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HIV protease inhibitors modulate apoptosis signaling *in vitro* and *in vivo*

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Abstract HIV protease inhibitors are an integral part of effective anti-HIV therapy. The drugs block HIV protease, prevent proper packaging of HIV virions, and decrease the HIV viral burden in the peripheral blood of infected individuals. In addition to direct anti-viral effects, the HIV protease inhibitors also modulate apoptosis. A growing body of work demonstrates the anti-apoptotic effects of HIV protease inhibitors on $CD4^+$ and $CD8^+$ T cells during HIV infection. The mechanism of this apoptosis inhibition is supported by several proposed hypotheses for how they alter the fate of the cell, including preventing adenine nucleotide translocator pore function, which consequently prevents loss of mitochondrial transmembrane potential. More recently, the anti-apoptotic effects of the HIV protease inhibitors have been tested in non-HIV, non-immune cell, whereby protease inhibitors prevent apoptosis, and disease in animal models of sepsis, hepatitis, pancreatitis and stroke. Interestingly, when HIV protease inhibitors are used at supra-therapeutic concentrations, they exert pro-apoptotic effects. This has been demonstrated in a number of tumor models. Although it is unclear how HIV protease inhibitors can induce apoptosis at increased concentrations, future research will define the targets of the immunomodulation and reveal the full clinical potential of this intriguing class of drugs.

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HIV infection causes a progressive depletion of $CD4^+$ T cells. There are likely many reasons for the $CD4^+$ T cell loss, including apoptosis. Current therapy for HIV-infected patients often includes a combination of reverse transcriptase inhibitors and HIV protease inhibitors. The first HIV protease inhibitor was FDA approved for anti-HIV therapy in humans in 1995. Currently, there are ten HIV protease inhibitors that are approved for clinical use in humans (saquinavir, indinavir, darunavir, nelfinavir, lopinavir, amprenavir, atazanavir, tipranavir, ritonavir, and fosamprenavir). These drugs are inhibitors of the HIV viral aspartyl protease. The compounds have a strong affinity for the active site of the HIV protease and irreversibly inhibit the catalytic activity of the enzyme.

When HIV protease is inhibited, viral particles are produced, but they are immature, and do not package properly into infectious virions. Moreover, naturally occurring HIV protease mutations, which arise during suboptimal antiretroviral therapy, result in impaired replication kinetics of progeny virions. In addition to its role in viral replication, HIV protease may also contribute to HIV pathogenesis. When transfected into a human or bacterial cell, HIV protease is cytotoxic and causes cleavage of a variety of host proteins including actin, Bcl_2 and procaspase 8. It remains unclear how HIV protease initiates cell death.

Although the HIV protease inhibitors have limited bioavailability and stability, they are the basis of effective anti-HIV therapy, and in combination with other antiretroviral agents, produce a sustained decrease in HIV viral load. Over the past ten years, evidence has accumulated that HIV protease inhibitors have non-viral effects on the host cells beyond the effect of blocking HIV protease enzymatic

activity. For example, HIV protease inhibitors also directly affect cellular apoptosis signaling.

The role of HIV protease inhibitors during treatment of HIV infection

Optimal anti-HIV therapy is called highly active antiretroviral therapy (HAART). This includes the combination of HIV reverse transcriptase inhibitors, often with an HIV protease inhibitor. Since the advent of HAART in late 1995, these regimens have been shown to increase $CD4^+$ T cell counts and reduce the amount of HIV virus quantitated in the peripheral blood, in infected individuals. However, in many cases, the increase in $CD4^+$ T cells that occurs during HIV therapy appeared to result from actions that were independent of the effect of the drug on viral replication. In one such instance, HIV-infected subjects were randomized in a clinical trial to receive therapy with three drugs, including one protease inhibitor (saquinivir, zidovudine, and zalitabine) or two drugs. One of the two drug regimens included a protease inhibitor (zidovudine and saquinivir), and the other two-drug regimen had no protease inhibitors (zidovudine and zalitabine) [1]. Two-hundred and eighty subjects completed 24 weeks of treatment. Even though there was worse virologic control in the two-drug, than the three-drug regimen group, the subjects in the two-drug group who received the HIV PI had improved $CD4^+$ T cell counts, compared to the two-drug group that did not receive an HIV PI. Subsequent clinical trials confirmed that HIV protease inhibitors improved $CD4^+$ T cell counts in HIV-infected individuals, independent of the viral suppression [2–5]. In a meta-analysis comparing anti-HIV drug regimens using a PI to those that switched the PI to a non-nucleoside reverse transcriptase inhibitor (NNRTI), PI-based therapy resulted in higher $CD4^+$ T cell counts [6]. The analysis required that both PI and non-PI based regimens have complete HIV viral suppression. Therefore, the $CD4^+$ T cell benefit with HIV protease inhibitor therapy appeared to be, in part, unrelated to the effect on HIV viral suppression. In a different trial comparing treatment with HIV protease containing highly active antiretroviral therapy (HAART) to protease inhibitor therapy alone, there was less virologic suppression of the subjects on PI mono-therapy. However, there was no decrease in $CD4^+$ T cell counts over one year, even in the group who received PI mono-therapy and virologically failed [7]. Additionally, in a comparison between PI-based anti-HIV therapy to NNRTI-based anti-HIV therapy, there was equal viral suppression in both groups; however, PI-based regimens had a greater increase in $CD4^+$ T cell counts [8].

In the absence of effective antiretroviral therapy, HIV-infected subjects have considerable lymphocyte apoptosis in lymphoid tissue. The lymphoid tissue apoptosis is reduced significantly when a patient is treated with HIV PI containing HAART, which correlates with a decrease in HIV peripheral blood viral load and an increase in peripheral blood $CD4^+$ T cells. In addition, $CD4^+$ and $CD8^+$ T cells isolated from HIV-infected individuals before and on days 1, 4, and 8, after starting treatment with HIV PI containing HAART, had decreased sensitivity to Fas-mediated apoptosis after being on HAART for as little as one day [9].

A recent trial by Landay et al. focused on the mechanism behind HIV protease inhibitor immune effects. The trial compared patients with suppressed HIV viral replication on a PI-based anti-HIV regimen or non-PI based drug regimen. One week after enrollment in the study, subjects in the PI containing arm had less spontaneous T cell apoptosis than those in the non-PI containing arm. Although there are likely multiple reasons for $CD4^+$ T cell decline during the course of HIV infection, these data suggest that patients on HIV protease inhibitors have less spontaneous $CD4^+$ T cell apoptosis and have improved $CD4^+$ T cell counts, further suggesting that the HIV protease inhibitors block $CD4^+$ T cell apoptosis, independent of their effect on HIV replication [10].

In vitro anti-apoptotic properties of HIV protease inhibitors

Due to findings that PI therapy reduced lymphocyte apoptosis, our group proposed in 1998 that highly active antiretroviral therapy (HAART) might independently block cellular apoptosis [9]. Subsequent investigations tested and confirmed this finding *ex vivo* and *in vitro* [11, 12]. HIV PIs anti-apoptotic effects were investigated using ritonavir in cultures of bone marrow cells from HIV-infected patients or normal controls. Hematopoietic colony forming unit replication, following addition of ritonavir, had 45% less apoptosis than untreated cultures. The authors also reported a decrease in caspase-1 expression after ritonavir treatment [13].

In additional experiments investigating the effects of HIV PIs on T cell death during HIV infection, $CD4^+$ T cells isolated from healthy uninfected individuals had increased Fas expression and Fas and anti-CD3-induced apoptosis when incubated with HIV virions. Inducible Fas expression and apoptosis were abrogated when the cells were pre-incubated with the HIV PI, saquinavir [14]. These findings were supported by subsequent investigations which reported that saquinavir and ritonavir inhibited TNF-mediated U937 cell apoptosis in a dose-dependent fashion with 38–60% reduction in apoptosis in PI treated cells [15]. During HIV infection, the HIV envelope gp120 binds to the CD4 and CXCR4 receptors on the surface of the $CD4^+$ T cell and signals the cell to undergo apoptosis. This bystander death is one of the ways that HIV depletes the immune system.

Matarrese et al. treated human $CD4^+$ T cells with HIV gp120 to make the cell sensitive to Fas-mediated apoptosis, which resulted in mitochondrial changes and apoptosis after Fas exposure. When the $CD4^+$ T cells were pretreated with a PI before HIV gp120, mitochondrial depolorization was blocked in a whole cell or cell-free system, or isolated mitochondria [16]. Taken together, this data indicates that patients who received HIV protease inhibitors had improved $CD4^+$ T cell counts independent of the state of HIV viral replication, and *in vitro* work confirmed that HIV PIs can inhibit T cell apoptosis, specifically that induced by HIV.

Mechanism of HIV protease inhibitors apoptosis inhibition

The mechanism of HIV protease inhibitor-mediated apoptosis inhibition is being actively investigated (Table 1). HIV PIs inhibit the proteolytic activity of HIV viral protease, therefore, it has been postulated that they might have a similar effect on other cellular proteases. Together with the observations that HIV PIs block Fas-mediated apoptosis, many investigators hypothesize that HIV PIs may inhibit the caspase family members and block apoptosis. Although this is an attractive model, caspases are cystine proteases and HIV protease is an aspartyle protease. Several groups have investigated the direct effect of HIV PIs on intracellular caspase activity. When recombinant caspases-1, -3, -6, -7, or -8 were incubated with a fluorogenic tetrapeptide substrate for each caspase in the presence of absence of nelfinavir, the HIV PI inhibited HIV protease cleavage of *gag/pol,* but did not inhibit the activity of any of the caspases [17]. These results were expanded in a study that demonstrated that caspases-3, -6, and -8 activity was not inhibited by indinavir in U937 cells at drug concentrations that effectively inhibited U937 apoptosis [18]. Nelfinavir did not block activation of caspases-1, -3, -4, -5, -9, and -8 in lysates of Jurkat T cells undergoing Fas-mediated apoptosis [19].

In addition to caspases, the calpain proteases have been considered as a possible site for HIV PIs to mediate apoptosis. Calpains are Ca^+-dependent cysteine proteases reported to be involved in several models of apoptosis, including U937 cells, but are not absolutely required for apoptosis [20]. Because HIV PIs are designed to inhibit the HIV cysteine protease, they may influence cellular apoptosis by blocking calpain activation and function. Ghibelli et al. demonstrated in a U937 model of apoptosis that indinavir and ritonavir directly inhibit apoptosis in cell systems where calpains are activated and block m-calpain activation [18]. Other investigators have demonstrated that ritonavir competitively inhibited activity of both m- and -μ calpain isoforms in PC12 cells [21]. Other investigators could not confirm this observation. These authors postulate that concentrations of ritonavir close to the maximum solubility of the drug, which was used in previous reports, may have artificially altered the results [22]. A calpain inhibitor could have significant therapeutic implications beyond HIV apoptosis, including neurodegenerative diseases; however, the evidence remains unclear whether HIV PIs have a significant effect on calpain activity.

An alternative model suggests that HIV protease inhibitors alter the expression of apoptotic regulatory proteins. Although early studies reported a change in Fas expression after PI treatment, subsequent work did not show a

Table 1 Possible methods for HIV protease inhibitor regulating apoptosis

Theoretical site of action	Support
Caspase activity	HIV PIs block Fas and TNF mediated apoptosis
	HIV PIs do not block caspase-1, -3, -4, -5, -6, -7, -8, or -9 activity in cell models
Calpain activity	HIV PIs inhibit apoptosis in cell systems where calpains are activated
	HIV PIs blocked m-calpain activation in U937 cells
	HIV PIs inhibited activity of both m- and -μ calpain isoforms in PC12 cells
	HIV PI did not inhibit m- or μ-calpain hydrolysis or activation at lower, physiologic, concentrations
Apoptosis regulatory proteins	No change in Fas protein levels after HIV PI treatment
	No change in RNA levels of Fas, Fas L, and TNF after HIV PI treatment
	No change in Bcl-2, Bax, and Bcl-X_L after HIV PI treatment
Mitochondrial transmembrane potential	HIV PIs maintain mitochondrial membrane integrity after apoptosis stimuli
	HIV PIs prevent cytochrome *c* release from mitochondria after apoptosis stimuli
	ANT (adenine nucleotide translocator) necessary for HIV PI to block mitochondria mediated apoptosis

change in Fas levels with HIV PI therapy [14, 23–25]. Bone marrow progenitor cells from HIV-infected individuals incubated with ritonavir and indinavir showed no change in RNA levels of Fas, or Fas L. Two reports have specifically investigated the levels of intracellular apoptotic regulatory proteins with and without HIV PI treatment. Protein levels of Bcl_2, Bax, and Bcl-X_L were evaluated by flow cytometry, and were unchanged after PI administration [17, 26]. Therefore, HIV PIs can block Fas- and TNF-mediated apoptosis; this effect does not appear to be due to changes in intra- or extracellular apoptotic regulatory protein levels [14–16].

HIV PIs may also block apoptosis at the level of the mitochondria by disrupting the transmitochondrial membrane potential. The mitochondrial transmembrane potential occurs from an asymmetric distribution of ions on both sides of the inner mitochondrial membrane that is maintained by the mitochrondrial permeability transition pore complex (PTPC). After an apoptotic signal, the PTPC opens, disrupts the membrane potential, and releases apoptogenic factors, including cytochrome *c* and procaspase-9. Thus, the mitochondria serves as a regulatory checkpoint of apoptotic signaling. Many regulatory proteins, including Bcl_2 family members and IAPs, interact at the level of the mitochondria to alter apoptosis. In the first report of the effects of HIV PI on mitochondrial integrity, Jurkat cell Fas-mediated apoptosis was inhibited with 10 μM of nelfinavir, a dose that would simulate physiologic levels if taken clinically. Nelfinavir treated cells maintained intact mitochondrial transmembrane potential, as determined by DioC6 staining, a lipophilic dye that stains the mitochondria [17]. The authors also reported that 10 μM of nelfinavir inhibited cytochrome *c* release during Fas-induced apoptosis. The HIV accessory molecule, Vpr, caused PTPC opening and loss of transmitochondrial potential when added directly to mitochondria. Nelfinavir pretreatment of Jurkat cells prevented Vpr-induced DioC6 release from the mitochondria and cell death. Other groups have confirmed that HIV protease inhibitors block mitochondrial transmembrane potential loss in multiple models of apoptosis [16, 17, 26–30]. In a recent report, Weaver et al. investigated how HIV PIs influence mitochondrial integrity by using yeast models. Wild type or yeast deficient in voltage-dependent anion channel (VDAC) or adenine nucleotide translocator (ANT) isoforms, two components of the PTPC, were treated with Vpr or H_2O_2 which induce mitochondrial apoptosis. Apoptosis only occurred after Vpr or H_2O_2 treatment when ANT was present. Furthermore, when Jurkat cells were pretreated with nelfinavir and then an agonist for VDAC, there was no inhibition of mitochondrial potential loss or apoptosis. However, 10 μM of nelfinavir blocked ANT agonist-induced mitochondrial transmembrane potential loss and apoptosis [31]. Lastly, proteoliposomes reconstituted with ANT, which release a fluorescent dye after pore opening, did not result in pore opening when pretreated with nelfinavir before the ANT agonist [31].

In summary, there are several theories regarding the mechanism by which HIV PIs inhibit cellular apoptosis. The most data suggest that HIV PIs block apoptosis by maintaining mitochondrial integrity, likely by HIV PIs preventing pore function of the adenine nucleotide translocator subunit of the mitochondrial permeability transition pore complex (Fig. 1).

HIV protease inhibitor anti-apoptotic properties during non-HIV disease states

The novel anti-apoptotic properties of HIV PIs are being evaluated in preclinical studies as a potential therapy for disease states associated with increased levels of apoptosis such as sepsis, the leading cause of death in critically ill patients. The original description of *in vivo* use of HIV PIs for non-HIV related disease was in a mouse sepsis model. Sepsis is the leading cause of death in critically ill patients. Despite advances in supportive care, mortality from sepsis remains high. Animal studies demonstrate that sepsis results in extensive lymphocyte apoptosis, as well as intestinal epithelial cell apoptosis [32, 33]. These findings have been confirmed during autopsy studies in humans who died of sepsis [34, 35]. In a mouse model of sepsis, created by cecal ligation and perforation, mice pretreated with HIV PIs had improved survival and reduced lymphocyte apoptosis [36]. The HIV PI treated mice had an increase in the Th1 cytokine TNFα and a reduction in the TH2 cytokines IL-6 and IL-10. It appears the beneficial effect of PI treatment was due to reduced lymphocyte apoptosis because lymphocyte deficient Rag 1 –/– mice had no benefit from HIV PI treatment. Follow-up work by Weaver et al. investigated the effect of HIV PIs during *Staphylococcal enterotoxin B*+DGal-induced shock. There was a 60% improvement in 24-h survival in mice pretreated with HIV PIs than those treated with vehicle control. In addition, the authors also demonstrated that HIV PI pretreatment reduced mouse death from Fas-induced fatal hepatitis and middle cerebral artery occlusion-induced stroke [31].

HIV protease inhibitors and stroke

Cerebral ischemia or stroke occurs when blood flow (and thus oxygen) to the brain is reduced through hemorrhage or clot-induced occlusion of blood vessels. The only therapy with proven clinical benefit is thrombolysis requiring administration of tissue plasminogen activator within 3 h of the onset of ischemic attack and/or oral aspirin within the first 48 h after stroke onset [37]. Although the pathophysiology

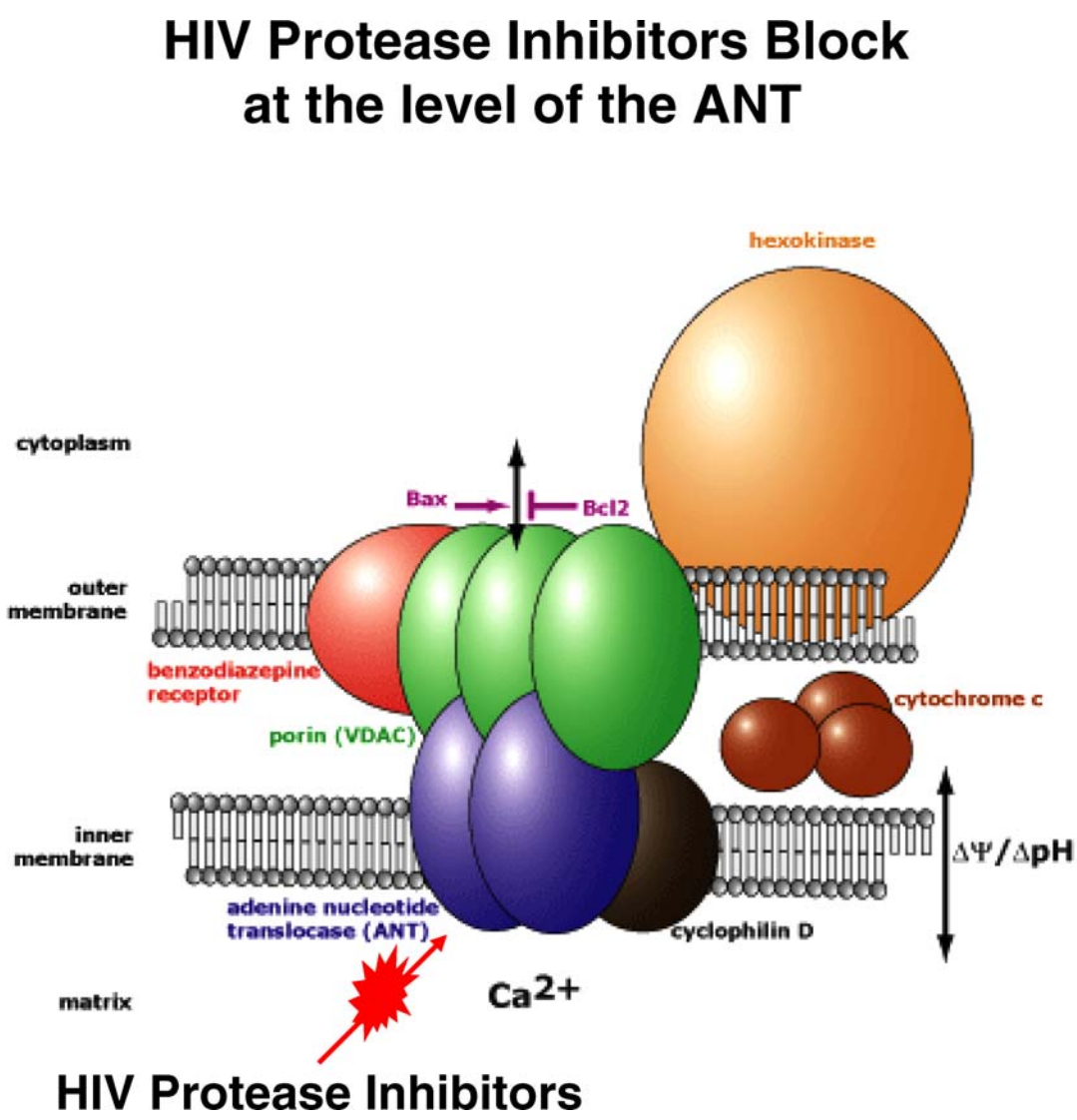

Fig. 1 HIV protease inhibitors block cellular apoptosis at the level of the adenine nucleotide translocase (ANT) in the mitochondrial pore complex. The figure has been adapted from the Mitosciences Inc. website

of stroke damage extends well beyond this time frame, no clinical intervention capable of targeting discrete molecular mechanisms of cell death has been shown to effectively protect neurons from subsequent neuronal loss. Elucidation of the complex and multiple cell death pathways initiated by ischemia has made it increasingly apparent that a broader therapeutic approach is required [37–40]. These findings are supported by correlative clinical evidence of neuroprotective activity in other central nervous system disorders following treatment with PIs [41, 42].

Stroke elicits rapid necrotic and excitotoxic mechanisms as well as delayed apoptotic-like responses. Cell death is initiated once core tissue is deprived of oxygen-rich blood. Immediately following vessel occlusion, cell viability is lost, in part, by a reduction in ATP levels resulting in an efflux of K^+ ions from compromised neurons and glia and an influx of Na^+, Cl^-, and Ca^{2+} ions. Neuronal depolarization resulting from a loss of calcium homeostasis is further exacerbated by the sustained release of glutamate and reduction in extracellular pH leading to excitotoxicity. Production of free fatty acids and oxyradicals are elevated, triggering oxidative remodeling of membrane lipids, impaired glial homeostatic functions and enhanced inflammatory cell activation [40, 43, 44].

Studies using primary hippocampal neurons indicate that HIV PIs can protect neurons from cell death triggered by membrane lipid remodeling associated with oxidative stress. Ritonavir has been shown to inhibit neuronal injury triggered *in vitro* by 4-hydroxynonenal, a lipid-soluble aldehydic product of membrane peroxidation that impairs Na^+, K^+, and -ATPase activity [45]. *In vivo,* administration of nelfinavir and ritonavir prior to ischemic insult effectively reduces infarct

size in mice following middle cerebral artery occlusion, resulting in functional recovery of ischemic neurons [31].

Mechanistic assessment suggests that HIV PIs act to reduce delayed neuronal ischemic death triggered also through mitochondrial-dependent pathways. Neurons located at the periphery of the necrotic core (dubbed the penumbra) are spared acute ischemic injury. Damage is not evident until hours, days, and weeks following reperfusion when cells begin to exhibit many of the morphological and biochemical characteristics of apoptosis [45]. Death is likely triggered during ischemia/reperfusion by downstream release of internal calcium stores from endoplasmic reticulum and mitochondria [43]. Additional death pathways are subsequently initiated by a complex cross-talk between extrinsic death receptor mediated-induction and intrinsic mitochondrial-dependent pathways. Briefly, extrinsic induction involves binding of apoptogens, such as Fas ligand and tumor necrosis factor α (TNFα), to death domain-containing receptors inducing oligomerization and recruitment of the adapter proteins FADD and TRADD [46]. Complex formation between adapter proteins, receptor death domains, and procaspases accelerates the autocatalytic activation of procaspase-8, -10, and -2. Once cleaved, these initiator caspases can cleave and activate executioner caspases-3, -6, and -7 responsible for the regulated disassembly of cellular proteins characteristic of apoptosis [47]. Intrinsic cell death is dictated by mitochondrial function. Release of reactive oxygen species following ischemic reperfusion triggers release of cytochrome *c* and ATP from compromised mitochondria into the cytosol, often with concomitant loss of loss of Δy_m. Activation of caspase 9 occurs with formation of the apoptosome composed of APAF-1, cytochrome *c*, ATP, and procaspase 9 resulting in downstream activation of caspase-3, -6, -7 [43, 46].

HIV PIs act at multiple mitochondrial branch points of these overlapping apoptotic cascades to reduce delayed neuronal death following ischemic insult. Pretreatment with nelfinavir prior to apoptotic challenge inhibits the pore function of the adenine nucleotide transporter, thereby reducing the loss of loss of Δy_m, apoptosome formation, and downstream caspase activation [31]. Because HIV PIs inhibit the downstream mitochondrial events elicited by caspase 8-mediaed cleavage of Bid, downstream events of the extrinsic pathway are also reduced [31].

Recent unpublished data from our laboratories indicate that this protection may also be of potential clinical benefit even if administered after the ischemic event—a key requirement for successful adjuvant therapies. Loss of CA1 pyramidal neurons in the hippocampus is a hallmark of global ischemia. Ischemic hippocampal injury can be experimentally designed by the two-vessel occlusion rodent model of global ischemia in which both internal carotid arteries are transiently ligated. As demonstrated in Fig. 2, we observed significant sparing of CA1 pyramidal neurons whether animals received nelfinavir boosted by ritonavir prior to or immediately after 2VO surgery (Fig. 2). Neuroprotection was still evident, albeit at reduced efficacy, when nelfinavir and ritonavir were administered up to 6 h after two-vessel carotid occlusion (Fig. 2), suggesting a wider therapeutic window than previously anticipated [31].

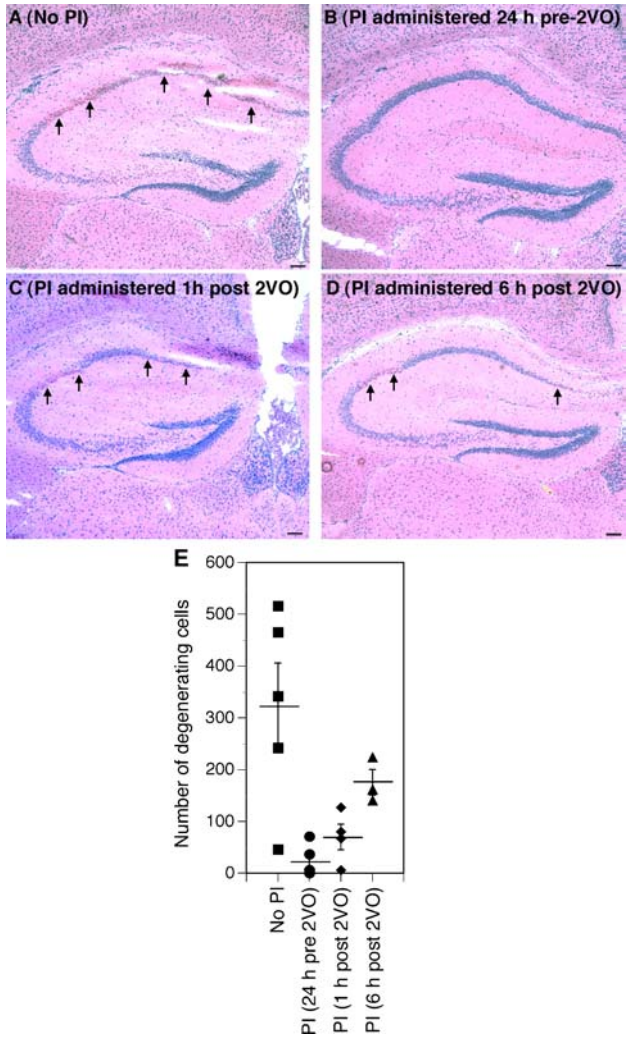

Fig. 2 Effects of nelfinavir and ritonavir on ischemic hippocampal damage induced by two vessel carotid occlusion. Mice subjected to two vessel occlusion (A) were compared to animals that received three oral gavages of either nelfinavir or ritonavir 8 h apart starting 24 h before (B), 1 h after (C), or 6 h after (D) carotid occlusion. Arrows indicate areas of ischemic damage. Viable cell number in the CA1 was counted 48 h after ischemic onset in hemotoxylin and eosin-stained sections (E). Data represent the average number of viable cells in both hippocampi and are expressed per animal. The mean number of viable cells per condition ± standard error of measurement is indicated. Neurodegeneration was completely attenuated by pre-treatment with PIs and partially attenuated by administration of PIs following carotid occlusion. Scale bars, 100 μm

While promising, mechanistic assessment of HIV PIs in stroke management is still in early days. Although HIV PIs appear to inhibit apoptosis in multiple organs at

concentrations comparable to that seen in the plasma following clinical application (reviewed in [48]), these same compounds trigger cell death at higher concentrations [49–51]. Moreover, chronic administration, as part of HIV treatment, has been associated with an enhanced risk of ischemia in both central and peripheral tissue. Chronic HIV PI treatment can elicit hyperlipidemia, accelerating atherosclerosis and represent a potential increased risk for myocardial infarction [52–54]. As such, it is unlikely that HIV PIs can be used prophylactically to prevent stroke damage in susceptible individuals. However, the promise of transient administration is promising. The impact of acute administration of HIV PIs following stroke requires a thorough evaluation of the therapeutic window open to mitochondrial manipulation and is essential to validate the promise of HIV PIs as a potential adjuvant strategy to promote neuronal survival following ischemia.

HIV protease inhibitors and pancreatitis

Accelerated apoptosis also contributes to cellular injury during acute pancreatitis. Our group investigated whether treatment with the HIV protease inhibitors nelfinavir/ritonavir would reduce the severity of pancreatitis using a mouse caerulein-induced pancreatitis model. Ritonavir was used to increase nelfinavir drug levels to a therapeutic level in the mice. Mice treated with nelfinavir/ritonavir before caerulein induced pancreatitis had reduced serum amylase levels and less acinar injury of the pancreas on histologic review, compared to mice pretreated with vehicle control.

HIV protease inhibitors and T cell production

The anti-apoptotic effects of HIV protease inhibitors may have indirect beneficial clinical effects as well. In a recent report by Graham et al., five out of seven HIV-negative patients treated with an HIV protease inhibitor containing HAART regimen for a needle-stick exposure experienced a 3-log increase in thymic-derived naïve T cells in the peripheral blood. The increase in naïve T cells, as determined by T cell receptor recombination excision circle levels (TREC), occurred after four weeks of therapy, suggesting, in yet another setting, that HIV PI can have beneficial immunemodulatory effects [55].

Paradoxical pro-apoptotic effect of HIV protease inhibitors

Under certain conditions HIV PIs may be pro-apoptotic as well. At increased doses of HIV PI, it is possible that the drug may interfere with cellular activation signals that result in transformation and protect the tumor cell from apoptosis. High concentrations of ritonavir (10–50 μM) inhibit the proliferation of murine and human tumor cell lines. DNA laddering demonstrated that 15–30 μM of ritonavir induced apoptosis in the same cell lines [56]. Of note, the concentrations of ritonavir used were over 15 times what most adults achieve at FDA-approved doses and at which the drug blocks apoptosis. This led to further work developing the pro-apoptotic effects of HIV PIs. Adult T-cell leukemia (ATL) is an aggressive malignancy associated with human T-cell leukemia virus (HTLV) and is very resistant to conventional chemotherapy. When ATL cells were incubated with 20–40 μM of ritonavir, there was a five-fold increase in spontaneous apoptosis, resulting in a similar decrease in tumor cell survival [57]. In ATL cell lines and primary ATL cells 40 μM of ritonavir inhibited transcriptional activation of NF-κB. In addition, HIV PIs inhibited the expression of the targets of NF-κB, Bcl-$X_{L,}$ survivin, c-Myc, and cyclin D2 [57].

There is also evidence that HIV protease inhibitors are pro-apoptotic in models of solid tumors. Freshly isolated multiple myeloma cells from patients under went apoptosis after incubation with 40–50 μM of ritonavir, saquinavir, and nelfinavir. This was associated with a decrease in the anti-apoptotic protein Mcl-1, and blocked IL-6 phosphorylation of ERK 1/2 and STAT 3 [58]. Different tumor types may have different mechanisms by which HIV PIs inhibit tumor growth and promote apoptosis. Ritonavir used at 20 μM in solid and hematologic tumor models caused an increase in the cellular concentrations of the antiproliferative and pro-apoptotic proteasome substrate cdk inhibitor, p21. This was associated with a block in proteolytic degradation [56], consistent with an earlier report that HIV PI impact proteasome activity [59]. Accumulation of intracellular p21 resulted in cell-cycle arrest in G_1 phase and subsequent apoptosis in ritonavir treated tumor cells.

HIV protease inhibitors are well-tolerated drugs with enticing possibilities for future use to alter apoptosis in many human disease states. Clinical trials of HIV PIs in disease states other than HIV are already underway investigating the *in vivo* effects on human cellular apoptosis (NCT00346619 and NCT00233948). Furthermore, now that the target for how HIV PIs block cellular apoptosis has been identified as the mitochondrial pore protein ANT, physiochemical optimization can be performed. Lastly, the mechanism by which high concentrations of HIV PIs induce apoptosis in transformed tumor models needs to be further clarified. Studies are needed to determine whether the HIV PIs alter proteins that modulate cellular proliferation or apoptosis, and whether there is a threshold to the pro-apoptotic effects.

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