HIV-1 Protease Inhibitors Nelfinavir and Atazanavir Induce Malignant Glioma Death by Triggering Endoplasmic Reticulum Stress

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Abstract

HIV type 1 (HIV-1) protease inhibitors (PI) have been shown to have anticancer activity in non–HIV-associated human cancer cells. The underlying mechanism of this effect is unclear. Here, we show that the PIs nelfinavir and atazanavir cause cell death in various malignant glioma cell lines in vitro. The underlying mechanism of this antitumor effect involves the potentiating stimulation of the endoplasmic reticulum (ER) stress response (ESR), as indicated by increased expression of two ESR markers, GRP78 and CHOP, and activation of ESR-associated caspase-4. Induction of ESR seems to play a central role in PI-induced cell death because small interfering RNA–mediated knockdown of the protective ER chaperone GRP78 sensitizes cells; whereas knockdown of proapoptotic caspase-4 protects cells from PI-induced cell death. Furthermore, the treatment of cells with PIs leads to aggresome formation and accumulation of polyubiquitinated proteins, implying proteasome inhibition. Thus, our results support a model whereby PIs cause tumor cell death via triggering of the ESR, inhibition of proteasome activity, and subsequent accumulation of misfolded proteins. Inhibition of glioma growth via ESR takes place in the in vivo setting as well, as nelfinavir inhibits the growth of xenografted human malignant glioma, with concomitant induction of the proapoptotic ER stress marker CHOP. Because ER stress has also been reported as the mechanism for insulin resistance and diabetes, our ER stress model of PI function may also explain why these drugs may induce insulin resistance as one of their most common side effects. [Cancer Res 2007;67(22):10920–8]

Introduction

HIV type 1 (HIV-1) protease inhibitors (PI) have become important tools in the treatment of HIV infection and include nelfinavir (Viracept), sequinavir (Invirase), ritonavir (Norvir), amprenavir (Agenerase), indinavir (Crixivan), and atazanavir (Reyataz). The addition of these drugs to highly active antiretroviral therapy (HAART) has improved patient outcomes and decreased viral resistance (1). Moreover, the treatment of prostate, melanoma cells (12). In addition, amprenavir and nelfinavir increased the effectiveness of radiotherapy in an animal xenograft tumor model (13). Although various mechanisms have been proposed to explain the antitumor effects of the different protease inhibitors, such as inhibition of nuclear factor κB (NF-κB; ref. 14), blockage of Akt signaling (4, 13), or inhibition of cytochrome P450 3A4 enzyme (CYP 3A4; ref. 9), this issue has not been convincingly clarified.

In this present study, we present evidence that protease inhibitors that might be beneficial for their potential use as anticancer agents is their ability to sensitize cancer cells to radio- and chemotherapy. For example, sequinavir, ritonavir, and indinavir were shown to potentiate the effects of all–trans-retinoic acid on human myelocytic leukemia cells (8), and ritonavir was shown to enhance the anticancer effects of docetaxel on prostate cancer cells in vitro (5). Furthermore, ritonavir was shown to inhibit EL4-T cell thymoma growth in syngeneic mice (6). However, in a study using an established glioblastoma cell line, Laurent et al. (7) observed that ritonavir failed to inhibit tumor growth in vivo, although the drug did display cytostatic and cytotoxic effects in vitro.

An additional feature of protease inhibitors that might be beneficial for their potential use as anticancer agents is their ability to sensitize cancer cells to radio- and chemotherapy. For example, sequinavir, ritonavir, and indinavir were shown to potentiate the effects of all–trans-retinoic acid on human myelocytic leukemia cells (8), and ritonavir was shown to enhance the anticancer effects of docetaxel on prostate cancer cells in vitro (5). Furthermore, ritonavir was shown to inhibit EL4-T cell thymoma growth in syngeneic mice (6). However, in a study using an established glioblastoma cell line, Laurent et al. (7) observed that ritonavir failed to inhibit tumor growth in vivo, although the drug did display cytostatic and cytotoxic effects in vitro.

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assembly, in targeting misfolded proteins for degradation, in ER Ca\(^{2+}\) binding, and in controlling the activation of transmembrane ER stress sensors (17). On the other hand, CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) and caspase-4 are critical executioners of the proapoptotic arm of the ESR (18, 19).

In this study, we show that nelfinavir and atazanavir are able to potently induce ESR in malignant glioma cells, as indicated by elevated levels of GRP78 and CHOP, and activation of caspase-4, which leads to cell death. Moreover, both drugs cause the accumulation of polyubiquitinated proteins and subsequent aggresome formation, consistent with our view that ER stress is due to proteasome inhibition, with resultant accumulation of misfolded proteins. This notion is further supported by our observation that inhibition of general protein synthesis (which prevents an increase in misfolded proteins) abrogates the ESR-stimulatory effects of both nelfinavir and atazanavir. Taken together, our study suggests that protease inhibitors might exert their anticancer activity via the stimulation of ER stress with resultant cell death.

**Materials and Methods**

**Materials.** Nelfinavir (Viracept, 625 mg) was purchased from Agouron Pharmaceuticals Inc., whereas atazanavir (Reyataz, 150 mg) was purchased from Bristol-Myers Squibb Company. The pills were ground and dissolved in 100% ethanol at 25 mmol/L (stock solution). Both drugs were added to the cell culture medium in a manner that kept the final concentration of solvent (ethanol) below 0.8%. Appropriate amount of ethanol (based on the highest concentration of solvent added with the drugs) was always added to the controls.

**Cell lines and culture conditions.** The malignant glioma cell lines U251 and LN229 were provided by Frank B. Furnari and Webster K. Cavenee (Ludwig Institute of Cancer Research, La Jolla, CA). T98G and U87 malignant glioma cell lines were obtained from the American Tissue Culture Collection. All cell lines were propagated in DMEM (Life Technologies BRL) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator at 37°C and a 5% CO\(_2\) atmosphere.

**Immunoblotting and antibodies.** The cells were lysed in radioimmunoprecipitation assay buffer (RIPA), and equal amounts of total cellular lysates were subjected to Western blot analysis with antibodies to ER stress markers GRP78, CHOP, and caspase-4. The levels of GRP78 protein were quantified using ImageJ software. Actin antibody was used as a loading control to verify equal amounts of lysate in each lane.
infiltrated in a 3:1 PO/Eponate resin mixture overnight. The pellets were then immersed in propylene oxide (PO) thrice for 2 min. Pellets were then dehydrated through an ethanol dilution series up to 100% ethanol and placed in 1 h. The cell pellets were then postfixed with 1% osmium tetroxide for 1 h. After fixation, cells were kept in culture undisturbed for 12 to 14 days, during which time the surviving cells spawned a colony of proliferating cells. Colonies were visualized by staining for 4 h with 1% methylene blue (in methanol) and then were counted.

Electron microscopy. Electron microscopy was done by the Doheny Eye Institute Specialized Microscopy Core Facility at the University of Southern California. Cells were spun down into a beam capsule with a clinical centrifuge. Pelleted cells were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/L phosphate buffer (pH, 7.4) at room temperature and then were pelleted. Pellets were then infiltrated in a 3:1 PO/Eponate resin mixture overnight. The pellets were then embedded in 100% Eponate resin (Ted Pel Inc.) in beam capsules and placed in a 60°C oven overnight. After hardening, tissue blocks were ultrathin sectioned at 75 nm thickness and placed on 300 mesh copper grids. Grids were next counterstained with saturated uranyl acetate and lead citrate and then analyzed on a Zeiss EM 10 electron microscope (Zeiss).

Immunohistochemistry. Immunohistochemical analysis of protein expression in tumor tissues was done with the use of the Vectastain ABC kit (Vector Laboratories) according to manufacturer's instructions.

Apoptosis in tumor sections was assessed with the use of the terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay (22). All components for this procedure were from the ApopTag In situ Apoptosis Detection kit (Chemicon), which was used according to the manufacturer's instructions. Hematoxylin was used for background staining.

Colony formation assay. Twenty-four hours after transfaction with small interfering RNA (siRNA), the cells were seeded into six-well plates at 200 cells per well. After complete cell adherence, the cells were exposed to drug treatment for 48 h. Thereafter, the drug was removed, fresh growth medium was added, and the cells were kept in culture undisturbed for 12 to 14 days, during which time the surviving cells spawned a colony of proliferating cells. Colonies were visualized by staining for 4 h with 1% methylene blue (in methanol) and then were counted.

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Transfections with siRNA. Cells were transfected in six-well plates with the use of LipofectAMINE 2000 (Invitrogen) according to manufacturer's instructions. The different siRNAs were synthesized at the microchemical core laboratory of the USC/K. Norris Jr. Comprehensive Cancer Center, and their sequences were as follows: si-GFP (sense): 5'-CAAAGUCUAGCUGGAGA- GUUCTT-3'; (antisense): 5'-GAUCUCAGGUGUCGUUGTT-3'; si-GRP78 (sense): 5'-GGAGCCGAUGAUCAUCAGATT-3'; (antisense): 5'-CGCAUGUA- CAGGCUUCCTT-3'; si-Caspase-4 (sense): 5'-AAGUGGCCUUCUACAGU- CAUTT-3'; (antisense): 5'-AAUGACUGAAGGCGCACC-3'.

Cytoplasmic calcium imaging. The cells were loaded by incubating them with 4 μmol/L Fura-2/AM (Invitrogen) for 30 min at room temperature in external solution containing 138 mmol/L NaCl, 5.6 mmol/L KCl, 1.2 mmol/L MgCl2, 2.6 mmol/L CaCl2, 10 mmol/L HEPES, and 4 mmol/L glucose (pH, 7.4). After loading, the cells were rinsed and transferred to the imaging setup. The cells were treated with individual drugs for 10 h, whereas fluorescence was elicited with the excitation wavelength alternating between 350 and 380 nm, using a Polychromator V (TILL Photonics GmbH) to provide illumination with a Zeiss Axiovert 100 microscope with a Zeiss Fluor 40× oil objective (Carl Zeiss). Images were captured using a Cascade 512B CCD camera (Photometrics) controlled with MetaFluor software (Molecular Devices) at 0.5 Hz acquisition frequency. Ratios of the images obtained at 350 nm and 380 nm excitation were used to illustrate changes in the cytoplasmic calcium concentration according to the principles developed by Tsien (23).

Tumor growth in nude mice. Male athymic nu/nu mice 4 to 6 weeks old were obtained from Harlan and implanted s.c. with 5 × 106 U87 glioblastoma cells as described in detail elsewhere (24). For the determination of tumor growth during continuous drug treatment, 40 mg/kg nelfinavir was given via direct administration into the stomach...

![Figure 2](image-url)
with a stainless-steel ball-head feeding needle (Popper and Sons Inc.) in 25% ethanol, and tumor growth was monitored and recorded as described (24). For the examination of short-term in vivo effects of drugs on CHOP expression, the animals received a daily dose (120 mg/kg) of the drug in the same way. Ninety-six hours later, the animals were sacrificed, and the tumors were harvested for analysis.

Results

To establish the effects of nelfinavir and atazanavir on malignant glioma cells in vitro, we did conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, which measured short-term growth and survival of the treated cell culture, as well as colony-forming assays, which revealed the ability of individual cells to survive and develop a new colony. We picked three glioblastoma cell lines (U251, LN229, T98G) based on their different genetic background. First, the cell lines were cultured in 96-well plates in the presence of nelfinavir and atazanavir for 48 h, and cell proliferation was determined using MTT assay. Both drugs inhibited cell growth in all cell lines in a concentration-dependent manner (Fig. 1A). Although the sensitivity to these drugs differed from cell line to cell line, the overall viability was significantly decreased in all cell lines used. In addition, the inhibitory effects of nelfinavir seemed to be more potent than those of atazanavir. Second, colony-forming assays were done with U251 and LN229 cells. As shown in Fig. 1B, both drugs decreased cell survival, and nelfinavir again was more effective than atazanavir.

Interestingly, we noted that the sensitivity of the various glioma cells to protease inhibitors correlated with their sensitivity to the model ER stress inducer thapsigargin.6 This observation prompted us to investigate the effects of nelfinavir and atazanavir on the expression of ER stress markers GRP78, CHOP, and caspase-4. Glioma cell lines were cultured in the presence of nelfinavir and atazanavir, and total cellular lysates were analyzed by Western blot with antibodies to GRP78, CHOP, and caspase-4. As shown in Fig. 1C, the treatment with nelfinavir and atazanavir strikingly increased GRP78 and CHOP protein levels in a concentration-dependent manner in all cell lines. Additionally, both drugs caused the activation of caspase-4, as indicated by the appearance of a cleaved caspase-4 protein with lower molecular weight.

To determine whether the ESR was involved in cell death induced by nelfinavir and atazanavir, we applied specific siRNAs to knock down the expression of either GRP78 or caspase-4. Glioma cells were transfected with siRNA and treated with the drug for 48 h, and the percentage of surviving cells was determined with the use of the colony formation assay. When GRP78 levels were reduced by siRNA, the cells became more sensitive, and there was less cell survival (Fig. 2A). In the case of caspase-4 siRNA, the opposite was observed; we found that the sensitivity of cells to drug treatment was significantly reduced (Fig. 2A). In addition, specific knockdown of the target was confirmed by Western blot analysis. As shown in Fig. 2B, siRNA to GRP78 reduced GRP78 protein levels; in the case of caspase-4, the specific siRNA not only reduced the overall amount of this target protein, but also prevented the appearance of the cleaved, i.e., activated, form of caspase-4.

Calcium homeostasis is an important function of the ER, and calcium disturbances result in ER stress. Thapsigargin, a widely used model inducer of ER stress, acts by inhibiting ER calcium ATPase activity, thereby causing an immediate spike in cytoplasmic calcium levels (25). To determine whether protease inhibitors would affect cytoplasmic calcium levels, we assessed the effect of acute exposure to nelfinavir and atazanavir. For this purpose, U251 cells were loaded with Fura-2/AM, exposed to 50 μmol/L nelfinavir or 100 μmol/L atazanavir, and the increase in cytoplasmic calcium levels was measured. As shown in Fig. 3, cells treated with nelfinavir and atazanavir did not display a rapid calcium spike, but rather maintained steady calcium levels after exposure to the drugs. In contrast, celecoxib, which is known to generate a rapid calcium spike (26, 27), was used as a positive control and caused an immediate cytoplasmic calcium spike (Fig. 3).

When tumor cells treated with nelfinavir or atazanavir were observed under light microscopy, we noticed striking morphologic changes (Fig. 4A). Following 24 h of treatment with either nelfinavir or atazanavir (only nelfinavir shown), large cytoplasmic vacuole-like structures could be observed. To investigate this phenomenon in greater detail, we applied transmission electron microscopy to nelfinavir-treated cells. Once again, large cytoplasmic vesicles were observed and were identified as grossly dilated, stressed ER. Similar effects on the ER were previously described by Dorner et al. (28) in Chinese hamster ovary cells, in which the synthesis of secreted proteins was excessively increased. In addition to the enlargement...

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6 Unpublished data.
of the ER, we detected that a majority of drug-treated cells contained a structure consistent in appearance with an aggresome (Fig. 4B). Aggresomes are presumed to consist of an aggregation of ubiquitinated proteins due to the inhibition of proteasome activity (29, 30). Therefore, to investigate if the accumulation of polyubiquitinated proteins would occur during protease inhibitor treatment, lysates of cells treated with nelfinavir and atazanavir were subjected to Western blot analysis with antibody against ubiquitin. A prominent increase of polyubiquitinated proteins of various different sizes, represented by a smear on the blot, was observed in drug-treated cells (Fig. 4C), indicating that proteasome activity was indeed inhibited by these protease inhibitors.

A prominent feature of the ESR is a general, transient down-regulation of overall protein synthesis, in combination with selectively increased translation of ER stress proteins, such as GRP78 (31, 32). We therefore investigated whether nelfinavir would impair cellular translation by determining the incorporation of 35S-methionine into newly translated proteins. As shown in Fig. 5A, 2 or 8 h of treatment with 30 μmol/L nelfinavir had very little effect on the rate of translation as compared with thapsigargin or the potent translational inhibitor cycloheximide, which both reduced ongoing translation by ~90% at 2 h. At 18 h, however, there was a noticeable decrease in protein translation rate in cells treated with nelfinavir; at 30 μmol/L, this reduction was 30% (Fig. 5A). Nelfinavir at 50 μmol/L and atazanavir at 80 μmol/L inhibited protein synthesis rate by >50% at 24 h (Fig. 5B). Interestingly, after 8, 18, and 24 h of treatment with nelfinavir and 24-h treatment with atazanavir, a prominent band with an apparent molecular weight of 78 kDa became visible. Via immunoprecipitation, we identified this band as GRP78 (Fig. 5C).

Thus far, our data indicated a model by which nelfinavir inhibited the proteasome, leading to the gradual accumulation of misfolded proteins, which caused ER stress and subsequent cell death. To further confirm this model, we employed the protein synthesis inhibitor cycloheximide, which would be expected to prevent the accumulation of newly synthesized, misfolded proteins, thereby averting the generation of ER stress. Toward this end, U251 glioma cells were treated with nelfinavir or atazanavir in the absence or presence of cycloheximide. After 24 h, light micrographs of all groups were taken. As shown in Fig. 5D, cell cultures treated with nelfinavir or atazanavir alone displayed striking morphologic changes in that the cells had lost most of their contact to the substratum, which led to cellular rounding and partial to complete detachment from the culture plate, consistent with the early phases of cellular death. In stark contrast, the presence of cycloheximide entirely prevented these drug-induced morphologic changes, and the cells maintained the same morphology as untreated control cells (Fig. 5D). We next investigated whether this obvious protective effect of cycloheximide could also be observed at the molecular level and, therefore, analyzed the amount of polyubiquitinated proteins and activation of caspase-4 by Western blot analysis. Consistent with the results above, cycloheximide prevented the accumulation of polyubiquitinated proteins, as well as the cleavage/activation of caspase-4, in nelfinavir- and atazanavir-treated cells (Fig. 5D).

Finally, in an effort to establish the antitumor effects of nelfinavir on malignant cells in vivo and to determine whether ER stress might be relevant in the in vivo setting as well, a xenograft nude mouse tumor model was used. U-87 malignant glioma cells were chosen because of their high in vivo tumorigenicity, and their genetic profile (p53 wild type, PTEN mutant) is consistent with a de novo glioblastoma (33–35). As shown in Fig. 6A, the daily treatment of tumor-bearing animals with nelfinavir significantly inhibited tumor growth during long-term (6 weeks) therapy. In
addition, after short-term treatment (96 h) of tumor-bearing animals with nelfinavir, highly elevated levels of the ER stress indicator protein CHOP could be detected in the tumor tissue from these animals; whereas non–drug-treated animals displayed only barely detectable amounts of this protein (Fig. 6B). In addition, the highly elevated amount of CHOP protein after nelfinavir treatment correlated with significantly increased apoptosis in the tumor tissue, whereas tumors from vehicle-treated animals did not display elevated levels of apoptosis (Fig. 6B).

Discussion

Despite growing interest in HIV-1 protease inhibitors (PI) as anti-cancer drugs, the anticancer mechanism of action of this group of chemical compounds is unknown. In our current study, we show that the PIs nelfinavir and atazanavir induce glioma cell death by triggering the ESR. In general, the main purpose of the ESR is to alleviate specific disturbances, for instance, changes in the ionic conditions of the ER or accumulation of misfolded proteins, and restore proper homeostasis. Major components of this
protective function are chaperone proteins, such as GRP78 (17). However, if the insult is too severe and cannot be eliminated, the ESR will initiate cell death via its proapoptotic components, which include CHOP and caspase-4 (18, 19).

Recently, Gills et al. (36) have evaluated seven Food and Drug Administration–approved HIV PIs (amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir) against lung cancer and found that nelfinavir was the most potent. Therefore, we used nelfinavir as one of the first-generation and atazanavir as one of the newer generation PIs, and we found that both of these drugs potently triggered ESR. At the molecular level, this effect was revealed by the robust induction of the ESR markers GRP78 and CHOP and the activation of ESR-associated caspase-4 (Fig. 1). In addition, both drugs caused dilated ER compartments (Fig. 4), which is a typical morphologic feature of cells experiencing severe ER stress (28).

Our siRNA experiments provided evidence that the induction of ESR indeed plays a critical role in the anticancer activity of these PIs. Specifically, knockdown of GRP78 (the protective component of the ESR) enhanced cell death by nelfinavir and atazanavir, whereas knockdown of caspase-4 (a proapoptotic component of the ESR) significantly increased cell survival after treatment with these drugs (Fig. 2). Both outcomes are consistent with the prosurvival role of GRP78 and the proapoptotic role of caspase-4 in the ESR and reveal a direct involvement of this process in PI action. Similar siRNA-type approaches by other investigators have shown a prominent role of the ESR in cell death induced by other categories of drugs as well. For example, knockdown of GRP78 resulted in decreased survival of gastric adenocarcinoma cells after treatment with celecoxib, a cyclooxygenase-2 inhibitor (37), and sensitized breast cancer cells to etoposide-mediated cell death (38). Conversely, knockdown of caspase-4 was shown to protect pancreatic cancer cells from apoptosis induced by bortezomib, a proteasome inhibitor that is clinically used in the therapy of multiple myeloma (29).

Our investigation of protein translation rates showed a moderate inhibition of overall protein synthesis after prolonged incubation of cells with PIs, concomitant with selectively increased expression of GRP78 (Fig. 5). Such differential effects are typical indicators of the ESR, although the severity and timing of inhibition may vary greatly depending on the insult. For example, shutdown of general translation by thapsigargin, a potent inhibitor of ER calcium pumps, is rapid and very efficient due to the immediate increase in cytosolic calcium concentrations (ref. 39; see also Fig. 5).

Figure 6. Nelfinavir inhibits tumor growth and stimulates ESR and apoptosis in tumor cells in vivo. A, nude mice were implanted s.c. with U87 glioblastoma cells. Once palpable tumors had formed, the animals received daily gavage with nelfinavir or the same volume of solvent (25% ethanol; 40 mg/kg). Tumor size was determined every 3 to 6 d. Points, mean tumor volume in each group; bars, SE; n = 5. P values between control and nelfinavir-treated animals on day 42 and 48 are indicated.

B, Nude mice were implanted s.c. with U87 glioblastoma cells. Once tumors had reached a volume of 500 mm3, two animals each received either nelfinavir or vehicle alone (25% ethanol) for 96 h (120 mg/kg). Thereafter, all four animals were sacrificed, and their tumors were analyzed by immunohistochemical staining for CHOP protein as well as TUNEL assay for cell death/apoptosis. Top, expression of CHOP protein. Small black rectangles, enlarged areas of the same photograph shown below in the middle. Bottom, cell death (arrows, examples of TUNEL-positive, i.e., apoptotic, cells). In all cases, representative sections are shown.
other hand, bortezomib, which triggers ESR via the inhibition of the proteasome, leads to a gradual buildup of misfolded protein levels and, therefore, requires longer incubation times and affects general translation rates only moderately (29). In comparison, we find that the kinetics of translational inhibition by nelfinavir resemble those of bortezomib and do not involve rapid changes in cytoplasmic calcium (Fig. 3). This similarity is noteworthy in light of our observation that treatment of glioblastoma cells with nelfinavir and atazanavir results in the accumulation of polyubiquitinated proteins and aggresome formation (Fig. 4), which are typical indicators of proteasome inhibition. Furthermore, reports by others have shown that nelfinavir impairs proteasome function in human hepatoma cells (40), and two further PIs, saquinavir and ritonavir, were shown to inhibit proteasomal activity in prostate carcinoma and thymoma cell lines (6, 10). Thus, our results provide additional support for the emerging view that some, if not all, HIV-1 PIs act as inhibitors of the proteasome. Furthermore, they suggest that this function might participate in triggering ESR, in agreement with several reports linking the inhibition of proteasomal activity by bortezomib and other established proteasome inhibitors to the accumulation of misfolded proteins and subsequent ESR (29, 41, 42).

The above results are consistent with the following proposed model of PI antitumor action: PIs inhibit the proteasome, which prevents the degradation of proteins; at the same time, general protein synthesis continues to proceed, which results in the accumulation of unfolded/misfolded proteins. This, in turn, triggers ESR as the cell’s attempt to neutralize impending proteotoxicity. As part of this protective response, the ER chaperone GRP78 is induced, and the rate of new protein synthesis is slowed. However, because proteins cannot be degraded due to constant inhibition of the proteasome in the continued presence of the drug, the protective arm of the ESR eventually is overwhelmed, and the balance is shifted toward its proapoptotic components (CHOP and caspase-4), which initiate cell death. This order of events is also supported by our finding that cycloheximide efficiently blocks the induction of ESR by PIs, i.e., PI-induced morphologic changes, accumulation of polyubiquitinated proteins, and cleavage of caspase-4 do not take place in the presence of this nondiscriminatory inhibitor of total protein synthesis (Fig. 5), quite likely due to the cell’s inability to accumulate newly synthesized unfolded/misfolded proteins under these conditions.

An important question resulting from our results was whether the effects observed in vitro would also take place in vivo. Two of our results indicate that the answer is yes. First, in our xenograft tumor model, nelfinavir significantly inhibited U87 glioma growth in vivo. Second, nelfinavir also caused robust induction of the ESR indicator CHOP and apoptosis in tumors from these animals (Fig. 6). Taken together, these results reveal that the antitumor and ESR-inducing effects of nelfinavir are not restricted to in vitro conditions, but also take place in the in vivo setting. Additionally, our in vitro results show that the induction of ESR markers, as well as the noticeable inhibition of glioblastoma cell proliferation, by nelfinavir can take place at concentrations of 6 μmol/L and below (Fig. 1A and B). Such concentrations are known to be attainable clinically (43, 44) and, therefore, open the prospect that antitumor effects of nelfinavir might be achievable in cancer patients as well. Cancer cells generally display elevated metabolic rates and increased rates of protein synthesis. Because of this distinction, the consequences of proteasome inhibition would be expected to be more deleterious in cancer cells than in normal cells. A few recent studies indeed seem to support this concept. For example, it was shown that the proteasome inhibitor bortezomib induced aggresome formation and apoptosis in pancreatic carcinoma cells, but did not exert these effects in normal pancreatic epithelial cells in vitro or in vivo (30). Similarly, the PI ritonavir potently stimulated apoptosis in various tumor cell lines, whereas non-transformed cell lines and terminally differentiated bone marrow macrophages were comparatively resistant to this effect (6).

The use of PIs in gliomas has a number of potential advantages and disadvantages. PIs are small molecules that should cross the blood-brain barrier (BBB) readily. They have already been shown (albeit, indirectly) to have activity in the central nervous system (CNS), as the number of HIV patients with CNS lymphomas has decreased significantly since the initiation of HAART (2). Laurent et al. have shown that ritonavir penetrated across the BBB and achieved adequate concentrations in the CNS in an intracranial glioma model (7). Yilmaz et al. (45) have measured cerebrospinal fluid (CSF) levels of both nelfinavir and saquinavir, with better CSF concentrations of nelfinavir, compared with saquinavir.

One major potential risk for PIs is the development of insulin resistance with progression to type II diabetes (46, 47). In this regard, it is interesting to note that several recent papers have established ER stress as a key link between obesity, insulin resistance, and type II diabetes (48, 49). In particular, it was shown that obese mice experienced chronic ESR, which lead to insulin resistance and type 2 diabetes, and these pathologic metabolic consequences could be prevented by an orally active chemical chaperone that alleviated ER stress in cells and whole animals (48, 49). The development of insulin resistance may be problematic in brain tumor patients who are often on systemic steroids. Many of these patients may have their systemic blood sugar level increased with the combination of Decadron and PIs; how significant this elevation becomes will need to be determined.

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