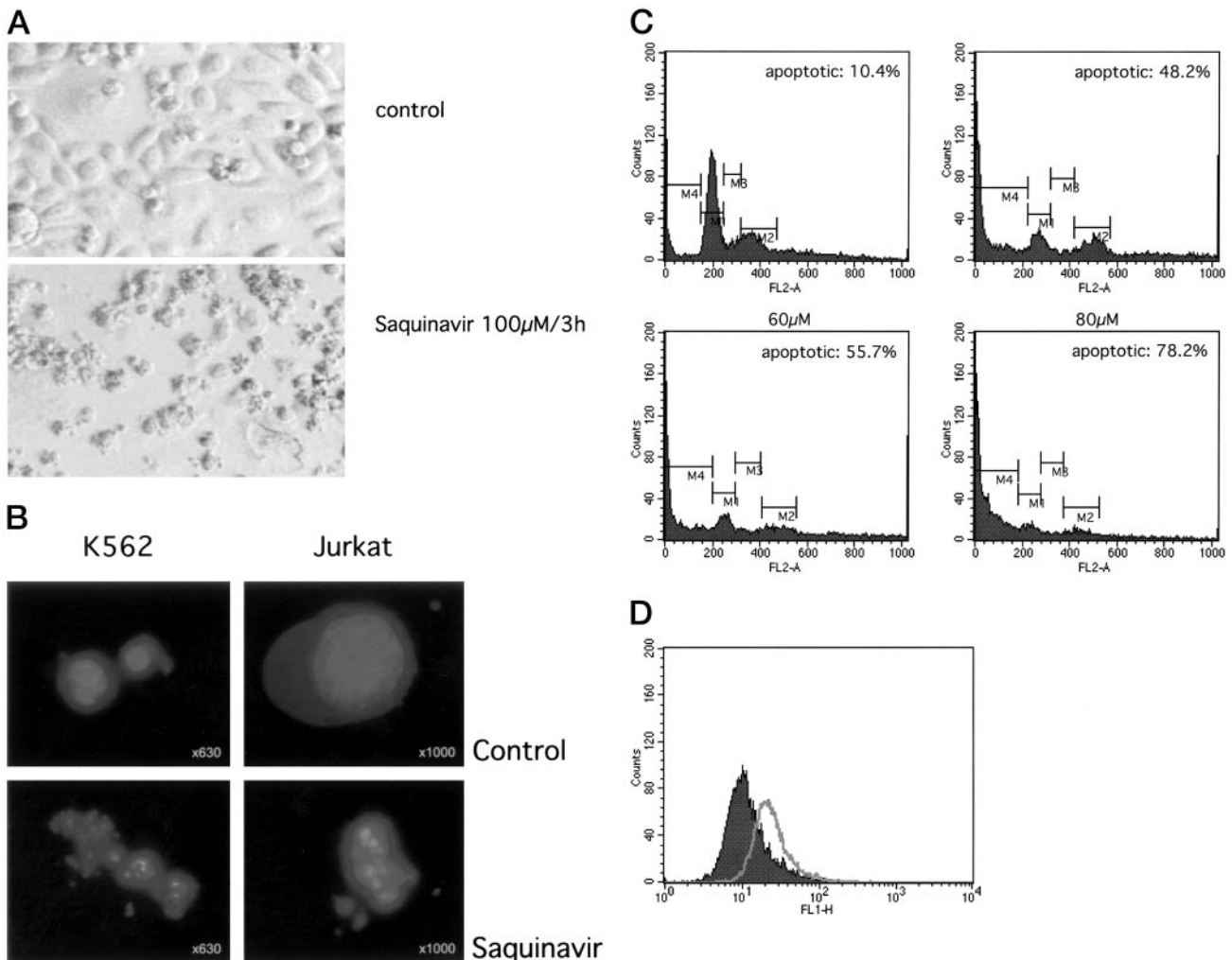


Fig. 4. Saquinavir induces apoptosis in human prostate cancer cells. In A, incubation of PC-3 cells with 100 μ M saquinavir caused a rapid induction of apoptosis within hours. Cells showed membrane blebbing and chromatin condensation as early as 3 h after the start of incubation. B, propidium iodide staining. Treatment of K562 and Jurkat leukemia cells with saquinavir caused membrane blebbing and chromatin condensation 24 h after the start of treatment. C, DNA content of PC-3 prostate cancer cells incubated for 48 h with different concentrations of saquinavir as determined by propidium iodide staining of ethanol-fixed cells. Incubation with saquinavir caused concentration-dependent induction of apoptosis. The percentages of apoptotic cells in the pro-G₁ peaks are indicated. In D, TUNEL staining of PC-3 cells 24 h after the start of incubation with 0 μ M (filled histogram) or 100 μ M Saquinavir (open histogram) detected TUNEL-positive cells with a shift in mean fluorescence from 13.5 to 25.

50 μM and 60 μM concentrations of saquinavir did not significantly alter the plating efficiency of PC-3 cells (control cells, $52.1 \pm 4.2\%$; saquinavir-treated cells, $47.3 \pm 1.7\%$) and DU-145 cells (control cells, $23.1 \pm 2.5\%$; saquinavir-treated cells, $26.0 \pm 2.6\%$). However, pretreatment with saquinavir sensitized the surviving cells to ionizing radiation (Fig. 5; PC-3: control cells, $\alpha = 0.35$, $\beta = 0.045$, $\alpha/\beta = 7.8$; saquinavir-treated cells: $\alpha = 0.334$, $\beta = 0.069$, $\alpha/\beta = 4.8$; DU-145 cells: control cells, $\alpha = 0.35$, $\beta = 0.034$, $\alpha/\beta = 10.3$; saquinavir-treated cells, $\alpha = 0.36$, $\beta = 0.075$, $\alpha/\beta = 4.8$).

DISCUSSION

The Ub/26s proteasome pathway is the major non-lysosomal proteolytic pathway in mammalian cells. It is responsible for the degradation of short-lived (15), and 70–90% of all long-lived proteins (15, 16). Inhibition of proteasome function has been shown to induce apoptosis in cancer cells (11, 17–22), and partial inhibition sensitizes surviving cells to the cytotoxic effects of ionizing radiation and chemotherapeutic drugs (11, 23). Proteolysis by the Ub/26s proteasome pathway is an important component of regulation of cellular functions like signal transduction, cell cycle control, and immune responses (24). Interestingly, the 20s core unit of the proteasome is the only mammalian protease known, thus far, to share specific cleavage action sites with the HIV-1 protease, which may be a pathogenic mechanism adopted by the virus. A recent report indicated that the HIV-1 PI ritonavir inhibits 20s proteasome function (25). Here, we investigated the effect of the HIV-1 PI saquinavir, which is clinically less toxic (1), on 20s and 26s proteasome function and the possible physiological consequences of such an inhibition in human cancer cells.

Prostate cancer cells in general show elevated constitutive DNA-binding activity of the antiapoptotic transcription factor NF- κB (26, 27), and we, and others, have demonstrated that the inhibition of NF- κB induces apoptosis in cancer cells (17, 20, 26, 28). NF- κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the I κB family. Activation of this pathway is normally achieved by phosphorylation, polyubiquitination, and subsequent degradation by the 26s proteasome of one of its most important inhibitors, I $\kappa\text{B}\alpha$. Degradation of I $\kappa\text{B}\alpha$ frees NF- κB for translocation to the nucleus and activation of its target genetic programs (reviewed in Ref. 29). We have shown that the HIV-1 PI saquinavir blocks NF- κB activation in the murine RAW macrophage and human PC-3 prostate cancer cell lines and stabilizes I $\kappa\text{B}\alpha$ in a concentration-dependent fashion. Because activation of NF- κB is an important precondition for replication and persistence of HIV (30), this suggests a pathway for action of saquinavir that is independent of direct viral protease inhibition.

Saquinavir, like ritonavir, was shown to directly inhibit 20s and 26s proteasome function *in vitro*. Because the inhibition of both 26s and 20s function showed similar drug concentration dependency, we conclude that it acts on the 20s core unit of the proteasome. Treatment of cells with saquinavir also inhibited proteasome function, although the IC₅₀ was markedly higher, perhaps because saquinavir is a substrate for the multidrug resistance (*mdr-1*) gene product P-glycoprotein, which is highly expressed in PC-3 human prostate cancer cells (12). Physiological inhibition of proteasome function by saquinavir was demonstrated by the finding of an accumulation of the Ub-R-GFP reporter of proteasome function (7) in living ECV 304 cells stably transfected with this construct.

A physiological consequence of the saquinavir treatment of PC-3 and DU-145 prostate cancer, U373 glioblastoma, and K562 and Jurkat leukemia cells was apoptosis, which occurred at concentrations that

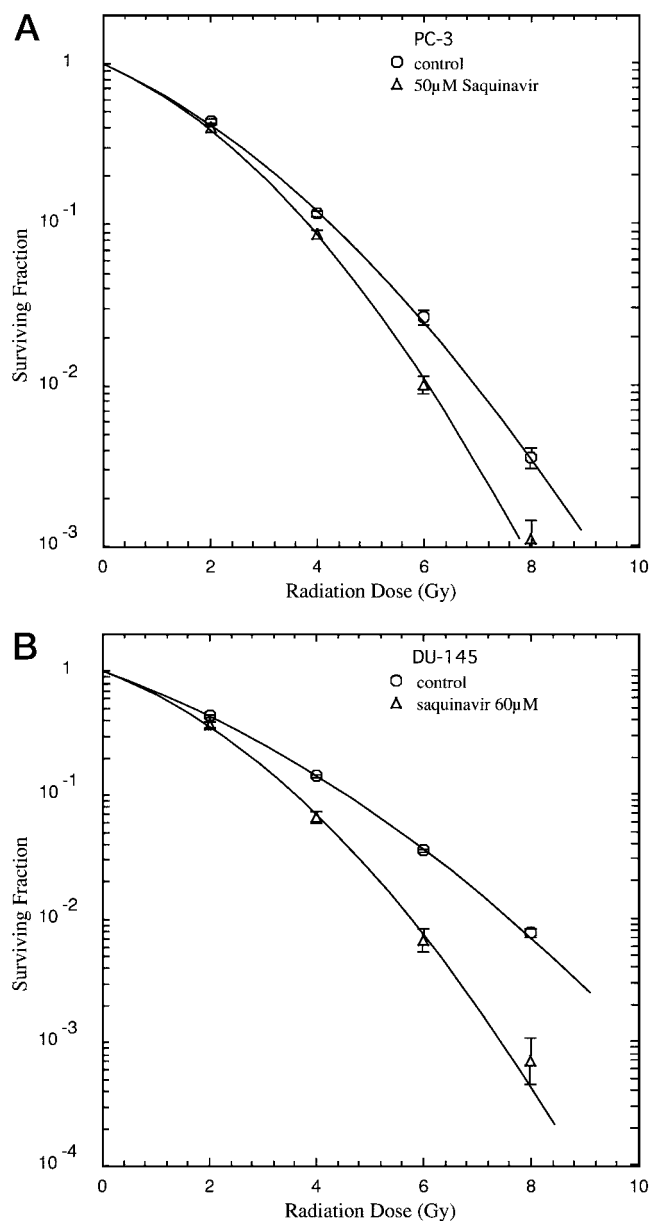


Fig. 5. Saquinavir sensitizes human prostate cancer cells to ionizing radiation. (A) PC-3 prostate cancer cells were incubated with 50 μM concentrations and (B) DU145 prostate cancer cells were incubated with 60 μM concentrations of saquinavir for one h. Cells were washed twice with PBS, trypsinized and irradiated with 0, 2, 4, 6 or 8 Gy. Cells were plated into Petri dishes in triplicate. After 14 days cells were fixed with 75% ethanol, stained with crystal violet and colonies that consisted of more than 50 cells were counted. The number of colonies of each dose point was normalized to the number of colonies of the corresponding unirradiated control. Resulting survival curves were fitted using a linear quadratic model. Pretreatment with saquinavir sensitized the surviving cells to ionizing radiation (PC-3: control cells: $\alpha = 0.35$, $\beta = 0.045$, $\alpha/\beta = 7.8$; saquinavir-treated cells: $\alpha = 0.334$, $\beta = 0.069$, $\alpha/\beta = 4.8$; DU145: control cells: $\alpha = 0.35$, $\beta = 0.034$, $\alpha/\beta = 10.3$; saquinavir-treated cells: $\alpha = 0.36$, $\beta = 0.075$, $\alpha/\beta = 4.8$).

were similar to those needed to inhibit proteasome function. These data are consistent with the hypothesis that saquinavir-induced apoptosis is the result of the inhibition of proteasome function and the blocking of NF- κB activation. We have previously shown that the inhibition of NF- κB activation in these cell lines by transduction with an I κB super-repressor gene also results in apoptosis (11).

The Ub/26s proteasome has recently been identified as a novel target for cancer therapy (11, 21, 31, 32). In this study, short-time preincubation with saquinavir clearly sensitized PC-3 and DU-145 prostate cancer cells to ionizing radiation, which resulted in a change

of the $\alpha:\beta$ ratio from 7.8 and 10.3, respectively, in control cells to 4.8 in saquinavir-treated cells. The clinical significance of these observations is supported by a recent report showing dramatically improved survival for AIDS patients suffering from HIV-related primary central nervous system lymphoma (PCNSL) treated with highly active antiretroviral therapy (HAART) and cranial irradiation when compared with cranial irradiation or HAART treatment alone (33). Results from two recent reports indicate that this effect is independent of the recovery of the immune system (34, 35). Because the inhibition of proteasome function, in general, sensitizes tumor cells to ionizing radiation (11, 23) and HIV-I PIs have been shown to inhibit P-glycoprotein-mediated multidrug resistance (3), HIV-I PIs may become a new class of chemotherapeutic agents in radiochemotherapy. As in HIV therapy, the use of radiation therapy combined with saquinavir and "baby-concentrations" of zidovudine may overcome the problem of low bioavailability of the drug (36).

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