Review

In vitro and *in vivo* effects of HIV protease inhibitors on apoptosis

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Abstract

Development of potent inhibitors of HIV protease has revolutionized the treatment of HIV infection. HIV protease inhibitors (PI) have caused more dramatic improvements in CD4 T-cell numbers than in other therapies that were available previously, prompting investigators to assess whether PI possess intrinsic immunomodulatory effects. An emerging body of data indicates that HIV PIs are antiapoptotic, although the exact molecular target responsible for this antiapoptotic effect remains to be defined *in vitro* and *in vivo*. Paradoxically, high-dose PI also may have proapoptotic effects, particularly when assessed *in vitro* in transformed cell lines and implanted mouse models. Future research will define molecular targets of PI that are responsible for their apoptotis modulatory effects (both pro- and anti-apoptotic). In addition, evaluation of the clinical utility of PI-based therapy in those non-HIV disease states that are characterized by excessive apoptotis will reveal the full clinical potential of this intriguing class of drugs.
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Role of HIV Protease in HIV Pathogenesis

The HIV genome consists of a single-stranded positive sense RNA molecule that is organized into three major coding elements: *gag, pol*, and *env*. The enzymes necessary for viral replication (protease, reverse transcriptase, and integrase) are included within the *pol* gene. During viral assembly and maturation, P55 *gag* precursor and P160 *gag/pol* precursors are protealitically cleaved by preformed protease, which is contained within the HIV virion and introduced into the cell at the time of initial infection. This preformed protease consequently generates new protease by virtue of new cleavages between the protease and reverse transcriptase regions of the *pol* gene. HIV protease is critical to the HIV life cycle, as demonstrated by observations that mutation or inhibition of HIV protease results in the formation of structurally disorganized, noninfectious viral particles. Moreover, naturally occurring mutations that arise as a consequence of sub-suppressive antiretroviral therapy results in generation of viral particles, which have impaired replication kinetics as well as impaired cleavage kinetics of mutant protease against the wild-type *gag/pol* substrate cleavage site.

In addition to its role in viral replication, HIV protease may also contribute to HIV pathogenesis itself. HIV protease is intrinsically cytotoxic and leads to death of both human and bacterial cells following transfection. In different contexts, the phenotype of HIV protease-mediated death is either apoptotic or necrotic. The mechanisms of cell death by HIV protease remain undefined, although HIV protease itself may directly cleave cell regulatory proteins to favor cell death. For example, Strack and co-workers[1,2] identified BCL-2 as a apoptosis regulatory protein, which is cleaved by HIV protease. More recently, other cellular proteins, including actin, laminin-B, Desmon, and Vimentin have been shown to be cleaved directly by HIV protease,[3–9] and when the eukaryotic initiation factor of translation (e1F4G-I) is directly cleaved by HIV protease, there is impaired translation of capped and uncapped mRNAs,[8,10,11] which may in turn lead to metabolic arrest, cellular respiration, and consequent death. Finally, studies derived from a cell-free system indicate that HIV protease may cleave procaspase 8, resulting in a classical mitochondrial-dependent pathway of apoptosis involving Bid cleavage, mitochondrial depolarization, apoptosome formation, nuclear fragmentation, and PARP cleavage.[12]

Importantly, the apoptotic pathology of HIV infection is complex, as cells directly infected with HIV, as well as cells that are not directly infected – succumb an apoptotic death. Virtually every HIV-encoded protein, in addition to HIV protease discussed above, has been suggested to modify host cell apoptosis, including gp120, gp160, Tat, Nef, Vpr, Vpu, and Vif. Details of the effects of individual proteins on apoptosis during HIV is beyond the scope of this review, but in most cases, there impact is cell type specific, and in many cases these proteins can be proapoptotic or antiapoptotic depending on the context.[13]

Role of HIV Protease Inhibitors in HIV Treatment

The optimal current management paradigm for HIV disease is highly active antiretroviral therapy (HAART), which involves concurrent administration of multiple antiviral drugs that

simultaneously target multiple aspects of the viral life cycle in order to both suppress viral replication and reduce the likelihood of generating viable escape mutants in treated individuals. HAART most often combines inhibitors of HIV reverse transcriptase with inhibitors of HIV protease. At present, there are eight currently approved protease inhibitors (PIs) (saquinivir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanivir, and fosamprenavir), and several others under development. The PI class of antiretroviral agents target the aspartyle protease of HIV, inhibiting the cleavage of viral polyproteins and the subsequent generation of individual viral proteins. This in turn prevents the formation of functional virions, although these drugs do not prevent upstream infection events, including entry reverse transcription and integration.

The introduction of HAART has been associated with dramatically reduced morbidity and mortality in patients infected with HIV, and has led to both quantitative and qualititative improvements in host immune function. The advent of PI-based antiretroviral agents stimulated considerable interest in evaluating changes in the CD4 T-cell kinetics, in particular, whether the principal cause of CD4 depletion is impaired production or enhanced destruction. Recent data from two independent groups using independent approaches each conclude that the principal cause of CD4 T-cell decline is enhanced T-cell death, rather than impaired production. Since both studies[14,15] showed that T-cell production rates in HIV infection actually increases, the degree of T-cell destruction is likely greater than that previously believed, as CD4 T-cell numbers decline even in the face of increased CD4 T-cell production rates. Moreover, T-cell production rates are highest in patients with the highest viral burdens, and initiation of antiretroviral therapy causes marked reductions in T-cell proliferative responses seen within 1–12 weeks. The changes that occur in naïve CD4 T-cell proliferation rates correlate with increases in the levels of T-cell receptor excision circles. Finally, the reduced proliferation which is seen in response to HAART is lost following HAART cessation.[16]

In HIV-infected patients who are not receiving antiretroviral therapy, CD4 T-cell numbers continue to decline gradually over the course of the disease. Numerous studies, both *in vitro* and *in vivo*, indicate that enhanced apoptosis is a dominant mechanism for this CD4 T-cell loss. Such apoptosis occurs both within cells directly infected with HIV, as well as in those cells that are uninfected with HIV through bystander mechanisms (reviewed in Phenix *et al.*[17]). The magnitude of the CD4 T-cell apoptosis occurring in untreated patients appears to correlate directly with levels of immune activation, degree of viral replication, and stage of HIV disease (reviewed in Gougeon[18]). This enhanced lymphocyte apoptosis can be directly demonstrated in peripheral blood lymphocytes from HIV-infected patients when examined *ex vivo*, as either fresh samples, samples that have been cultured overnight, or samples that have been stimulated with either apoptosis inducing ligands, mitogen, or T-cell receptor ligation. In all situations, cells from HIV-infected patients have exaggerated apoptotic responses compared to cells from HIV uninfected patients. Not only is enhanced CD4 and CD8 T-cell apoptosis present in the peripheral blood compartment but also within lymphoid tissues (reviewed in Badley *et al.*[19]).

Since PI-based HAART dramatically increases CD4 T-cell numbers, it became of interest to determine what, if any, effect such therapies have on CD4 T-cell apoptosis. With remarkable consistency, numerous studies have now demonstrated that CD4 T-cell apoptosis is dramatically reduced in response to PI-based therapy. Such reductions are observed in the peripheral blood, in cells cultured *in vitro* either in the presence or absence of activating and/or apoptotic stimuli, as well as in the lymphatic compartments. Such reductions in apoptosis are accompanied with reductions in HIV viral burden, decreases in HIV-associated immune activation, and improvements in patient's health (reviewed in Phenix *et al.*[17]). Moreover, where there is poor CD4 T-cell recovery persistently high levels of apoptosis are maintained.[20]

The mechanisms underlying the reduction in exaggerated apoptosis that follow HAART are almost certainly due in part to reductions in viral burden that result from treatment. Indeed, reductions in apoptosis are seen in patients who received non-PI-based therapies, indicating that reduced viral replication in and of itself is associated with reduced apoptosis.[21] In addition, however, it is possible that PIs may possess intrinsic antiapoptotic properties, and a variety of indirect evidences suggest that this is the case:

1. CD4 T-cell numbers increase more with PI-based HAART compared to treatment that does not use PI, even when with equivalent viral suppression.[22,23]
2. PI-based therapy may cause CD4 T-cell increase despite failure to suppress HIV viremia significantly.[24,25]
3. Regimens containing PI produce a greater CD8 T-cell increase than therapies using reverse transcriptase inhibitors alone.[26]
4. CD4 T-cell number continues to increase in patients with suppressed viral replication who continue PIs compared to those who switch off PI to start nonnucleoside-based reverse transcriptase inhibitors.[27]
5. Apoptosis is reduced following PI monotherapy, even in the absence of significant antiviral effect.[28]
6. Significant CD4 T-cell increases following PI-containing regimen compared to non-PI-containing regimens, even in the face of inferior antiviral outcomes[29–31] (see Figure 1).

This abundant indirect data suggest that PI-based therapy may result in improved CD4 T-cell reconstitution compared to non-PI-containing antiretroviral therapy. In a study designed to assess this question directly, 55 HIV-infected patients who were naïve to prior antiretroviral therapy were randomized to receive lopinavir/ritonavir, and nevaripine (PI containing) or lamivudine, stavudine, abacavir, plus nevaripine (PI sparing). Viral load reductions were comparable between groups at weeks 8 and 48. An 8-week follow-up demonstrated similar CD4 T-cell change, CD8 T-cell change, as well as no differences in lymphocyte subset analyses comparing naïve and memory T-cell subsets. On the basis of these findings, the authors conclude that CD4 T-cell restoration is comparable between PI-containing *versus* PI-sparing antiretroviral regimens. However, also included in that manuscript is a comparison of spontaneous apoptosis between groups. In the PI-sparing treatment arm, spontaneous apoptosis reduces gradually during the course of therapy. In contrast,

in the PI-containing treatment arm, apoptosis is dramatically reduced after 1 week of treatment, a change which is not observed in the NRTI arm.[32] Of note, this dramatic reduction in apoptosis that is seen prior to significant reduction in viral burden is entirely consistent with previous publications in a cohort of nonrandomized patients[28] where dramatic reductions in apoptosis are seen within the first 4 days of treatment.
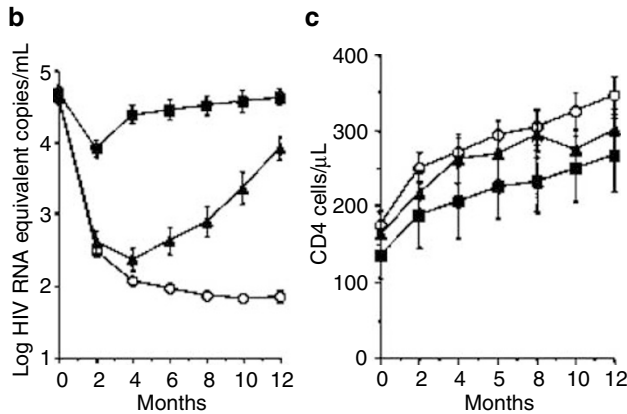

Figure 1 A total of 99 HIV seropositive, PI naïve, adults were treated with one or two PI and two nucleoside reverse transcriptase inhibitors and followed for 12 months. Every 2 months, patients blood was analyzed for viral replication and for CD4 T-cell count. Patients were classified as virologic responders (white circles), virologic nonresponders (black squares), or virologic transient responders (black triangles). CD4 T-cell recovery was comparable between groups, indicating a lack of dependence on viral suppression. Reprinted with permission. This research was originally published in Lu *et al*. HIV protease inhibitors restore impaired T-cell proliferative response *in vivo* and *in vitro*: a viral-suppression-independent mechanism. *Blood* 2000; **96**(1):250–258. © The American Society of Hematology

Mechanisms of PI-Mediated Apoptosis Inhibition

At least five distinct mechanisms have been proposed to account for the antiapoptotic effects of PI (Figure 2).

Decreased expression of apoptosis regulatory molecules

One of the first papers to demonstrate reduced apoptosis following PI treatment *in vitro*,[33] demonstrated convincingly that addition of ritonavir to hematopoietic colony-forming units augmented colony-forming unit replication; in cultures from HIV-infected patients, the addition of ritonavir resulted in a four-fold increase of both myeloid and erythroid colonies. To assess the putative mechanisms behind such reduced apoptosis and improved survival, the authors measured the expression of caspase 1 to determine whether the caspase transduction cascade was altered by ritonavir. In CD34-positive hematopoietic progenerative cells, caspase 1 appears to be important and necessary for various forms of apoptosis.[34] In that study, ritonavir was found to reduce the expression of caspase 1 as measured by intracellular flow cytometry, prompting the suggestion that PIs may modify apoptosis by virtue of altered expression, at either the transcriptional or translational level of caspase 1 or potentially other caspases. In a conceptually similar study, it has also been reported that membrane expression of Fas ligand in PHA-stimulated peripheral blood lymphocytes is decreased by ritonavir.[35] In another study, the effect of saquinivir on anti-Fas- or anti-CD3-induced death of PBMCs from healthy donors was investigated. Pretreatment with drug for 4 days reduces susceptibility to both Fas-induced and
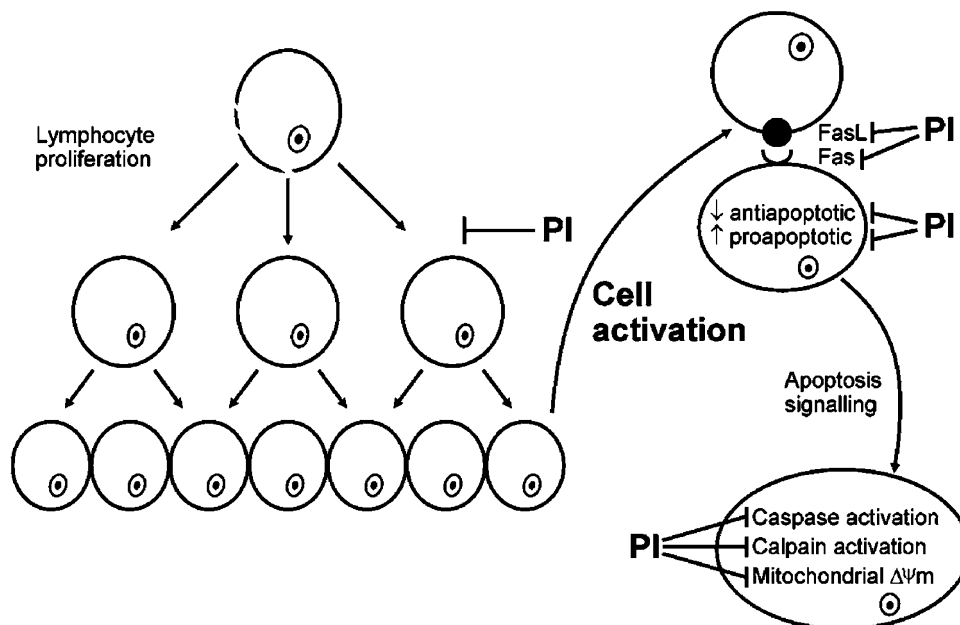

Figure 2 Conceptual model of proposed mechanisms by which PI inhibit apoptosis

anti-CD3-induced apoptosis. This effect occurred in addition to the antiapoptotic effects of interleukin-2 (IL-2) treatment, and was associated with a decreased expression of Fas-receptor. The authors conclude that a potential mechanism of apoptosis inhibition is the reduced CD95 expression.[36]

Subsequent investigation does not support the model that PI are antiapoptotic by virtue of reduced expression of apoptosis regulatory molecules. Inhibition of protein synthesis by cyclohexamide did not reduce the ability of nelfinavir to inhibit camphothecin or Fas-induced apoptosis of Jurkat T cells.[37] Moreover, the effect of nelfinavir on mRNA or protein level for a variety of apoptosis regulatory proteins was not altered in these experiments. Similarly, subsequent work using saquinavir, indinavir, or lopinavir treatment of activated T cells reduced apoptosis but did not alter intracellular levels a BCL-2, Bax, BCL_{XL}, or HSP-70 as assessed by intracellular flow cytometry.[38]

Caspase inhibition

An alternative model to account for the observed inhibition of apoptosis by HIV PIs suggests that caspase family members may be directly or indirectly inhibited by such drugs.[35,39] Indeed, this proposal is intuitively appealing since HIV PIs themselves are likely to inhibit not only the aspartyle protease of HIV but also other cellular proteases. This model has been widely adopted by the HIV research community. Of note, however, is the observation that HIV protease itself is an aspartyle protease whereas caspases are cystine proteases, raising concerns as to the accuracy of this model from a stoichiometric perspective. At least three publications have directly assessed the impact of HIV PIs on caspase activity. Recombinant active caspases 1, 3, 6, 7, and 8 were incubated in the presence or absence of nelfinavir, vehicle control, or z-VAD-Fmk, in the presence of a fluorogenic caspase-specific substrate. In those experiments, addition of nelfinavir did not alter the ability of recombinant active caspases 1, 3, 6, 7, or 8 to cleave their respective fluorogenic substrates while nelfinavir maintained the ability to block HIV protease-mediated cleavage of a fluorogenic *gag/pol* consensus site.[37] Such results have been reproduced using recombinant caspases 3, 6, and 8, incubated in the presence of indinavir or ritonavir where the activity of these caspases is not altered by either drug.[40] Finally, the ability of indinavir to inhibit active caspases generated *in vivo* has also been assessed. Jurkat T-cells were stimulated to undergo apoptosis by the Fas agonists CH-11 and cell lysates prepared. Caspase-specific fluorogenic substrate for caspases 1, 3, 4, 5, 8, and 9 were added to cell lysates in the presence or absence of indinavir and, consistent with previous observations, the activity of these caspases was unaltered by indinavir but potently inhibited by peptidomimetic caspase inhibitors.[41]

Altered proliferation

A third model of how PIs may alter cell survival suggests that PIs themselves may alter proliferative responses of lymphocytes *in vitro*. Since the principal observation which spurred the suggestion that PIs might be antiapoptotic is the improved CD4 T-cell number seen in patients on PI-based therapies,

this model would argue that T-cell increases are a result of enhanced proliferation as opposed to impaired death. Data that indirectly supports this model were presented in one of the first publications to describe the immunomodulatory effects of HIV PIs. Normal CD4 T cells were activated with IL-2 and PHA and then exposed to a Fas agonist. These cells showed increased viability and decreased apoptosis in the presence of ritonavir.[35] Other investigators subsequently directly assessed such a possibility. Peripheral blood mononuclear cells (PBMCs) from HIV-infected but untreated patients were harvested and incubated with indinavir or saquinivir and subsequently treated with PMA and anti-CD3 antibodies. Cells were then cultured *in vitro* with new PI added at each passage, and cumulative cell number monitored until day 16. The addition of PI dramatically enhanced cumulative cell number in this patient population, as well as in cells from HAART-treated patients. Importantly, such improved cell proliferation occurred even in those patients who did not manifest a virologic response to antiretroviral therapy.[31] Such observations prompted the speculation that PIs enhanced CD4 T-cell number by virtue of improving proliferative responses, and since these authors did not observe modified apoptotic responses to TCR or Fas ligation-induced apoptosis by HIV PIs that the effect on CD4 T-cell number is solely an effect on proliferation as opposed to apoptosis inhibition. However, Chavan and colleagues assessed lymphoproliferation in PBMCs from healthy human volunteers precultured in media alone or media supplemented with different concentrations of indinavir followed by anti-CD3, PHA, Con-A, or PMA plus ionomycin. In these studies, indinavir resulted in a dose-dependent reduction of proliferative responses to all stimuli tested. Moreover, such treatments led to cell cycle arrest in the G-0 or G-1 phase.[41] Consistent with the suggestion that PIs may inhibit lymphoproliferative responses, another study examined foot pad swelling of C57BL6 mice infected with lymphocytic choriomeningitis virus (LCMV). Foot pad swelling reactions were markedly inhibited by ritonavir treatment in a dose-dependent manner, and this effect was conserved among a variety of different virus strains, different mouse strains, and both by parenteral and oral administration of the drug.[42] These authors conclude that ritonavir (and potentially other PIs) may prolong cell survival indirectly by inhibiting their entry into cell cycle and thereby modifying the baseline susceptibility towards apoptotic signaling.

Calpain inhibition

Since apoptosis is commonly associated with abnormal calpain activation, it has also been suggested that HIV PIs may modify apoptotic signaling by virtue of direct inhibition of calpain.[43,44] Using indinavir and ritonavir, apoptosis induced by purimyocin or etoposide in U937 cells was investigated. These authors found no impact of these drugs on caspase activity, and therefore analyzed calpain activity in lysates from U937 cells treated with these agents. Using a fluorogenic substrate assay, calpain-like activity was reduced by incubation of U937 cells with indinavir and ritonavir. Moreover, it has been described that indinavir and ritonavir directly inhibited recombinant μ calpane and M calpane.[40] However, results from a different lab failed to demonstrate an effect of ritonavir

on either autolytic or catalytic activity of μ calpain or M calpain.[45] Other investigators have also reported *in vitro* results that ritonavir can inhibit calpains.[46]

Inhibited loss of mitochondrial transmembrane potential ($\Delta\Psi_m$)

A final proposed mechanism by which HIV PIs might inhibit apoptotic signaling involves prevention of mitochondrial transmembrane potential loss. Over the past decade, the role of mitochondria in apoptosis has become better defined, and it is now generally agreed that mitochondria serve as central regulators that coordinate the initiation phase with the executionary phases of apoptosis. Mitochondria serve as regulatory checkpoints for apoptotic signaling and, in fact, numerous apoptotic regulatory proteins including BCL-2 family members, IAPs, and SMAC/DIABLO function at the level of mitochondria to modify apoptotic responses. The first paper to evaluate the impact of PIs on mitochondrial apoptotic signaling performed multiparameter flow cytometry of Jurkat T cells stimulated with actinomycin D, campothecin, or CH-11 in the presence or absence of nelfinavir. These studies demonstrated that nelfinavir-treated cells stimulated to undergo apoptosis have less loss of mitochondrial transmembrane potential than cells treated with control agents. Moreover, mitochondrial release of cytochrome *c* was inhibited in nelfinavir pretreated cells compared to control. Such observations that prompted the speculation that PIs might act at the level of mitochondria were then directly tested in two independent models. First isolated mitochondria were treated with nelfinavir and stimulated with the direct mitochondrial apoptotic agents atractyloside or VPR, demonstrating that nelfinavir but not vehicle control reduced mitochondrial transmembrane potential in these mitochondria. Further, nelfinavir was shown to inhibit mitochondrial-dependent apoptotic signaling but not mitochondrial-independent signaling.[37]

A second group evaluated the impact of PI on mitochondrial function by treating resting lymphocytes with lopinavir, indinavir and saquinivir, and stimulating the cells with anti-Fas, TNF-α, TRAIL or UV irradiation, and measuring mitochondrial transmembrane potential. PI pre-exposure exerted a significant protection from $\Delta\Psi_m$. To confirm this observation, since mitochondrial hyperpolarization has been related to reactive oxygen intermediate (ROI) hyperproduction, these investigators measured ROI production from cells stimulated in such a manner. All drugs tested significantly reduced the ROI production. These observations were supported by observations that PI prevent AZT (a mitochondrio-toxic HIV drug)-induced apoptosis of lymphocytes, and that PIs reduce caspase 9 and 2 activity, but not caspase 8 activity.[38] Altogether, these studies suggest that PIs exert their antiapoptotic effect at a mitochondrial location, but the specific mitochondrial target is unknown.

Effects of HIV PIs on Apoptosis *In Vivo* in Non-HIV Models

Most studies that evaluate changes in apoptosis in response to antiretroviral therapies are confounded by at least two factors. First, the majority of these studies evaluate apoptosis changes that occur in patients receiving multiple anti-HIV drugs, commonly one or two PIs in addition to one or two reverse transcriptase inhibitors. Secondly, most of these studies evaluate changes in apoptosis that occur in HIV-infected patients, in whom a decrease in viral replication occurs almost universally, although the magnitude of these viral reductions is highly variable. Since reduced viral burden is likely to impact apoptosis, one cannot confidently ascribe reduced apoptosis solely either to direct drug effect or to reduced virus burden.

In light of these confounders, interest has developed in evaluating changes in apoptosis that occur in systems where HIV is not involved. One such study evaluated two HIV uninfected volunteers who received indinavir daily for 30 days. In both patients, shortly following the initiation of indinavir treatment, CD4 T-cell numbers dropped from baseline levels of approximately 1100 cells/μl to untreatment levels of approximately 900 cells/μl. Apoptosis itself was not measured in that study.[47] More recently, this question has been readdressed. Eight healthy health-care workers with potential exposure to HIV were initiated on antiretroviral postexposure prophylaxis consisting of AZT, 3TC, and nelfinavir. Cells were harvested before therapy, during therapy, and at least 1 month after postexposure prophylaxis ended. Peripheral blood T-cell number and percentage did not change, nor did proliferative responses to mitogen or recall antigen. However, apoptosis susceptibility in response to campothecin was significantly reduced during antiretroviral therapy. Changes in Fas-induced apoptosis were observed, yet, due to the low level of Fas-induced killing these changes did not reach significance.[48]

Since disordered apoptosis is involved in the pathogenesis of numerous disease states in addition to HIV, we have recently evaluated the effects of PI therapy in a murine model of sepsis. Sepsis is associated with extensive lymphocyte and intestinal epithelial cell apoptosis, both in animal models, as well as in patients with sepsis (reviewed in Hotchkiss *et al.*[49]), and inhibition of apoptosis with pan caspase inhibitors significantly improves survival from sepsis in murine models.[50] Given differences in drug metabolism between mouse and human, in order to achieve pharmacologically relevant serum levels of nelfinavir in mice, it was necessary to co-dose nelfinavir with ritonavir. Crude 48-h survival rates in mice treated with vehicle control was 17% compared to 67% in mice that received nelfinavir/ritonavir prior to cecal ligation and perforation, and 50% when nelfinavir/ritonavir treatment was begun 4 h following sepsis. Improved survival was correlated with a reduction in lymphocyte apoptosis, and an improvement in proinflammatory cytokine production (see Figure 3).[51]

Paradoxical Pro-Apoptotic Effects of HIV PIs

In addition to the well-described antiapoptotic effects of HIV PIs, an emerging body of literature suggests that these drugs may also be proapoptotic. Indeed, while HIV PIs appear to be antiapoptotic at concentrations of drug as low as 10 nM (AD Badley, unpublished observations), whereas at significantly higher doses they may paradoxically induce apoptosis; treatment of monocytes and CD4 T cells with 10 μM indinavir
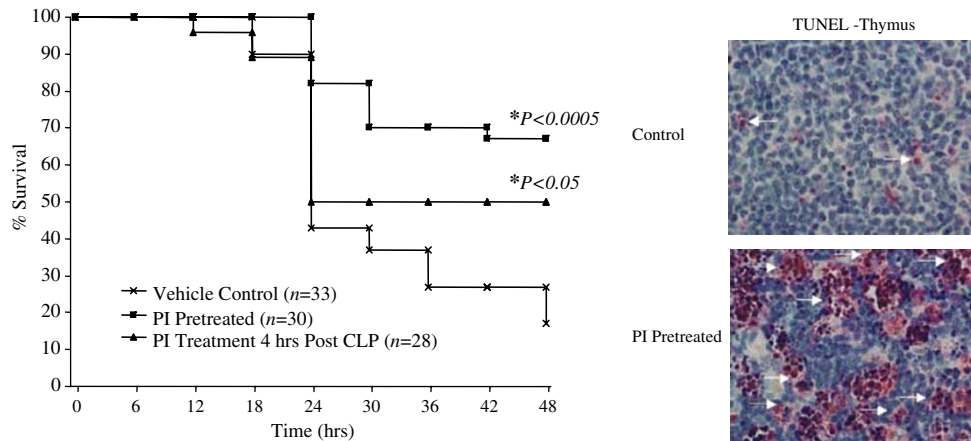
Figure 3 Female ND4 mice were anesthetized, the cecum externalized, punctured (cecal ligation and perforation, CLP), and replaced into the peritoneum. Mice received control vehicle gavage, or PI (nelfinavir/ritonavir) either before or after the CLP as indicated. Mice were followed for 48 h, and survival monitored. In separate mice, 12 h following CLP, thymus was harvested and analyzed by TUNEL for apoptosis. Reprinted with permission. This research was originally published in Weaver *et al.* Improved survival in experimental sepsis with an orally administered inhibitor of apoptosis. *The FASEB Journal* 2004; **18**:1185–1191
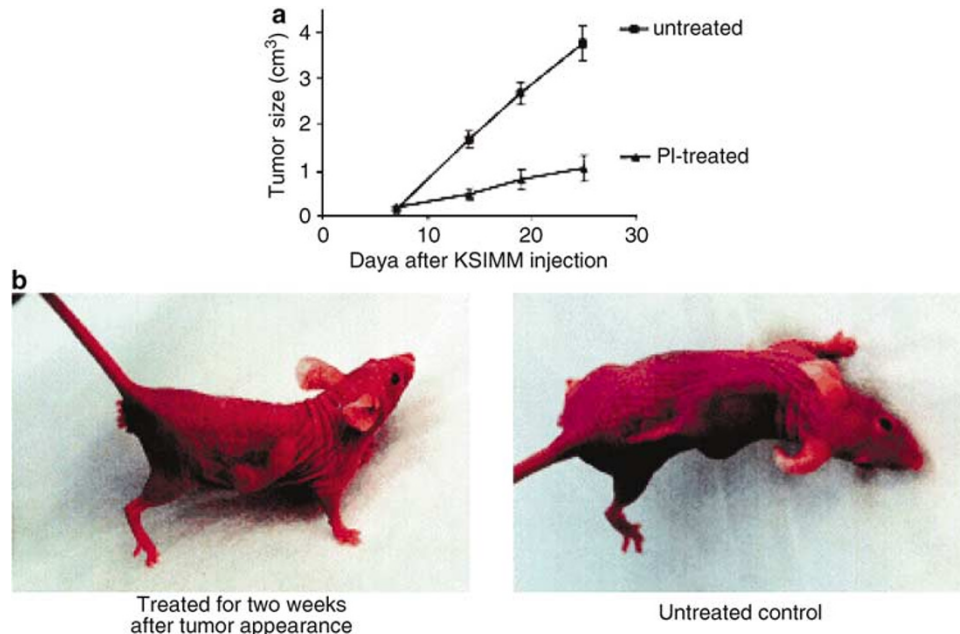

Figure 4 Male BNX mice were implanted with the KSIMM Kaposi's sarcoma cell line, and treated with ritonavir as indicated. Tumor size was monitored biweekly. Reprinted with permission. This research was originally published in Pati *et al.* Antitumorigenic effects of HIV protease inhibitor ritonavir: inhibition of Kaposi's sarcoma. *Blood.* 2002; **99**(10): 3771–3779. © The American Society of Hematology

or saquinivir significantly enhances the proportion of cells that bind annexin V-FITC. Moreover, we have observed enhanced apoptosis in Jurkat T-cell lines incubated with doses of lopinavir, ritonavir, and nelfinavir that exceed 10 μM (AD Badley, unpublished data).

Consistent with the proposed proapoptotic effects of HIV PIs, several groups have evaluated the effect of ritonavir on tumor cell viability. Since prior publications have indicated that the HIV PI ritonavir can inhibit the peptidase activities of the 20S proteosome,[42] and since other proteosome inhibitors are both antiproliferative and induce apoptosis of tumor cells,[52] the ability of ritonavir to induce tumor cell apoptosis was investigated. Using doses of ritonavir between 15 and 50 μM, a dose-dependent death of transformed cell lines characterized by intranucleosomal DNA laddering was observed. In contrast, nontransformed murine cells did not demonstrate DNA laddering in response to these doses of ritonavir. Furthermore, administration of 6 mg of ritonavir per day to C57BL6 mice implanted with EL4 thymomas resulted in 76–79% growth inhibition of the thymoma compared to control animals.[53] Of note, this dose of ritonavir is roughly equivalent to 300 mg/kg/day, whereas human dosing of ritonavir is approximately 17 mg/kg/day for a 70 kg patient. In the same publication, cells from treated animals had an accumulation of

cells arrested in G1 phase that expressed high levels of P21^{WAF1}. Since P21 may be proapoptotic in different contexts,[54] the authors speculate that the accumulation of P21 in the presence of ritonavir is likely due to inhibited proteosome activity, and that P21 accumulation may contribute to proapoptotic effects of this treatment.

In a separate study, the antitumor effects of ritonavir on Kaposi's sarcoma (KS) cells were investigated. The theoretical basis for this study was not only the proteosome inhibitory effects of ritonavir, but also observations that KS lesions can improve significantly in patients treated with PI-based regimens, even in the absence of a reduction in HIV viral loads.[55] In vitro data demonstrated that ritonavir treatment of a KS cell line can induce caspase-3-independent apoptosis. However, similar treatment of primary human umbilical veins endothelial cells did not. Such treatments were also associated with reduced production of proinflammatory cytokines, including interferon-γ, TNF-α, interleukin-1β, and IL-6, as well as decreased expression of endothelial cell adhesion molecules ICAM-1, VCAM-1, and E-selectin. Finally, when the KS cell line is implanted into an immunodeficient BNX mouse, treatments with ritonavir at doses of 30 mg/kg/day for 15 days resulted in decreased tumor size in treated animals compared to controls (see Figure 4).[56]

It is apparent, therefore, that under certain circumstances, particularly with high-dose PI, a paradoxical proapoptotic effect can be observed. While the mechanisms underlying this proapoptotic effect are undefined, it is theoretically possible that these drugs may possess both an agonistic activity at high dose, yet an antagonistic activity at low dose, potentially by interacting with the same apoptosis regulatory protein. Such a mixed agonist/antagonist effect has been well described in other circumstances. For example, raloxisene is a mixed estrogen agonist/antagonist, which coincidentally can induce TUNEL-positive apoptosis of selected transformed cell lines.[57] Moreover, other estrogen agonists/antagonists may upregulate proteins involved in the control of cell proliferation, transcription, and survival in some contexts, but alternately upregulate proteins involved in apoptosis in other contexts;[58] raising the possibility that PI may function in a similar manner.

Acknowledgements

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