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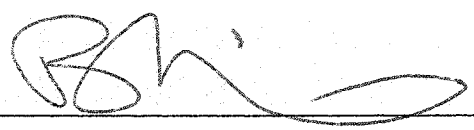

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A New Role for Human Immunodeficiency Virus (HIV)-1 Protease Inhibitors: Suppression of Apoptosis

By

Barbara N. Phenix

THESIS
Submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology,
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ABSTRACT

Protease inhibitor (PI)-induced improvements in CD4 T cell counts may be in part independent of PI effects on HIV replication. Since HIV-associated CD4 T cell depletion occurs by apoptosis, we analysed the effect of PIs on apoptosis in peripheral blood lymphocytes (PBLs) from HIV-infected patients and in an uninfected T cell line. The *in vivo* effects of PIs were monitored in an animal model of stroke and in HIV negative patients taking anti-retroviral therapy (ART) in the context of post-exposure prophylaxis (PEP).

Patient PBLs and Jurkat T cells were cultured with PIs. Following stimulation, apoptosis was measured by annexin-V, and loss of mitochondrial membrane permeability ($\Delta\psi_m$) was assessed in cells and isolated mitochondria using $DiOC_6(3)$. The mechanism of inhibition was determined at the level of caspase activity, and of protein and messenger ribonucleic acid (mRNA) synthesis of various pro- or antiapoptotic factors. For *in vivo* experiments, mice were given PIs by gastric lavage at various time points prior to and following transient forebrain ischæmia. Histological analyses were performed on hippocampal sections. Apoptosis of *ex vivo* peripheral blood mononuclear cells (PBMCs) of patients taking PEP (AZT, lamivudine, nelfinavir) was assessed prior to, on, and post-therapy in response to a variety of stimuli.

Results revealed that PIs reduced spontaneous and anti-Fas induced apoptosis both in CD4 and CD8 T cells from HIV patients. Jurkat T cell apoptosis, $\Delta\psi_m$, cytochrome *c* release, and caspase 8 cleavage were also inhibited by PIs. The mechanism responsible for inhibition of apoptosis does not involve modification of caspase activity, protein, or mRNA synthesis. Mitochondrial involvement was confirmed following inhibition of viral protein R (Vpr)- and atractyloside (Atr)-induced $\Delta\psi_m$ of isolated mitochondria. Apoptosis in hippocampal sections of mice having undergone transient forebrain ischæmia was inhibited by PI treatment, as was camptothecin-induced apoptosis in PBMCs from patients taking PEP.

In conclusion, PIs inhibit apoptosis in PBLs from HIV-infected patients and in uninfected Jurkat T cells. The mechanism appears to involve mitochondria, as inhibition of Vpr- and Atr-induced $\Delta\psi_m$ of isolated mitochondria was observed. Stroke-induced apoptosis may be inhibited *in vivo* by PIs, and importantly, survival following anti-Fas challenge may be positively influenced.

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Last, but certainly not least as they say, I would like to dedicate this thesis to my parents, Rick and Madelene, without whose unfailing support this work would have never been done. You always believed in me, and more importantly, taught me to believe in myself. I can't imagine that anyone has a better example to look up to.

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LIST OF ABBREVIATIONS

3TC: lamivudine
ActD: actinomycin D
AFC: 7-amino-4-trifluoromethyl coumaride
AICD: activation-induced cell death
AIDS: acquired immunodeficiency syndrome
AIF: apooptosis inducing factor
ANT: adenine nucleotide translocator
Apaf-1: apoptosis protease-activating factor-1
ApoB: apolipoprotein B
APV: amprenavir
ART: anti-retroviral therapy
AZT: azidothymidine
BA: bongkrekic acid
Bcl-2: B-cell lymphoma-2
BH: Bcl-2 homology
BIR: baculoviral IAP repeat
BRUCE: BIR repeat-containing ubiquitin conjugating enzyme
BSA: bovine serum albumin
CA: capsid
CARD: caspase recruitment domain
Caspase: cysteine aspartyl protease
CCR: C-C chemokine receptor
CD: cluster differentiation
cFLIP: cellular FLICE-like inhibitory protein
CH11: anti-Fas antobody, clone CH11
CHX: cycloheximide
cIAP: cellular IAP
CPT: camptothecin
CRABP: cytoplasmic retinoic-acid binding protein
CSF: cerebrospinal fluid
CTL: cytotoxic T lymphocyte
CXCR: chemokine C-X-C receptor
CYP3A4: cytochrome P450 3A4
DC: dendritic cell
DcR: decoy receptor
DD: death domain
ddC: dideoxycytidine
ddG: dideoxyguanosine
ddI: dideoxyinosine
DED: death effector domain
DEVD-FMK: Z-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-FMK
DFF: DNA fragmentation factor
DIABLO: direct IAP binding protein with low pI
$DiOC_6(3)$: 3,3'-dihexyloxacarbocyanine iodide
DISC: death-inducing signalling complex

DNA: deoxyribonucleic acid
DNP: 2,4-dinitro phenyl
DR4: death receptor 4
dsRNA: double-stranded RNA
DTT: dithiothreitol
ECL: enhanced chemiluminescence
EDTA: ethylenediamine tetra-acetic acid
ELISA: enzyme-linked immunosorbent assay
Env: envelope
FACScan: fluorescence-activated cell Scan
FADD: Fas-associated DD protein
FAP: Fass-associated phosphatase
Fas: Fas receotor or CD95
FLICE: Fas-associated ICE-like protease or caspase 8
FMK: fluoromethyl ketone
Gag: group-specific antigen
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
Glut4: Glucose transporter 4
gp120: glycoprotein 120
HAART: highly-active ART
HEPES: hydroxyethyl piperazineethanesulfonic acid
HIV: human immunodeficiency virus
hsp: heat-shock protein
IAP: inhibitor of apoptosis protein
ICE: interleukin-1β-converting enzyme
IDV: indinavir
IETD-FMK: Z-Ile-Glu)Ome)-Thr-Asp(Ome)-FMK
IMM: inner mitochondrial membrane
IRS: insulin receptor substrate
LPV: lopinavir
LTNP: long-term non-progressor
MA: matrix
MCA: (7-methoxycoumarin-4-yl) acetyl
MDM: monocyte-derived macrophage
mRNA: messenger RNA
MRP: multidrug resistance associated protein
NAIP: neuronal apoptosis inhibitor protein
NFV: nelfinavir
NGF: nerve growth factor
NNRTI: non-nucleoside RTI
NRTI: nucleoside RTI
OAS: 2'-5' oligoadenylate synthetase
OMM: outer mitochondrial membrane
OxPhos: oxidative phosphorylation
Par-4: prostate apoptosis response protein 4
PARP: poly(ADP-ribose)polymerase

PBL: peripheral blood lymphocyte
PBMC: peripheral blood mononuclear cell
PCNA: proliferating cell nuclear antigen
PEP: post-exposure prophylaxis
PI: protease inhibitor
PKB: protein kinase B or Akt
PMSF: phenylmethylsulfonyl fluoride
Pol: polymerase
PR: protease
PS: phosphatidyl serine
PTPC: permeability transition pore complex
PVDF: polyvinylidene difluoride
RIP: receptor interacting protein
RNA: ribonucleic acid
RNAse L: 2-5A-dependent RNAse
ROS: reactive oxygen species
RPA: ribonuclease protection assay
RTI: reverse transcriptase inhibitor
RTV: ritonavir
Smac: second mitochondria-derived activator of caspase
SQV: saquinavir
SREBP: sterol-regulatory-element binding protein
SU: surface
TAR: transactivating response element
Tat: transactivator protein
tBid: truncated Bid
TBST: Tris-buffered saline Tween 20
TcR: T cell receptor
TM: transmembrane
TNF: tumour necrosis factor
TNFR: TNF receptor
TNFβ: lymphotoxin-β
TRADD: TNFR-associated DD protein
TRAIL/Apo2L: TRAIL/Apo2 ligand
TRAILR: TNF-related apoptosis-inducing ligand receptor
TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
VDAC: voltage-dependent anion channel
VEID-FMK: Ac-Val-Glu-Ile-Asp7-FMK
Vif: virion infectivity factor
VLDL: very-low density lipoprotein
Vpr: viral protein R
wt: wild-type
XIAP: x-linked IAP
ZVAD-FMK: Z-Val-Ala-Asp(Ome)-FMK
$\Delta\psi_m$: loss of mitochondrial transmembrane permeability

1. INTRODUCTION

1.1 APOPTOSIS

Modeling of tissue morphology and function involves numerous cellular processes, including cell growth, replication, and differentiation. Historically, one additional and important aspect, that of cell death, has often been overlooked.

Ultrastructural studies suggest that most cell death fall into two distinct categories. [1, 2] Necrosis is typically characterised by mitochondrial swelling and dissolution of internal and plasma membranes. [3] Necrotic cells die in an unregulated fashion, resulting in tissue damage and inflammation. In contrast, the other pattern of cell death is a highly regulated signal-induced process requiring gene expression. [4] Shrinkage of the cell, chromatin condensation, and fragmentation of the cell into membrane-bound vesicles are morphologic features associated with this phenomenon often referred to as *programmed cell death*. Apoptosis, a Greek expression which means, "falling off of petals from a flower", is the term used by Kerr, Wyllie and Currie to designate this process in 1972. [5] Unlike necrosis, apoptosis usually occurs without activation of inflammatory responses. [1, 6]

Apoptosis is fundamentally involved in the regulation of the immune system. Regulation of apoptosis itself occurs at many different levels, including receptor expression, intracellular apoptosis modifiers such as caspases, B-cell lymphoma (Bcl)-2 family members, inhibitor of apoptosis proteins (IAPs), and at the level of the mitochondria. Loss of regulation can potentially lead to severe diseases. Many extracellular death signals (extrinsic) and intracellular insults

(intrinsic) can trigger the genetically programmed pathway of apoptosis. [7] (Figure 1)

1.1.1 The Intrinsic Pathway

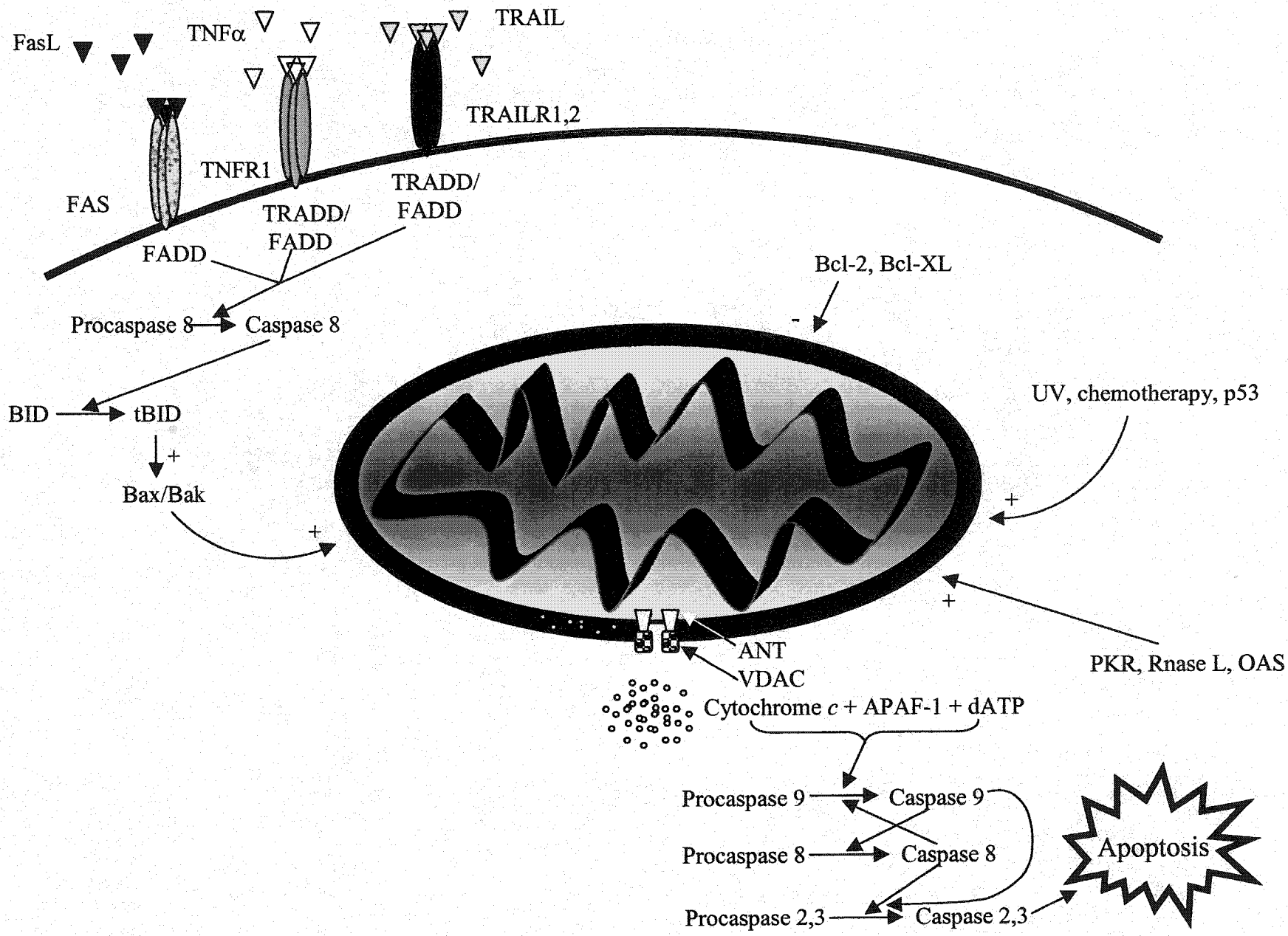
The intrinsic pathway allows a cell to respond to a variety of intracellular stresses, including deoxyribonucleic acid (DNA) damage, protein kinase inhibition, p53, c-myc, Bax, and loss of survival signalling. Integration of these apoptotic signals occurs at the level of the mitochondrion.

Mitochondria are the powerhouses of the human cell and produce energy through oxidative phosphorylation (OxPhos). [8] These organelles are encased in two phospholipid bilayer membranes. The outer mitochondrial membrane (OMM) is permeable to most small molecules and ions, but does not allow proteins and other macromolecules to pass. Conversely, the highly folded inner mitochondrial membrane (IMM) does not allow the passage of small ions, and consequently maintains an enclosed intra mitochondrial space (matrix).

The permeability transition pore complex (PTPC) is a multiprotein channel that consists of both matrix and mitochondrial membrane proteins including voltage-dependent anion channel (VDAC; OMM), peripheral benzodiazepine receptor (PBR; OMM), adenine nucleotide translocator (ANT; IMM), and cyclophillin D (matrix) amongst others. [9, 10] The PTPC normally maintains the mitochondrial

Figure 1. Regulation of apoptosis as it pertains to HIV.

Schematic representation of both the extrinsic pathway of apoptosis, mediated at the level of the cellular membrane by members of the TNF family of death-inducing receptors, and the intrinsic pathway of apoptosis, involving direct targeting of mitochondria.


FasL
TNFα
TRAIL
TRAILR1,2
TNFR1
FAS
TRADD/
FADD
TRADD/
FADD
FADD
Procaspase 8
Caspase 8
Bcl-2, Bcl-XL
-
BID
tBID
+
Bax/Bak
+
UV, chemotherapy, p53
+
+
ANT
VDAC
PKR, Rnase L, OAS
Cytochrome c + APAF-1 + dATP
Procaspase 9
Caspase 9
Procaspase 8
Caspase 8
Procaspase 2,3
Caspase 2,3
Apoptosis

transmembrane potential that results from the asymmetric distribution of ions on both sides of the IMM. [11, 12]

Soon after receiving an apoptotic signal, mitochondrial PTPC open, which disrupts the inner transmembrane potential. [13-16] Loss of mitochondrial transmembrane potential ($\Delta\psi_m$) is associated with the release of apoptogenic factors from the mitochondria [9, 13, 17] including cytochrome *c*, [18] pro-caspases 2 and 9, [19] second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO), [20, 21] apoptosis-inducing factor (AIF), [13] and endonuclease G [22]. Cytochrome *c*, together with apoptosis protease-activating factor (Apaf)-1 and in the presence of dATP, forms the apoptosome [23] that initiates apoptotic signalling downstream of the mitochondria by mediating auto-catalysis of pro-caspase 9. [24]

1.1.2 The Extrinsic Pathway

The extrinsic apoptosis pathway allows a cell to react to extracellular stimuli and usually involves members of the tumour necrosis factor (TNF) receptor superfamily and their ligands. This family of type I integral membrane glycoproteins contain extracellular cysteine-rich domains which exhibit sequence homology. [25] The p55 and p75 forms of TNF receptor (TNF-R1 and TNF-R2 respectively), [26-28] nerve growth factor (NGF), [29] cluster differentiation (CD) 40, [30] CD27, [31] CD30, [32] OX40, [33] 4-1BB, [34] the Fas receptor (CD95) [35] and the TNF-related apoptosis-inducing ligand receptors (TRAILRs) [36-42] have all been identified as members of this family.

The ligands for these receptors are all type II transmembrane proteins and display sequence identity with TNFα and lymphotoxin-α (TNFβ), two prototype ligands for this receptor family. [43]

The Tumour Necrosis Factor Receptor

A variety of cell types, with the exception of resting lymphocytes and erythrocytes, express the Tumour Necrosis Factor Receptor (TNFR). [43] Two distinct receptors exist: TNF-R1 (p55; CD120a; 60kDa) and TNF-R2 (p75; CD120b; 80kDa). [26-28, 44]

TNF-R1 shares structural and functional homology with Fas, and is capable of inducing apoptosis or necrosis depending on the cell type. Fas and TNF-R1 also share a common 80 amino acid intracellular motif - the "death domain" (DD). [45] Receptor oligomerisation causes aggregation of the DDs, which in turn initiates the apoptosis signalling pathway. [46, 47] The clustered DDs of TNF-R1 recruit TNFR-associated DD protein (TRADD), [48, 49] an adaptor protein that forms a docking site for Fas-associated DD protein (FADD). [50, 51] FADD in turn binds to and activates caspase 8 (Fas-associated ICE-like protease; FLICE) by a homotypic interaction involving the "death effector domains" (DEDs) of each protein. [52-54]

In contrast, TNF-R2 is primarily associated with cellular proliferation and cell signalling. Recent efforts to characterise TNF-R2 have shown that this receptor is capable of mediating apoptosis, [55, 56] despite its lack of a DD. Though the exact mechanism by which TNF-R2-mediated apoptosis occurs

remains unclear, TNF-R2-mediated death does involve the serine/threonine kinase receptor interacting protein (RIP). [57]

Reports that maximal induction of apoptosis is observed when both TNFRs are present [58] support the *in vitro* evidence that optimal TNF responses occur as the result of reciprocal interactions between the two TNFRs. (Reviewed in [59]) These interactions include "ligand passing" by R2 to R1, [60] TNF-R2-mediated induction of endogenous production of TNF, [61] and/or intracellular interactions of signal transduction molecules known as TNFR-associated factors (TRAFs). [62-67]

Many hematopoietic and non-hematopoietic cells express the 26kDa TNFα molecule, one of the principal ligands of the TNFRs. The membrane-associated form can kill target cells through direct contact [68] or can provide a co-stimulatory signal for B cells. [69] Processing of the membrane-bound form of this molecule gives an active 17kDa soluble form [70] that mediates a range of inflammatory and cellular immune responses. [71]

Apoptosis signalling through TNF-R1 may involve mitochondria, depending on the cell type. Active caspase 8 generated following receptor oligomerisation can cleave cytosolic, inactive p22 Bid, a member of the Bcl-2 family, into a major p15 (truncated Bid; tBid) and minor p13 and p11 fragments. [72] Truncated Bid targets mitochondria and releases cytochrome *c*, [73] which then mediates activation of caspase 3, poly(ADP-ribose)polymerase (PARP) cleavage and DNA fragmentation. [74]

Loss of mitochondrial membrane permeability ($\Delta\psi_m$) is involved in TNF-R1-mediated apoptosis, [75] as is production of reactive oxygen species (ROS). [76] Interestingly, the membrane proximal region of TNF-R1 can mediate clustering of mitochondria. [77] Absence of mitochondrial translocation can result in a delay in apoptosis. This occurs when a membrane-proximal region-dependent signal impedes mitochondria-associated kinesin. [77]

Another link established between mitochondria and the cell surface involves delivery of the TNFα cytokine to mitochondria. This demonstrates the presence of a pathway capable of delivering TNFα from the cell surface to mitochondria. [78] In addition to its role in inducing apoptosis, TNFα also induces the phosphorylation and translocation of Bad through a phosphatidylinositide-3-OH kinase-dependent pathway. [79] Phosphorylation prevents the interaction of Bad with the antiapoptotic protein Bcl-x_L, thus allowing Bcl-x_L to mediate its antiapoptotic activities. Additionally, A1, a member of the Bcl-2 family, may be upregulated in response to TNFα. [80, 81] This molecule is capable of maintaining mitochondrial viability and function in response to TNFα-induced apoptosis. [82]

The Fas Receptor

The Fas receptor (Fas or CD95) is a 45kDa molecule expressed on a variety of cell types, including activated T and B cells, [83, 84] macrophages [85] and neutrophils. [85] Apoptotic signalling through Fas mimics that seen with TNF-R1, with the exception that following receptor clustering, FADD directly

associates through its own DD to that of Fas. [46, 86] The intracellular structure composed of the cytoplasmic portion of the trimerised Fas, FADD and caspase 8 is termed the "death-inducing signalling complex" (DISC). Interestingly, oligomerisation of Fas may activate one of two different pathways leading to apoptosis – only one of which utilizes a mitochondrial signal amplification loop. [87-89] Cells with a type I Fas signalling pathway display optimal DISC formation. The active caspase 8 generated following optimal DISC formation immediately activates caspase 3, thereby bypassing the requirement for mitochondria. As a result of the bypass, antiapoptotic Bcl-2 family members cannot block apoptosis signalling in these cells. [87]

Cells with a type II signalling pathway recruit FADD to trimerised Fas; however in these cells DISC formation is minimal. An amplification step requiring mitochondria and the pro-apoptotic Bcl-2 family member Bid is required for caspase activation. Active caspase 8 cleaves and activates cytosolic Bid, which then targets to mitochondria. [73] Mitochondrial PTPC opening, $\Delta\psi_m$, release of cytochrome *c*, activation of caspases 9, 7, and 3, DNA fragmentation and cell death occur sequentially. [90-92] Antiapoptotic members of the Bcl-2 family inhibit this second pathway. [87] Though mitochondria are "activated" in both type I and type II cells, they are not strictly necessary for the death of type I cells.

The TNF-Related Apoptosis-Inducing Ligand Receptor

The TNF-related apoptosis-inducing ligand receptors (TRAILRs) are relatively new members of the TNFR superfamily. Five different TRAILR isoforms exist, though only two, TRAIL-R1 (DR4), and TRAIL-R2 (DR5), induce apoptosis.

[36-39] TRAIL-R3 (Decoy receptor 1; DcR1), which lacks a cytoplasmic domain, and TRAIL-R4 (Decoy receptor 2; DcR2), which contains a truncated cytoplasmic domain, may act as decoy receptors to inhibit TRAIL signalling [37, 38, 42] and do not mediate apoptosis upon ligation with TRAIL. [37, 38, 40-42]

TRAIL-R1 and -R2 share the intracellular DD that is common to the other members of the TNFR superfamily. Research indicates that TRAIL induces apoptosis through a caspase-dependent signalling cascade. [93] TRAIL-R1 and -R2 signalling pathways may involve the recruitment of both TRADD and FADD since a dominant negative form of FADD blocks death signals; [94, 95] however, the specifics of this signalling pathway remain controversial. [36, 96] Both TRAIL-R1 and -R2 activate NF-κB, presumably through a TRADD-dependent pathway. [94, 95] FADD- and caspase-dependent c-Jun amino (N)-terminal kinase (JNK) activity also contribute to TRAIL-induced apoptosis. [97] Interestingly, cellular FLICE-like inhibitory protein (cFLIP) abrogates TRAIL-mediated apoptosis. [94]

The cytotoxic ligand TRAIL/Apo2L shows a broad tissue distribution, [98] induces rapid apoptosis of various cell lines, [96, 98, 99] and is a potent mediator of tumour cell apoptosis. The fact that TRAIL/Apo2L exhibits minimal cytotoxicity toward normal tissues *in vitro* and *in vivo* has generated much interest. [93, 100, 101] Though the exact mechanism by which TRAIL induces apoptosis remains to be clarified, Bid-mediated mitochondrial involvement has been confirmed. [102, 103] Controversy remains however, since recent work regarding the effect of overexpression of Bcl-2 or Bcl-x_L on TRAIL-induced apoptosis show conflicting results. [104, 105]

1.1.3 Major Players in Apoptosis

The identification of major functional groups of proteins that affect cell death has facilitated apoptosis research. These proteins are common to the extrinsic and the intrinsic pathway, and include caspases, the Bcl-2 family members, inhibitor of apoptosis proteins and heat shock proteins.

The Caspase Family

Initiation of the apoptosis signal at the level of the cellular membrane translates intracellularly into activation of a family of cysteine aspartyl proteases termed caspases. Caspases cleave their target proteins at specific aspartate residues, [106, 107] and are important mediators of the apoptosis cascade.

At present, 14 mammalian caspases exist. Some participate in apoptosis, while others are involved in the processing of pro-inflammatory cytokines. [108, 109] Caspases exist as inactive zymogens and contain a pro-domain and a protease domain. [108, 110] The protease domain of caspases consists of a large and a small subunit. Activation of caspases involves release of the smaller subunit of the protease domain at the C-terminus, followed by removal of the pro-domain from the large subunit of the protease at the N-terminus. [110] Crystallography studies suggest that active caspases exist as heterotetramers composed of two large and two small subunits. [111-113]

Caspases with long pro-domains, such as caspases 8 and 10, function as upstream initiators, and contain two tandem DED repeats within their pro-domains. Homotypic interactions between these DEDs and those of adaptor

molecules such as FADD are responsible for caspase recruitment. [114] Caspases 1, 2, 4, and 9 also have long pro-domains, but instead of DEDs contain a caspase recruitment domain (CARD) that interacts with CARDs contained within other adaptor molecules. [114] Downstream effector caspases have short pro-domains and depend on the upstream initiator caspases for activation. These include caspases 3, 6 and 7.

The Bcl-2 Family

Organelle dysfunction, in particular mitochondrial function, is a common downstream effect of apoptosis. (For reviews, see [10, 110]) Bcl-2 family members function mostly at the level of mitochondria, and influence whether a cell will live or die. The Bcl-2 family includes both pro- and antiapoptotic molecules, and it is the ratio between these two subsets that helps determine, in part, the susceptibility of a cell to undergo apoptosis. [115] Members of the Bcl-2 family often function as homo- and heterodimers, and are characterised by their ability to become integral membrane proteins. The prototype antiapoptotic Bcl-2 inhibits apoptosis by forming heterodimers with pro-apoptotic Bax, thus preventing insertion of Bax in the mitochondrial membrane. [116-119]

Structurally, members of the Bcl-2 family can possess up to four conserved Bcl-2 homology (BH1-4) domains corresponding to α-helical segments. [120-122] Antiapoptotic members display sequence conservation in all four domains, whereas pro-apoptotic molecules display less sequence conservation within BH4. Deletion and mutagenic studies identified the amphipathic α-helical BH3 domain of pro-apoptotic members as a critical death

domain. This is also evidenced in the subset of "BH3-domain only" members displaying pro-apoptotic features. Another feature shared by certain members is the C-terminal hydrophobic domain, which has been shown to be essential for targeting molecules to membranes such as the outer mitochondrial membrane. [123]

Inhibitor of Apoptosis Proteins

IAPs are another family of molecules that exert their influence on apoptosis. To date, at least six vertebrate IAPs have been identified, including neuronal apoptosis inhibitory protein (NAIP), cellular IAP1 (cIAP-1), cIAP-2, X-linked IAP (XIAP), Survivin and BIR-repeat containing ubiquitin conjugating enzyme (BRUCE). [124-128]

IAPs are characterised by their baculoviral IAP repeat (BIR). BIR domains are named in accordance with their initial discovery in the genomes of baculoviruses. [129, 130] Three tandem copies of BIR domains can occur within IAPs; interestingly structure-function studies have demonstrated that a minimum of one BIR domain is required for inhibition of apoptosis. Other domains such as the C-terminal RING domain found in some mammalian, fly and viral IAPs may also be required under certain circumstances. Of note, the necessity of RING domains in the context of inhibition of apoptosis varies.

Overexpression of IAPs suppresses apoptosis induced by a variety of stimuli including TNFα and FasL. [125-127, 131] Mechanistically, certain IAPs – XIAP, cIAP-1, and cIAP-2 – function to inhibit caspases directly. [132, 133] These IAPs bind caspase 3, thus arresting the apoptotic cascade and providing

protection from Fas/caspase 8-induced apoptosis. [132-134] IAPs also bind to and inhibit pro-caspase 9. [134]

Heat Shock Proteins

In addition to Bcl-2 family and IAP family members, cells can also respond to a variety of stimuli by activating or accumulating members of the heat shock protein (hsp) family. [135-137] Under normal circumstances, these molecular chaperones regulate protein folding, however elevated expression of certain hsps can inhibit particular death processes.

Hsp 70 inhibits cleavage of the caspase 3 substrate PARP by reducing the processing of pro-caspase 3. [138-140] Another member of this family, hsp 90, forms a cytosolic complex with Apaf-1 and limits its ability to form an active complex. This results in inhibition of caspase 9 activation. [141] Additionally, hsp 27 has the ability to bind cytochrome *c* released from mitochondria. [142]

1.1.4 Animal Models and Apoptosis

Apoptosis is involved in the pathogenesis of a variety of human diseases. Historically, cell culture (*in vitro*) models have helped elucidate the cellular and biochemical pathways involved in apoptosis. *In vivo* efforts to study apoptosis in human disease have encountered many obstacles, including the unavailability of post-mortem tissue within the time frame of cell death. [143] Consequently, animal models have become useful in the study of the mechanisms involved in apoptotic cell death.

Human Neurological Disorders

Apoptosis is involved in the pathophysiology of a variety of human neurological disorders, including Alzheimer's, Parkinson's and Huntington's diseases, stroke, and multiple and amyotrophic lateral sclerosis. (For review see [144] and references therein) Animal models have proven invaluable as research tools in characterising pathways involved in neuronal death, which include mitochondrial alterations, caspase activation, ROS production, and perturbed calcium homeostasis. [144] Stroke research in particular has benefited from the increasing availability of animal models to identify the pathways involved in ischæmic brain injury and to develop novel therapeutic strategies that can minimise brain damage following a stroke.

Ischæmia

Throughout the world, stroke is a major cause of death and disability. Reduction in blood flow to the brain deprives neuronal cells of oxygen and glucose, and results in ischæmic injury. Cell death in the severely ischæmic core of injured tissue is typically necrotic in origin, whereas morphological and biochemical evidence of apoptosis has been documented in the less ischæmic (penumbral) region that surrounds the core. [145]

Evidence supporting a role for apoptosis in ischæmic injury includes terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL)-positive neurons in brains from human patients following stroke. Interestingly, levels of PARP are increased in these regions, which usually include the CA1 region of hippocampus. Neurons undergoing apoptosis exhibit:

$\Delta\psi_m$, [146] cytochrome *c* release, [146] increased prostate apoptosis response (Par)-4 expression, [147, 148] translocation of one or more members of the Bcl-2 protein family to mitochondrial membranes, [149] DNA fragmentation and externalisation of phosphatidyl serine (PS). [150, 151] Additional evidence supporting apoptotic death following stroke includes caspase 3 activation [152] and differential regulation of a variety of apoptosis mediators such as bcl-2 and bax following ischæmic injury. In cells that are destined to survive, the antiapoptotic *bcl-2* gene is upregulated, [153] whereas pro-apoptotic *bax* is induced in neurons destined to die. [154] The fact that certain manipulations prevent neuronal apoptosis, including Bcl-2 or Bcl-x_L overexpression [155-159] inhibition of protein synthesis, [160] and addition of caspase inhibitors further lend support to the role of apoptosis in neuronal death.

Replicating the pathologic state of the brains of stroke victims is possible, in part, thanks to two animal models. These include the transient global forebrain ischæmia model, where transient interruption of all the blood supply to the brain induces a stroke, and the focal cerebral ischæmia model, where damage to cerebral cortex and striatum occur following occlusion of the middle cerebral artery. (See [161, 162] for reviews)

1.2 HIV AND APOPTOSIS

Elimination of mature T lymphocytes which are no longer required or which may be harmful is essential in order to maintain homeostasis of the immune system. [163] However, excessive deletion of cells, such as CD4 T cells

in the context of the acquired immunodeficiency syndrome (AIDS), may lead to the loss of certain antigenic reactivities. [164] Studies suggest that increased apoptosis may play an important role in the ætiology of depletion of these cells during infection with HIV-1. [165] Various mechanisms may bring about the decline of CD4 T cells.

1.2.1 Mechanisms of Cell Death

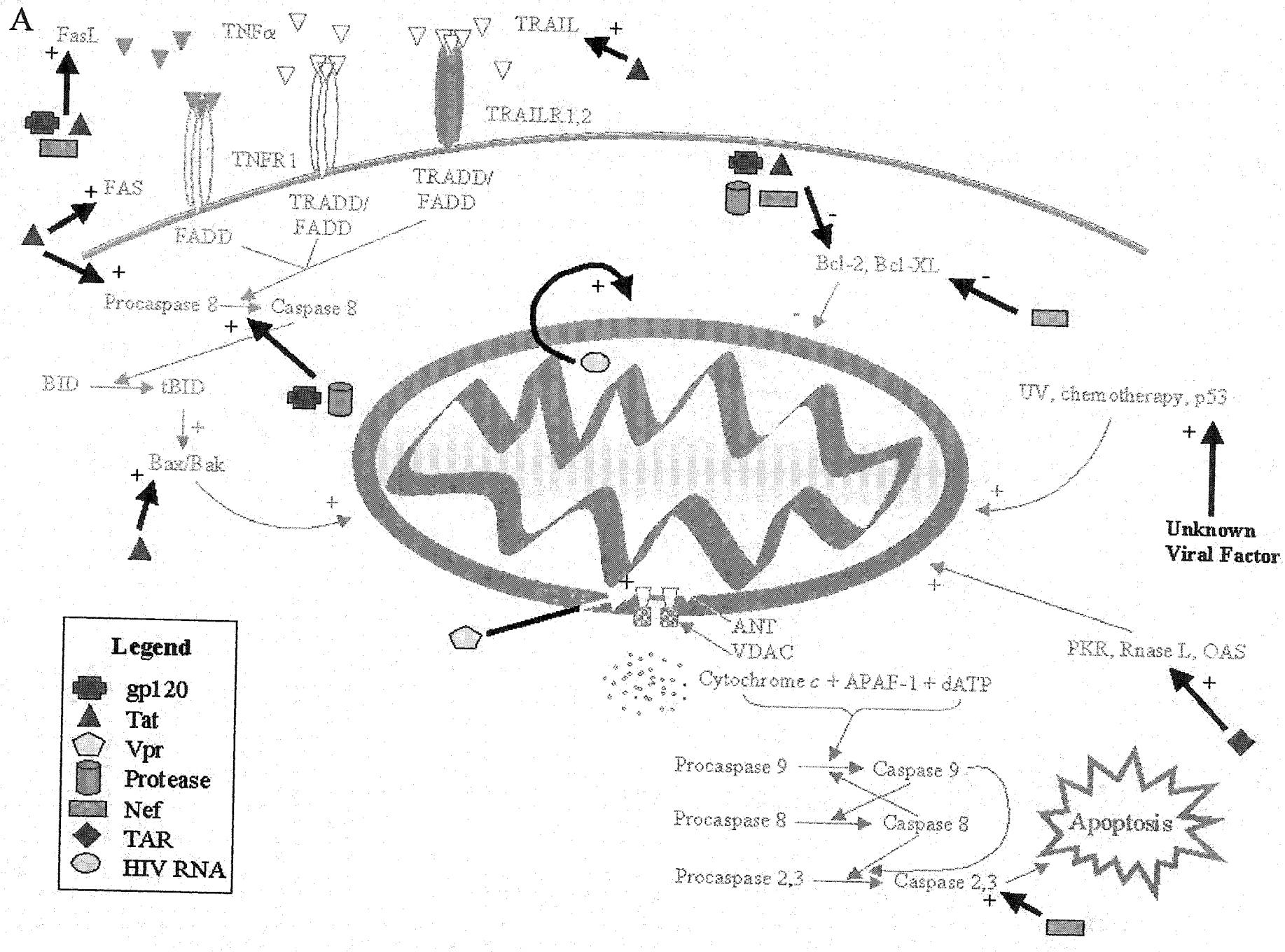
In persons infected with HIV, both infected (See Figure 2A) and uninfected (See Figure 2B) cells undergo accelerated apoptosis *in vitro* and *in vivo.* Apoptosis of cells directly infected with HIV accounts for only a small percentage of the apoptosis observed, as the vast majority of cells undergoing apoptosis are uninfected. [166, 167] The mechanisms proposed to mediate apoptosis in HIV include (1) gp120 cross-linking of CD4 or co-receptors, (2) activation-induced cell death (AICD), (3) direct infection of T cells, and (4) autologous cell-mediated killing of uninfected CD4 T cells ("bystander effect").

gp120 Cross-Linking of CD4 or Co-Receptors

The envelope (Env) polyprotein, which is comprised of the surface (SU or gp120) and transmembrane (TM or gp41) proteins, is a structural component that makes up – along with the gag proteins matrix (MA), capsid (CA), nucleocapsid (NC), p2, p1 and p6 – the core of the virion and outer membrane envelope. [168] gp120 is present on the surface of infected cells, on viral particles, or as a soluble protein. [169, 170]

Figure 2. Mechanisms of apoptosis in HIV.

(A) HIV-encoded proteins influence apoptosis at a variety of different levels. These inclde the following effects: gp120 on Bcl-2, FasL and caspase activation; Tat on Fas/FasL, caspase 8, Bcl-2, Bax and Trail; Vpr on ANT; protease on Bcl-2 and caspase activation; Nef on FasL, Bcl-2 Bcl-xL and caspase 3 activation; TAR on various dsRNA-dependent enzymes, which include the interferon-inducible protein kinase PKR, 2-5A-dependent RNAse (Rnase L) and 2'-5' oligoadenylate synthetase (OAS), as well as HIV RNA on mitochondria. (B) Many virally-encoded proteins are able to mediate apoptosis in uninfected T cells. These include the ability of gp120 to influence Bcl-2, FasL and caspase activation; Vpr and its effects on ANT; Tat's effects on Fas, as well as Nef's influence on FasL, Trail, Bcl-2, Bcl-xL and caspase 3 activation.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FADD
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TRADD/
FADD
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Procaspase 8
Caspase 8
BID
tBID
Bax/Bak
UV, chemotherapy, p53
Unknown
Viral Factor
ANT
VDAC
PKR, Rnase L, OAS
Cytochrome c + APAF-1 + dATP
Procaspase 9
Caspase 9
Procaspase 8
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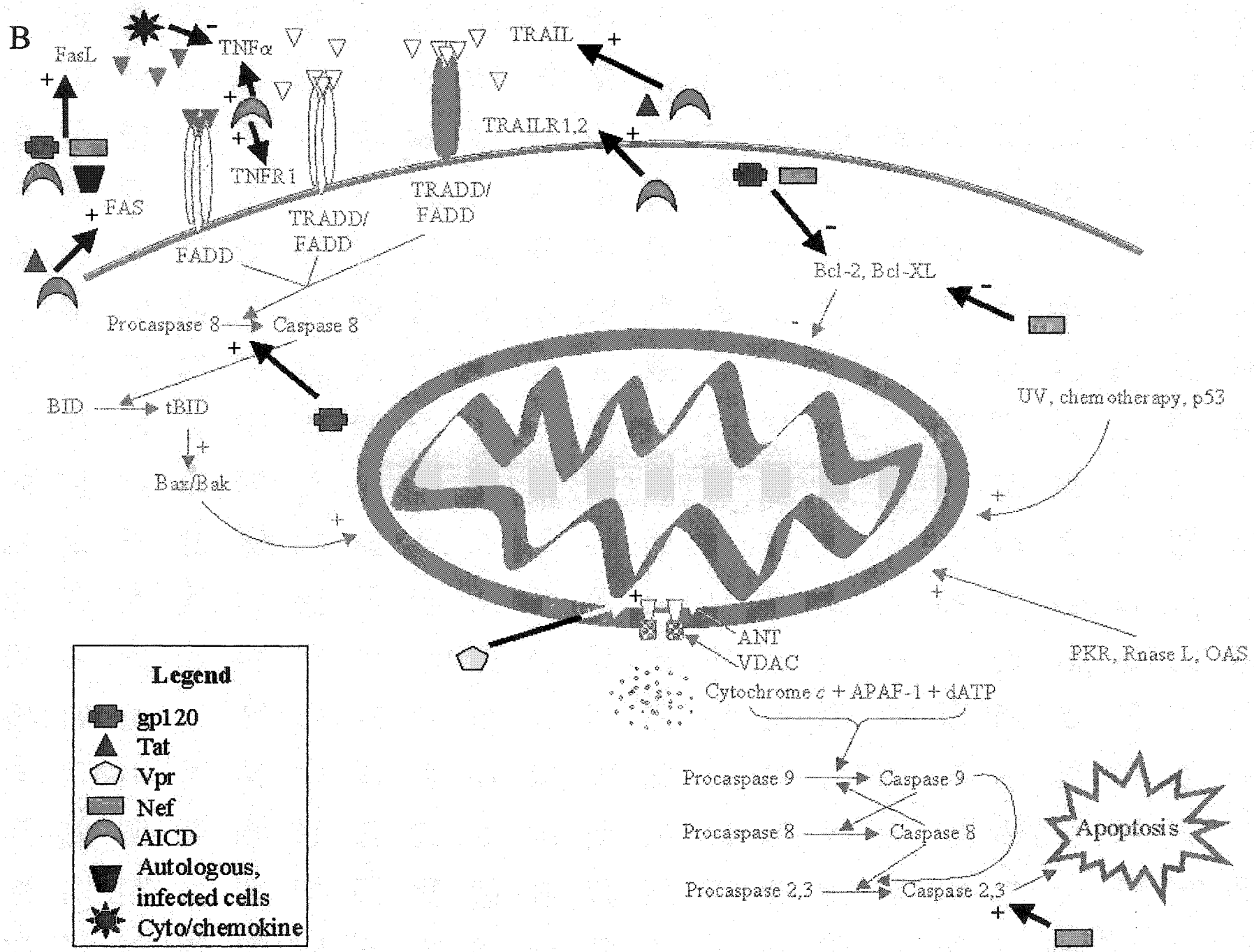
gp120
Tat
Vpr
Protease
Nef
TAR
HIV RNA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FasL
TNFα
TRAIL
TRAILR1,2
TNFR1
FAS
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FADD
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FADD
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Procaspase 8
Caspase 8
BID
tBID
Bax/Bak
UV, chemotherapy, p53
ANT
VDAC
PKR, Rnase L, OAS
Cytochrome c + APAF-1 + dATP
Procaspase 9
Caspase 9
Procaspase 8
Caspase 8
Procaspase 2,3
Caspase 2,3
Apoptosis
Legend
gp120
Tat
Vpr
Nef
AICD
Autologous, infected cells
Cyto/chemokine

Mechanistically, gp120 can bind to and cross-link CD4 and the chemokine co-receptors. Since CD4 stimulation in the absence of CD3 activation results in apoptosis, [171] gp120 binding to CD4 may trigger a similar outcome. [172] The interaction between gp120 and CD4 results in activation and enhancement of susceptibility to Fas-mediated killing in infected as well as uninfected cells. [173] This occurs as the result of increased expression of both Fas and FasL following gp120-mediated cross-linking of CD4 or co-receptors. [174] Controversy exists however, as both Fas-dependent [174-176] and Fas-independent [177-179] mechanisms have been demonstrated.

Intracellularly, gp120 signalling via CD4/chemokine C-X-C receptor (CXCR) 4 induces caspase activation, [180-184] leads to $\Delta\psi_m$, [185, 186] down-regulates Bcl-2 expression, [187] and increases Bax expression. gp120 may also initiate an alternate rapid form of cell death of CD4 and CD8 T cells by a mechanism that is independent of caspases and that does not lead to oligonucleosomal DNA fragmentation but that involves $\Delta\psi_m$. [188] Death of both infected and uninfected CD4 T cells has been attributed to gp120. [173, 174, 189]

Activation-Induced Cell Death

Activation-induced cell death (AICD) results from the sequential activation of CD4 T cells. Whether the primary stimulus is infection with HIV, or is mediated by cytokines, stimulation results in an increase in the level of susceptibility to Fas-mediated apoptosis. [190-192] In HIV-negative cells activation is associated with decreased cFLIP expression. [193] Interestingly, expression of cFLIP in

peripheral blood mononuclear cells (PBMCs) is no different in HIV-infected *versus* -uninfected persons. [194]

Following a second stimulus, cells express FasL, which can induce apoptosis in both autocrine and paracrine fashions. [195-199] Both *in vivo* and *in vitro*, HIV infection is associated with an activated T cell phenotype, [200-204] elevated expression of Fas, enhanced susceptibility to Fas-mediated killing, [174, 191, 205-208] as well as increased T cell expression of FasL following T cell receptor stimulation. [190-192] Although these observations implicate the Fas/FasL pathway, other studies have shown that AICD may occur without the participation of Fas in HIV-infected persons. [178, 209]

Administration of TRAIL/Apo2L and TNFα antagonists reduces AICD in cells from patients infected with HIV, suggesting that all three death receptors, Fas, TRAIL and TNF-R1, are involved. Though TRAIL does not induce apoptosis in normal peripheral blood T and B cells, [210] a neutralising monoclonal antibody to TRAIL effectively inhibits AICD in some, but not all, HIV-infected patients. [211] TRAIL agonists also induce apoptosis of *in vitro* HIV-infected monocyte-derived macrophages (MDMs) as well as peripheral blood lymphocytes (PBLs) from HIV-infected patients. [212]

Infected Cell Death

Infected cell death is dependent upon viral replication [170] but is independent of the Fas/FasL pathway as the kinetics of T cell apoptosis from patients expressing undetectable levels of Fas are unchanged compared to controls. [178, 213-215] Many different HIV-encoded proteins are involved in

infected cell death. These include Env, the transactivator protein (Tat), viral protein R (Vpr), HIV protease (PR), the numerous effector functions (Nef) protein, and the trans-activating response element (TAR). Some of these proteins may also be involved in uninfected cell death.

Envelope

In addition to Env's involvement in cell-surface-mediated apoptosis involving CD4 and the co-receptor, gp120 – a soluble product of Env – has also been implicated in apoptosis of syncytia. Syncytia form as the result of the fusion between infected gp120/gp41 expressing cells and uninfected cells expressing CD4/CXCR4. [169, 216, 217] Env-induced apoptosis in syncytia involves $\Delta\psi_m$; however inhibition of caspase activity does not abrogate release of AIF and cytochrome *c* from mitochondria. Conversely, nuclear condensation and fragmentation do not occur following inhibition of caspase activity in this model. Inhibition of $\Delta\psi_m$ by Bcl-2 does however suppress nuclear condensation and fragmentation. [188]

Transactivator Protein

HIV-1 transactivator protein (Tat) induces apoptosis both in T cell lines and in cultured PBMCs from uninfected donors. [218-221] Tat-induced apoptosis is characterised by enhanced expression of Fas/FasL, [174, 222] caspase 8 [223] and Bax, [224] and by decreased Bcl-2 and, possibly, Par-4 expression. Par-4 induces apoptosis by a mitochondrial-dependent pathway. [225, 226]

Stably transfected HIV Tat cells develop enhanced susceptibility to serum withdrawal-induced apoptosis, which is characterised by $\Delta\psi_m$, increased production of ROS and loss of nuclear DNA. Tat's suppressive effect on messenger ribonucleic acid (mRNA) [227] and/or protein expression [228] of the mitochondrial isoform of superoxide dismutase (SOD2) may, in part, explain this phenomenon. Decreased levels of SOD2 cause decreased levels of glutathione and facilitate oxidative conditions that generate ROS and mitochondrial death. [228] The fact that Tat translocates to mitochondria to cause $\Delta\psi_m$ [229] confirms mitochondrial involvement in Tat-mediated apoptosis.

Viral Protein R

HIV-1 Vpr directly induces $\Delta\psi_m$ in both intact cells and in isolated mitochondria. Vpr localises to the mitochondria, where it specifically binds ANT with an affinity in the nanomolar range. [230] Accordingly, overexpression of Bcl-2 abrogates Vpr's effects on mitochondria and prevents the induction of nuclear condensation and fragmentation. Studies using deletion mutants established that the C-terminal portion of the Vpr molecule (Vpr52-96) is the segment responsible for release of cytochrome *c* and AIF, uncoupling of the electron transport chain, OxPhos collapse, and caspase-dependent apoptosis. [231, 232]

In addition to its effects on mitochondria, Vpr has been implicated in the induction of G_2/M cell cycle arrest leading to apoptosis; [233-235] however since apoptosis is seen in all phases of the cell cycle, this concept remains controversial. [215] In some instances Vpr may even protect from apoptosis,

[236, 237] but this may only occur early in infection since in later stages of infection Vpr induces apoptosis. [238]

The frequency of mutations in the apoptosis-inducing domain of Vpr in long-term non-progressors (LTNPs) hints at the relative contribution of Vpr in apoptosis. *In vitro*, cells treated with mutant R77Q Vpr peptides exhibit less apoptosis, and isolated mitochondria have less $\Delta\psi_m$ compared to cells and mitochondria treated with wild type (wt) peptides. [239] R77Q mutations exist in as many as 80% of LTNPs, whereas only 33% of patients with progressive disease have such mutations. [239] Since Vpr is cell permeable, it may be involved in killing both infected and uninfected cells.

HIV Protease

HIV-encoded protease (PR) is a cytotoxic protein that causes apoptosis of human and bacterial cells following transfection [240-243] by virtue of activating cellular factors which, in turn, induce apoptosis. Consistent with this hypothesis, HIV PR cleaves Bcl-2, thus shifting the balance of pro- and antiapoptotic proteins in favour of apoptosis. [244] In addition, HIV-1 PR can activate caspase 8, [245] thereby leading to Bid cleavage and mitochondrial activation. This ultimately leads to cleavage of PARP, DNA fragmentation factor (DFF) and to nuclear fragmentation. [245]

Numerous effector functions protein

The numerous effector functions (Nef) protein is essential for viral pathogenicity. This fact may suggest a role for Nef as a mediator of apoptosis.

Human infection with naturally occurring Nef deletion mutants leads to less rapid CD4 T cell depletion. [246, 247] Further, Nef synergistically enhances the activating effects of T cell receptor (TcR) ligation [248-250] in a stimulus-dependent manner. These differential effects may be due to differences in the intracellular localisation of Nef [251] or to the resultant decrease in efficiency of viral replication. [252-255]

Stably transfected Nef-expressing T cells express FasL. [256] Interactions between Nef and the TcR ζ chain induce FasL expression [257] and reduce expression of Bcl-2 and Bcl-x_L. Interaction between Nef and the CD4 receptor results in downregulation of the CD4 molecule. [258, 259] This occurs as the result of associations between the C-terminus of the CD4 receptor and both Nef and p56lck. [260]

Nef also mediates cytolysis of uninfected CD4 [261] and CD8 T cells, [262] and functions in a manner that requires cell contact, and is major histocompatibility complex (MHC) unrestricted. [261] The characteristics of this form of cell death include $\Delta\psi_m$, activation of caspase 3, and cleavage of PARP. The partial reduction of apoptosis that follows addition of a broad-spectrum caspase inhibitor suggests the existence of an additional caspase-independent pathway. [263] Interestingly, Nef may also exert an apoptotic effect on uninfected CD4 T cells by Fas-independent binding to an unidentified receptor(s). [264]

Trans-Activating Response Element

Cellular systems possess innate immunity against viral infections. The presence of intracellular double-stranded RNA (dsRNA) of viral origin may

activate enzymes including the interferon-inducible protein kinase R (PKR), 2-5A-dependent RNAse (RNAse L), and 2'-5' oligoadenylate synthetase (OAS). [265, 266] The activation of these enzymes leads to mitochondrial release of cytochrome *c*, followed by caspase activation and apoptosis. [267] In HIV infection, the viral transactivating response element (TAR), an RNA hairpin located at the 5' end of the nascent viral transcript, acts like a dsRNA fragment and induces PKR-mediated apoptosis. [268]

Autologous Infected Cell-Mediated Killing

Cells derived from HIV-infected patients – including macrophages, [269, 270] monocytes, [175, 271, 272] PBMCs, [273] CD4, [274] and CD8 T cells [275] – induce death of autologous, uninfected CD4 T lymphocytes. Two mechanisms contribute to autologous infected cell-mediated killing. First, increases in FasL and/or TNFα expression in infected cells can result in the induction of apoptosis of surrounding activated CD4 and CD8 T cells. [270, 276] Second, interactions between gp120 and macrophage CXCR4 can lead to apoptosis of CD8 T cells through a TNFα/TNFR2 interaction. [270] The direct correlations of FasL expression and tissue apoptosis in tonsillar biopsies obtained from HIV-infected but untreated patients highlights the significance of macrophage-mediated death of bystander cells *in vivo*. [277, 278]

1.2.2 HIV and Mitochondria

Mitochondrial dysfunction, characterised by $\Delta\psi_m$ and enhanced production of mitochondrial ROS has been observed in circulating T lymphocytes from

persons infected with HIV. [232] In addition, a p53-initiated mitochondrial pathway in which increased levels of total and phosphorylated p53 and Bax are observed was recently identified. [279] p53 expression correlates with disease progression in both rapid and slow progressors. [280] Alternately, mitochondrial damage in HIV disease has also been hypothesised to be the result of a direct cytolytic effect of HIV RNA (genomic or mRNA transcripts) on the infected cell. [281] In one report, high concentrations of viral RNA, found preferentially in mitochondria relative to the cytoplasm and nucleus, were associated with a decrease in mitochondrial viability. [281]

1.2.3 Apoptosis and Disease Progression

The enhanced rate of T cell apoptosis seen in HIV infection correlates directly with the rate of disease progression and suggests that apoptosis plays a critical role in the pathogenesis of HIV disease. The rate at which HIV-1 infection progresses to AIDS is dependent upon host factors such as age, [282-285] and genetic background, [286-289] as well as on viral factors including *nef*, virion infectivity factor (*vif*), *vpr*, *vpu*, *tat* and *rev*. [246, 290-294]

In contrast, a subset of HIV-1-infected LTNPs remains asymptomatic and immunologically normal despite prolonged HIV-1 infection. As many as 25 - 30% of LTNPs have specific homozygous mutations in the HIV-1 C-C chemokine co-receptor (CCR) 5 and CCR2. These mutations result in impaired viral attachment and diminished viral infectivity. [295] In other LTNPs, reduction of viral replication to undetectable levels occurs because of mutations in viral genes (such as *nef* [296]) that influence replication.

Other factors that may modify HIV disease progression include MHC haplotypes (B27, B57, and B51) that are strongly associated with long-term non-progression. Finally, HIV-specific CD8 cytotoxic T lymphocytes (CTLs) may retard disease progression and favour long-term non-progression. [297]

Spontaneous apoptosis is greater in patients with progressive HIV disease than in LTNPs. The frequency of T cells exhibiting $\Delta\psi_m$ and increased ROS production is also lower in LTNPs. [298] These findings indicate that the degree of apoptosis correlates inversely with CD4 T cell depletion, and suggest that apoptosis is both necessary and sufficient for HIV-induced immunodeficiency.

Supportive evidence that apoptosis contributes to loss of CD4 T cells in HIV-1-infection of humans comes from the normal levels of apoptosis in apathogenic lentivirus infections of non-human primates, including HIV-1 infection of chimpanzees. [299-301] Chimpanzees infected with LAI/LAV-1b strain of HIV develop progressive loss of CD4 T cells with high viral burdens and increased levels of CD4 T cell apoptosis both *in vitro* and *in vivo*. [302] By contrast SIV-infected chimpanzees do not lose CD4 T cells and have no increases in apoptosis compared to control groups.

1.2.4 Apoptotic Changes During Therapy

Changes in apoptosis in response to anti-retroviral therapy (ART) also suggest a link between disease progression and apoptosis. Numerous studies evaluating apoptosis in lymph nodes, rectal mucosa and PBL subsets in HIV-infected patients have shown dramatic decreases in apoptosis in response to therapy. Of note, these decreases may occur as early as four days following the

start of protease inhibitor (PI)-based ART. [277, 278] Patients receiving only the PI component of highly active anti-retroviral therapy (HAART) also show similar decreases in apoptosis. [194] HAART typically combines the use of two reverse transcriptase inhibitors (RTIs) with one or more PI.

Many different mechanisms contribute to the increase in CD4 T cell numbers observed within two weeks following institution of HAART. These include redistribution from lymphoid tissue, [303] cellular proliferation of the peripheral T cell pool, [304] new T cell synthesis from a thymic source, [305-307] and reduced levels of apoptosis. [194, 278] One particular study that investigated the effects of HAART on spontaneous T cell apoptosis observed that the rapid and sustained increase of both naïve and memory CD4 T cells and, to a lesser extent CD8 T cells was associated with a significant decrease of apoptosis. [308] Thus, effective viral suppression can contribute to a decrease in apoptosis, which in turn may contribute to immune reconstitution.

1.3 HIV PR INHIBITORS

The first generation of licensed anti-HIV drugs were inhibitors of the HIV-encoded reverse transcriptase (RT). These drugs, RTIs, resemble nucleic acids (RNA and DNA), but differ from their natural analogues by virtue of a 3' hydroxyl group that cannot form the 5' to 3' phosphodiester link. They function therefore both as competitive inhibitors of reverse transcriptase and as DNA chain terminators.

In initial human trials, treatment with Zidovudine (ZDV) monotherapy improved CD4 T cell numbers, caused weight gain, decreased the incidence of opportunistic infections and AIDS-defining events, and increased survival. [309-313] Unfortunately these beneficial effects were transient [314] due to the rapid development of viral mutations, and the consequent decrease in susceptibility to inhibition by this family of drugs. In addition, frequent toxic side effects limit the tolerability of RTIs. Consequently, the search for novel targets for antiretroviral drug therapy continued.

1.3.1 Identification and Targeting of HIV PR

The successful addition of HIV PIs to the existing arsenal of anti-HIV drugs was the direct result of the early recognition of the central role of HIV PR in the viral life cycle, and the intensive effort made toward gaining an understanding of its structure and function.

In 1985, it was suggested [315] that HIV, similar to other retroviruses, encoded a protease within the *pol* region of the viral genome. Homology to cellular aspartyl proteases, biochemical inhibition by pepstatin, and inactivation by mutagenesis of the active site aspartate residues prompted the classification of this enzyme as an aspartyl protease. [316-319]

The HIV PR sequence identified an open reading frame that coded for a protein that was only 99 amino acids in length - considerably shorter than that of other known aspartyl proteases such as pepsin and rennin. [320] The active site of other aspartyl proteases is composed of two aspartic acids, one from each half of the molecule; by contrast HIV PR exists as a homodimer with each molecule

contributing an aspartyl residue to the active site. [321, 322] Pearl and Taylor [323] modeled the putative HIV PR dimer and published a structure that fits remarkably well with the X-ray structure that has now been determined for the enzyme.

1.3.2 Mechanism of PR and Role in the Viral Life Cycle

The HIV genome resembles that of other members of the retrovirus family and consists of a single-stranded, positive-sense RNA molecule that is organised into three major coding elements: *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope) genes.

The 55KDa gag polyprotein (Pr55gag) contains the structural proteins of the virion – matrix (MA), capsid (CA) and nucleocapsid (NC) – in addition to the peptides p2, p1, and p6, which are involved in the assembly, and morphogenesis of mature capsids. (Refer to [324] and references therein)

Encoded within the *pol* gene are the enzymes necessary for viral replication, including PR, RT, and integrase (IN). PR160gag-pol, a larger polyprotein translated within the context of a ribosomal frameshift and read-through of the *gag* gene, also contains these proteins. During viral assembly and maturation, preformed PR contained within the HIV virion and introduced into the cell at the time of infection proteolytically cleaves the Pr55gag and Pr160gag-pol polyproteins. [325, 326] Preformed PR also generates new PR by virtue of new cleavages at its C-terminus between PR and RT. [327] The critical role of PR in the HIV life cycle is demonstrated by observations that mutation or inhibition of

PR results in the formation of structurally disorganized and non-infectious viral particles. [328-331]

Experiments designed to identify what elements of a peptide sequence define it as a substrate suggest that a pattern of hydrophobic/hydrophilic amino acids across a stretch of 8 amino acids surrounding the scissile bond are key to the specificity of PR; however enormous versatility exists regarding substrate sequence specificity. [332, 333] Interestingly, the substrate specificity HIV PR is not restricted to viral proteins, as numerous host proteins are also cleaved by PR. [332, 333] Identification of the substrate specificities of HIV PR has led to the development of synthetic substrates, and consequently the design of quantitative assays of PR activity. [334, 335]

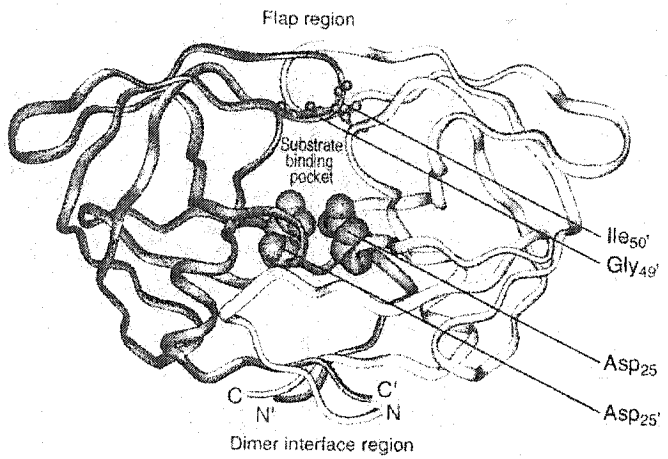
1.3.3 HIV PR Inhibitor Design

In view of the structural and functional similarities between the retroviral and cellular aspartyl proteases (such as rennin), previous efforts to design peptidomimetic substrates containing non-cleavable bonds paved the way for the design of HIV PR inhibitors. [336] Identification of the structure of HIV PR (Figure 3A) by high-resolution 3D crystallography [337] in the presence and the absence of peptidomimetic inhibitors allowed for the development of a new generation of inhibitors that did not rely strictly on substrate or peptide mimicry. [336] Furthermore, computational modeling led to increased antiviral potency and improved bioavailability. [338] By combining these approaches, newly identified HIV PR inhibitors (Figure 3B-D) now include competitive small-molecule

Figure 3. Structures.

(A) Structure of HIV Protease; [341] Chemical structures of (B) nelfinavir (NFV), [359] (C) ritonavir (RTV) [359] and (D) saquinavir (SQV) [359].

A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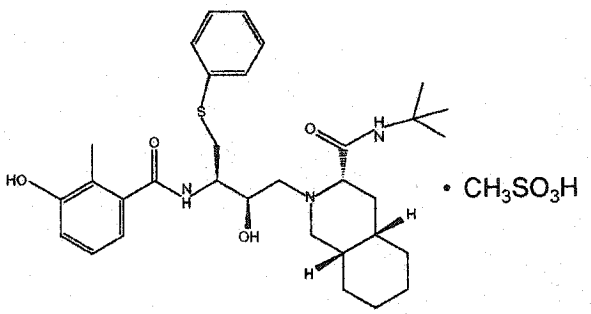
Asp25
Asp25'
C
N'
C'
N
Dimer interface region

B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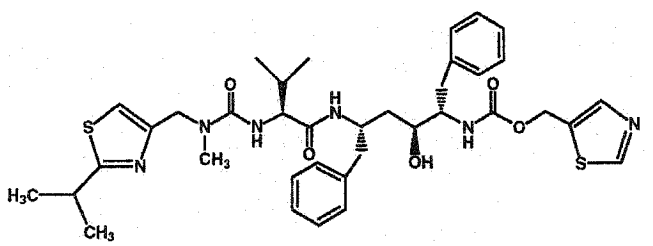
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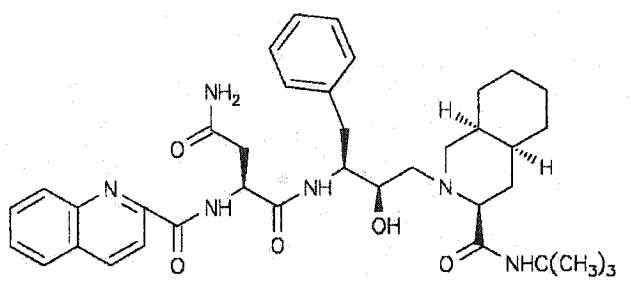
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inhibitors, irreversible small-molecule inhibitors, dimer disrupters and dominant-negative inhibitors.

1.3.4 Mechanism of Inhibition

All compounds currently in clinical use as inhibitors of HIV PR belong to the competitive small-molecule inhibitor family of drugs. These include saquinavir (SQV; Hoffman-LaRoche), ritonavir (RTV; Abbott), indinavir (IDV; Merck), nelfinavir (NFV; Agouron/Pfizer), amprenavir (APV; Vertex/Glaxo-Wellcome) and lopinavir (LPV; Abbott)]. All six drugs are nonscissile substrate analogues for the HIV protease and share the same structural determinant – a hydroxyethylene bond. [339] They function as peptidomimetic inhibitors. [340]

Other PR inhibitors are in various stages of development and testing. [340] They include: (1) agents that irreversibly modify the active site aspartates, [341] (2) drugs which target the dimer interface region of the enzyme, [342-345] and (3) defective PR monomers. [346-349]

1.3.5 Impact of PIs on HIV Disease

The development of clinically effective PIs allows clinicians to administer combinations of drugs that target different steps within the HIV viral life cycle. HAART typically causes CD4 T cell counts to increase and plasma HIV RNA levels to decrease, and has greatly reduced the morbidity and mortality associated with HIV infection. [350-352] Several factors influence the ability of PIs to impact HIV replication, including pharmacokinetics, and drug resistance.

Pharmacokinetics of PIs

The clinical utility of HIV PIs [353, 354] is influenced by their absorption, distribution, metabolism, elimination, and interaction with other drugs. Some PIs are poorly absorbed, (e.g. SQV: 4% for a 600mg dose) [340] yet food may enhance the absorption of other PIs, depending upon the specific drug, its formulation, and meal composition. [355, 356]

PIs are also tightly protein-bound in the plasma, and only the free drug fraction is available to penetrate the various anatomical compartments in which HIV replicates, including into the cerebrospinal fluid (CSF). [357] The CSF to plasma ratios for APV, [358] NFV, [359] RTV, [360] and SQV [360] are 1% or less, whereas the comparable ratio for IDV is at least 12%. [361]

The cytochrome P450 enzyme system exists predominantly in the gut and liver. [362, 363] Among its many functions, cytochrome P450 converts PI compounds into their metabolites, which may or may not retain activity against HIV PR. Consequently, inhibition of P450 enzymes increases the bioavailability of the parent drugs. [364-366] Ingestion of grapefruit juice inhibits the P450 isoform CYP3A4, which is the isoform largely responsible for PI metabolism. [367, 368] Therefore co-administration of PIs with grapefruit juice increases the bioavailability of a variety of most PIs. [369] Since the PI RTV also inhibits CYP3A4, [370] its co-administration with other PIs enhances the bioavailability of that second PI, as well as providing additive or synergistic antiviral effects.

Resistance

The emergence of drug-resistant viral variants has reduced the clinical effectiveness of PIs. [371] Viral resistance to PIs emerges as the result of the rapidity of HIV-1 viral replication [372, 373] combined with the high error rate of HIV-1 RT (1 mutation *per* 10,000 bases) [374] and incomplete viral suppression by the currently available antiretroviral agents. [375] Thus, given the size of the HIV genome (~9kb), and the daily HIV turnover of up to 10^{10} virions per day, the likelihood of developing a specific mutation becomes a near certainty given enough time. To date, drug resistance has been associated with 42 substitutions occurring at 27 sites within the 99 amino acids of HIV PR, [376] including mutations that occur within the substrate-binding domain of the enzyme. [377, 378]

1.4 INDIRECT EVIDENCE THAT PIs HAVE INTRINSIC IMMUNOLOGIC EFFECTS

Over the course of the past few years, an increasing amount of indirect evidence has accumulated suggesting that PIs have immunologic effects that are independent of their effects on viral suppression. The suggestion that PIs directly influence CD4 T cell number surfaced following studies in which increases in CD4 T cell counts were greater in the PI-based versus the non-PI-based therapies given similar amounts of viral suppression.

Epidemiological studies had until then established a correlation between suppression of viral replication and improvements in CD4 T cell counts. [379-382]

This relationship proved to be true in early studies of RTI-based therapies; however the rapport between viral replication and the rate of CD4 T cell decline in PI therapies suggested that this was not always the case. [383-385]

Certain subsets of patients manifest discordant responses to therapy. In these patients, who represent as many as one-fifth of individuals receiving ART, [386] improvements in CD4 T cell counts occur despite incomplete suppression of HIV plasma viremia and/or persistent high-grade viral replication. [386-394] Two studies of discordant subjects confirmed the dependence of discordance to the presence of PIs. In these studies, discontinuation of PI-based therapy coincided with a rapid decline in CD4 T cell counts, even though only modest increases in HIV plasma viremia occurred. [395, 396]

PI-containing regimens are also associated with greater CD8 T cell increases than non-PI-containing regimens. [397] Peak increases in CD8 T cell numbers correlate with peak CD4 T cell responses. In contrast, no correlations have been observed between CD8 T cell counts and peak viral load. [397]

Recently, several studies have investigated the impact of switching the PI component of a PI plus dual nucleoside RTI (NRTI) regimen to a non-nucleoside RTI (NNRTI). While CD4 counts remained stable before and after the "switch", five studies reported a decrease in CD4 T cell counts in patients whose drug regimen involved a switch from PI to NNRTI. [398-402]

1.5 NON-VIROLOGIC EFFECTS OF HIV PIs

Experiments probing the effects of the PI class of drugs on cellular systems have shown that PIs influence many different aspects of cellular function independently of their effects on viral replication. One of the first such reports involved inhibition of cytochrome P450 isoforms. [403] As previously discussed, RTV is a potent inhibitor of CYP3A4-mediated reactions, and other PIs, namely SQV and IDV, also display inhibitory effects with respect to this family of enzymes. In addition, experiments performed by André and colleagues showed that RTV was able to modulate proteasome activity, antigen presentation, and T cell responses. [404] P-glycoprotein (P-gp) activity is also subject to modulation by HIV PIs. [405]

1.6 RATIONALE AND RESEARCH OBJECTIVES

Advances in treatment have transformed HIV infection from a progressive and ultimately fatal disease to one that is manageable with the help of chronic suppressive ART. PIs used in the management of HIV infection not only inhibit viral replication; indirect evidence suggests that these compounds also have intrinsic immunologic effects.

Our hypothesis is that HIV PIs can inhibit a cell's ability to undergo apoptosis. The purpose of our experiments is to determine whether PIs inhibit apoptosis in infected and/or uninfected cells, or whether administration of PIs inhibits apoptosis within the context of an animal model of disease in which apoptosis plays a role. Defining the potential molecular mechanism underlying

this phenomenon is also critical. In order to address these issues, we propose the following objectives:

1. Evaluate the effect of HIV PIs on spontaneous and stimulus-induced apoptosis in cells from HIV-infected patients and in uninfected cell lines.

2. Investigate the mechanism by which HIV PIs modulate the ability of cells to undergo apoptosis.

3. Assess whether HIV PIs can modulate apoptosis *in vivo* in animal models of disease in which apoptosis plays a role.

2. MATERIALS AND METHODS

Patients

HIV-infected patients attending the Immunodeficiency Clinic at the Ottawa Hospital, General Campus, healthy adult volunteers initiating post-exposure prophylaxis (PEP) and healthy controls were invited to participate. HIV-infected patients were excluded if they had been treated with RTIs for more than six months, or had been previously treated with HIV PIs. Blood (20-40mLs) was collected in heparinised tubes. For the PEP study, immunologic evaluation occurred immediately prior to the initiation of, during, and at least one month following PEP. These studies were reviewed and approved by the Ottawa Hospital Research Ethics Board and the University of Ottawa Ethics Committee.

Cells

PBLs from HIV-infected and -uninfected donors were isolated by density gradient centrifugation (Heræus Megafuge 1.0, Raleigh, NC; 500g, 15 minutes, no brake), using Ficoll Paque Plus. Following centrifugation (320g, 5 minutes), cells were washed and resuspended in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin and L-glutamine. Monocytes were removed by plastic adherence, and the resultant PBL population ($0.5x10^6$ cells/mL) was incubated at 37°C in a 5% CO_2 humidified environment. PBLs were cultured in media supplemented with drugs as indicated for 16 hours, and then stimulated with either an agonistic anti-Fas antibody (Clone CH11) or an isotype-matched control antibody for four hours and assayed for apoptosis (see below).

PBMCs from patients receiving post-exposure prophylaxis (PEP; azidothymidine [AZT], lamivudine [3TC], NFV) were isolated on days 0 (prior to start of PEP), day 7 and 6 weeks following discontinuation of therapy by density gradient centrifugation using Ficoll Paque Plus. Following centrifugation (320g, 5 minutes), cells were washed and resuspended in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin and L-glutamine.

Jurkat T (Clone E6-1) and H9 T cells (derivative of HuT 78) were cultured in RPMI 1640 supplemented with 10% or 20% fetal calf serum respectively, together with penicillin, streptomycin, and L-glutamine, and were incubated at 37°C in a 5% CO_2 humidified environment. Cells were seeded in media supplemented with drugs as indicated at 0.3 $x10^6$ cells/mL and passed every 2 days.

Drugs

AZT, dideoxyguanosine (ddG), and dideoxycytidine (ddC) were purchasedfrom Sigma-Aldrich (Oakville, ON). Dideoxyinosine (ddI) was a generous gift of Bristol Myers Laboratories. Ritonavir (RTV) was a generous gift from Abbott Laboratories, whereas nelfinavir (NFV) and saquinavir (SQV) were generous gifts from Agouron Pharmaceuticals and Roche Laboratories respectively. All drugs were reconstituted in either methanol (NFV, RTV, and SQV) or water, and frozen at -80°C until use. Control cells were treated with an equivalent volume of either methanol or water, as appropriate.

Induction and Measurement of Apoptosis and Mitochondrial Membrane Permeability Changes

Following the indicated drug treatments, patient and control PBLs were stimulated with either agonistic anti-Fas antibody (CH11; 0.5µg/mL) or with an isotype matched control IgM antibody. Following 4 hours of stimulation, PBLs were gently harvested, washed with phosphate buffered saline (PBS), resuspended in 400 µL binding buffer (10mM Hepes/NaOH pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$) and incubated sequentially with 10µg/10^6 cells γ-immune globulin, 1µg/10^6 cells of anti-CD4-APC, 1µg/10^6 cells of anti-CD8-PerCP (or appropriate isotype control antibodies) and 5 µL annexin-V-FITC. Samples were incubated in the dark for 20 minutes at room temperature.

After staining, PBLs were fixed with 0.25% paraformaldehyde and stored in the dark at 4°C until flow cytometry. Annexin-V positive cells were determined by setting the annexin-V gate to zero using lymphocytes from an HIV negative control processed in parallel. To exclude dead cells, forward versus side scatter plots were gated to include only the viable PBL population, and the expression of CD4, CD8 and annexin-V was determined only within this viable population as previously described. [194, 406] For each analysis, 20,000-30,000 total events were collected using a FACScan. (BD Biosciences, Mississauga, ON)

Apoptosis of PBMCs from patients taking PEP was induced with actinomycin D (ActD; 10µg/mL), camptothecin (CPT; 10µM), or CH11 (0.5µg/mL). Control cells were cultured in media only. Following a 16-hour incubation, apoptosis was measured by annexin-V and propidium iodide staining. Cells (10^6) were washed in PBS and resuspended in 400µL binding buffer prior

to adding 5 µL annexin-V-FITC. Samples were incubated in the dark for 20 minutes at room temperature prior to adding 10µg/mL propidium iodide. Samples were analysed using an Epics ELITE flow cytometer, (Beckman-Coulter, Mississauga, ON) and 10,000 events were counted for each sample.

Jurkat or H9 cells were cultured in the presence of AZT, NFV, RTV, SQV or diluent at indicated concentrations for periods of time ranging from 1 hour to 5 days prior to induction of apoptosis. Apoptosis was induced with ActD (10µg/mL), CPT (10µM), anti-Fas antibody (CH11; 0.5µg/mL) or recombinant synthetic Vpr peptide. For Vpr stimulation, cells were incubated in isotonic buffer (2.4% glucose, 13mM hydroxyelthy piperazineethanesulfonic acid (HEPES), 68mM NaCl, 1.3mM KCl, 4mM Na_2HPO_4 and 0.7mM KH_2PO_4, pH 7.2) with 2.5µM synthetic Vpr peptide (residues 52-96) or bovine serum albumin (BSA) for 30 minutes or overnight followed by culture in complete media. Following treatment, apoptosis was measured by annexin-V and propidium iodide staining, whereas mitochondrial membrane permeability changes were determined using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; 40nM). [9, 407] Cells (10^6) were washed in PBS and resuspended in 400µL binding buffer prior to adding 5 µL annexin-V-FITC and 40nM $DiOC_6(3)$. Samples were incubated in the dark for 20 minutes at 37°C prior to adding 10µg/mL propidium iodide. Samples were analysed using an Epics ELITE flow cytometer, and 10,000 events were acquired for each sample.

Where indicated, protein synthesis was inhibited by a 1 hour pre-treatment with cycloheximide (CHX; 10µg/mL). [408] Protein synthesis was evaluated by

biosynthetic labelling with ^{35}S-labelled methionine. ^{35}S incorporation was measured using the 1450 microbeta liquid scintillation counter. (Wallac, Turku, Finland) Cell viability was assessed by the trypan blue exclusion assay.

Measurement of p24 Antigen and Viral Load

Measurement of p24 antigen concentration in culture supernatants was performed using a commercial p24 ELISA assay according to the manufacturer's instructions. Control standards were included in parallel to the test samples to verify the sensitivity of the assay. The levels of HIV-1 RNA in tissue culture supernatants were quantified using a nucleic acid sequence-based amplification (NASBA) assay. This assay has a quantification limit of 20 copies/mL of supernatant.

Ribonuclease Protection Assays

Total RNA was isolated from cells treated with methanol, AZT or NFV for 3 days using an RNeasy mini kit as per manufacturer's instructions. For the ribonucleotide protection assay (RPA), 40µg RNA was probed using 2 custom templates containing probes for caspase-3, -8, -9, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TNFR1, Fas-associated phosphatase (FAP)-1, Bid, Bfl-1, XIAP, NAIP, cIAP-1, cIAP-2, Bcl-2, Bcl-x_L and Bcl-x_S. Following gel resolution, band intensities were quantitated using the ImageQuant software (Molecular Dynamics, Amersham, Baie d'Urfe, QC) and normalized to band intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunoblotting

Following indicated treatments, protein expression of pro-caspases 3 and 8 were assessed by washing cells in cold PBS and lysing them in buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM ethylenediamine tetra-acetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride (PMSF), 1µg/mL aprotinin, leupeptin and pepstatin, 1mM Na_3VO_4, 1mM NaF) prior to determining the protein concentration. Subsequently, 40µg of protein was subjected to 4-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked overnight with 5% non-fat dry milk powder in Tris-buffered saline Tween 20 (TBST; 50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween 20) and immunoblotted for 1-hour at room temperature using antibodies specific for caspase 3 (1:1000) or caspase 8 (1:15). [409] Membranes were washed five times with TBST for 5 minutes and exposed to various dilutions of anti-IgG-horseradish peroxidase conjugate for 1 hour at room temperature. Proteins were visualised by enhanced chemiluminescence (ECL). The loading and transfer of equal amounts of protein was confirmed by blotting with an anti-proliferating cell nuclear antigen (PCNA) antibody (PC-10, 1:1000).

Cytochrome *c* Release Assay

Jurkat cells were treated overnight with methanol, AZT or NFV prior to being stimulated to undergo apoptosis with CPT. Following induction of apoptosis, cells were washed twice with ice-cold PBS, (pH 7.4) and pelleted by centrifugation at 200g for 5 minutes. The pellet was resuspended in caspase

buffer (100mM HEPES pH 7.5, 250mM sucrose (w/v), 0.5mM EDTA and 10mM dithiothreitol (DTT) and protease inhibitors) before being lysed in a dounce homogenizer. Cytosolic and mitochondrial fractions were separated by centrifugation at 14,000g for 15 minutes, [410] and blotted for cytochrome *c* (1:500). To ensure the cytosolic fraction contained only cytochrome *c* that was released from mitochondria, both cytosolic and mitochondrial fractions were blotted for the mitochondrial protein hsp 70. [411]

Determination of Caspase 1, 3, 6, 7, and 8 Activity

The effect of NFV on the activity of recombinant active caspases 1, 3, 6, and 8 was assayed using 7-amino-4-trifluoromethyl coumaride (AFC)-conjugated substrates, and the activity of recombinant active caspase 7 was assessed using a (7-methoxycoumarin-4-yl) acetyl/2,4-dinitro phenyl (MCA/DNP)-conjugated substrate. NFV, methanol or caspase inhibitor (100μm zVAD-FMK for caspases 1, 6 and 7, 100μm DEVD-FMK for caspase 3 and 100μm IETD-FMK for caspase 8) were added to caspase buffer containing 50U of recombinant enzyme and fluorogenic caspase-specific substrate (WEHD-AFC for caspase 1, DEVD-AFC for caspase 3, VEID-AFC for caspase 6, IETD-AFC for caspase 8, and MCA-VDQVDGW[K-DNP] for caspase 7). Caspase activity was determined after excitation at 400nm or 325nm by measuring the fluorescence emission at 505nm or 392nm for AFC or DNP conjugates respectively, using a Cytofluor 2300 Series (Millipore, Mississauga, ON). Results are shown as relative fluorescence relative to controls. As a control for NFV activity, the ability of NFV to inhibit cleavage of a fluorogenic gag-pol substrate by HIV PR was also assayed. Recombinant HIV-1

PR (50U) was added to PR buffer (50mM NaOAc, pH 4.9, 200mM NaCl, 5mM DTT and 10% glycerol) containing the fluorogenic HIV PR gag-pol substrate and either NFV or methanol.

Isolation of Jurkat T Cell Mitochondria

Mitochondria from Jurkat T cells were isolated as previously described. [412, 413] Briefly, Jurkat T cells were resuspended in isolation buffer (0.32M sucrose, 1.0mM EDTA, 10mM Tris-HCl, pH 7.4) and homogenised by hand in a dounce homogeniser using 4 up-and-down strokes with the A pestle (0.12mm clearance) and 8 strokes with the B pestle (0.05mm clearance). Homogenates were centrifuged at 2,000g for 3 minutes, and supernatants were transferred into a clean centrifuge tube. The pellet was resuspended in isolation buffer and spun at 2,000g for 3 minutes. Supernatants were combined and spun at 31,600g for 10 minutes. The pellet was resuspended in 15% Percoll, and layered on a discontinuous density gradient (bottom 40%, middle 23%). Tubes were centrifuged at 44,600g for 5 minutes, and the material at the interface of the lower two Percoll layers was collected and diluted 1:4 with isolation buffer and centrifuged at 24,200g for 10 minutes. The pellet was then resuspended in incubation buffer [100mM KCl, 75mM mannitol, 25mM sucrose, 5mM phosphate (adjusted to pH 7.4 with Tris), 0.05mM EDTA, and 10mM Tris-HCl, pH 7.4]. All steps were performed at 4°C or on ice.

Mice and Animal Care

Mice used in the stroke model were male C57Bl/6 (16-18g) from Charles River. Animals were maintained in a temperature- and light-controlled environment with a 12:12 hour light/dark cycle and were given access to standard laboratory chow and water *ad libitum*. The water supplied to the C57Bl/6 mice contained 0.1% Tylenol. The protocols were approved by the University of Ottawa Animal Care and Veterinary Services ethics committee.

Administration of PIs and Sublethal Global Ischemia in Mice

The ability of PIs to inhibit apoptosis *in vivo* was assessed in a mouse model of sublethal forebrain ischæmia. [414, 415] Four experimental conditions were assessed: two groups of mice were pre-treated with PIs, one for 24 hours, and another one hour prior to forebrain ischæmia; a third group received PIs 6 hours following forebrain ischæmia, and the last group was treated with vehicle control. Mice were given a mixture of 160 mg/Kg NFV and 13 mg/Kg RTV in 1% ethanol/water every 8 hours or an equivalent volume of 1% ethanol/water by gastric lavage using an 18-gage stainless steel rounded-end biomedical needle. Ischæmic animals were subjected to a temporary (10 minutes) bilateral occlusion of the common carotid arteries. Briefly, animals were anesthetised with halothane, and a ventral midline neck incision was made, exposing the trachea and the bilateral common carotid arteries. Blood flow was blocked by wrapping silk suture (5.0) around the carotids. Following the occlusion, carotid blood flow was allowed to resume, and the incision was sutured. Neurological damage was monitored by histological analysis of animals killed 48-hours following forebrain

ischæmia. Two mice in the 24 hour pre-treatment group were monitored for 30 days prior to being sacrificed and assessed for neurological damage.

Histopathological Analysis

Neuronal damage was assessed 48-hours after ischæmia by histological examination of brain sections at the level of the hippocampus. Briefly, animals were anesthetised with halothane and euthanised by decapitation. Brains were rapidly removed, frozen in isopentane, and stored at -80°C. Sections (10µm) were cut on a microtome cryostat (HM 500 OM; Carl Zeiss Canada Ltd., North York, ON) at -20°C. Fresh frozen sections were fixed in 1% glutaradehyde for 20 minutes at room temperature and washed with PBS for 30 minutes at room temberature. Samples were permeabilised with methanol/acetone for 2 minutes on ice, washed with PBS and treated with 20µg/mL proteinase K for 15 minutes at room temperature. Hoechst 33258, a membrane-permeable, fluorescent DNA stain, was used to visualise nuclei. Slides were incubated with 1µg/mL for 7 minutes at 37°C in the dark and washed with PBS. Apoptosis was assessed by TUNEL. [416] Slides were incubated with TUNEL reaction mixture containing label solution (nucleotide mixture in reaction buffer) and enzyme solution (terminal deoxynucleotidyl transferase) for 1 hour at 37°C in the dark and washed with PBS prior to being visualised by fluorescent microscopy.

Drug Level Determination

Mice were anesthetised with halothane and sacrificed by decapitation. Brains were rapidly removed and thoroughly rinsed with PBS. Excess liquid was

removed using filter paper prior to weighing the brain. The tissue was transferred to a 15mL screw-capped culture tube, 5.0mL of 0.1M HCl was added, and the tissue was homogenised using a polytron (Kinematica, Switzerland) at 20,000t/minute for 1 minute. Plasma concentrations of NFV and RTV were measured simultaneously using liquid chromatography with tandem mass-spectrometry (LC/MS/MS) at the Ottawa Hospital, General Campus. The lower limit of quantitation for NFV and RTV was 2.5 ng/mL.

Statistics

In order to compare the amount of apoptosis seen in drug treated *versus* untreated PBLs following *in vitro* treatment, the following approach was used. Each patient sample was treated with differing doses of either RTIs or PIs as indicated, or with no drug. The absolute amount of apoptosis seen in the no drug aliquot was used as a control value, and the effects of drug treatments compared to that value. This approach of normalising apoptosis to percent of maximal apoptosis has been previously used to minimize the effects of wide inter-subject variation. [278, 417] To compare apoptosis between treatments, data was first analysed by ANOVA to determine if a significant difference was present followed by a post-hoc TUKEYS HSD test with correction for multiple comparisons to identify the treatment responsible for the difference. Only the results of the TUKEYS test are reported in the text. For analysis of apoptosis at different times during the clinical trial, paired Student's t tests were performed. For comparisons of apoptosis between Jurkat or H9 cell treatment groups, Student's t tests were performed. Data presented represent means of experiments with standard

deviations. The Wilcoxon Log Rank test was used to compare differences in apoptosis levels in the different patient samples for the PEP study.

3. RESULTS

3.1 EFFECT OF PIs ON APOPTOSIS

3.1.1 PIs Inhibit Apoptosis in PBLs from HIV-Infected Patients

In order to establish whether anti-HIV drugs influence apoptosis, PBLs from HIV-infected but treatment naïve patients were isolated and incubated in the presence or absence of escalating doses of either the RTIs AZT and ddG (non-PI antiviral controls), or with the PIs RTV and SQV. PBLs from seven patients were analysed. The mean CD4 T cell count was 354 cells/mm^3 (range 115-655), and the mean viral load was 26,220 copies/mL (range 680-104,523).

Apoptosis was determined by flow cytometry following annexin-V and propidium iodide staining. Annexin-V, is a 35–36Kd Ca^{2+}-dependent high affinity PS-binding protein. [418] In viable cells, PS is located on the cytoplasmic membrane surface; however, during apoptosis this molecule translocates from the inner to the outer plasma membrane leaflet, and consequently can be detected by annexin-V conjugates. Annexin-V conjugates can be used in combination with other dyes including nucleic acid stains such as propidium iodide to accurately assess mixed populations of apoptotic and non-apoptotic cells. [419]

Consistent with prior reports, significant amounts of spontaneous apoptosis [194, 200] were observed in both the CD4 and CD8 T cells subsets (mean 20.2% range 4-49%). Neither AZT nor ddG, at concentrations ranging

from 0.1μM to 10μM, resulted in significant alterations in spontaneous apoptosis. (Figure 4) In contrast, RTV and SQV at doses ranging from 0.1μM to 10μM reduced the observed spontaneous apoptosis in comparison with untreated controls. In CD4 T cells treated with 10μM RTV or SQV, spontaneous apoptosis was reduced by 25% (p=0.02) and 31% (p=0.002) respectively (Figure 4). Similarly, RTV and SQV reduced spontaneous CD8 T cell apoptosis by 32% (p=0.006), and 41% (p=0.001) respectively; however no reductions in apoptosis were observed with AZT or ddG. Lesser concentrations of PIs resulted in smaller degrees of apoptosis inhibition, thereby demonstrating a dose-dependent phenomenon.

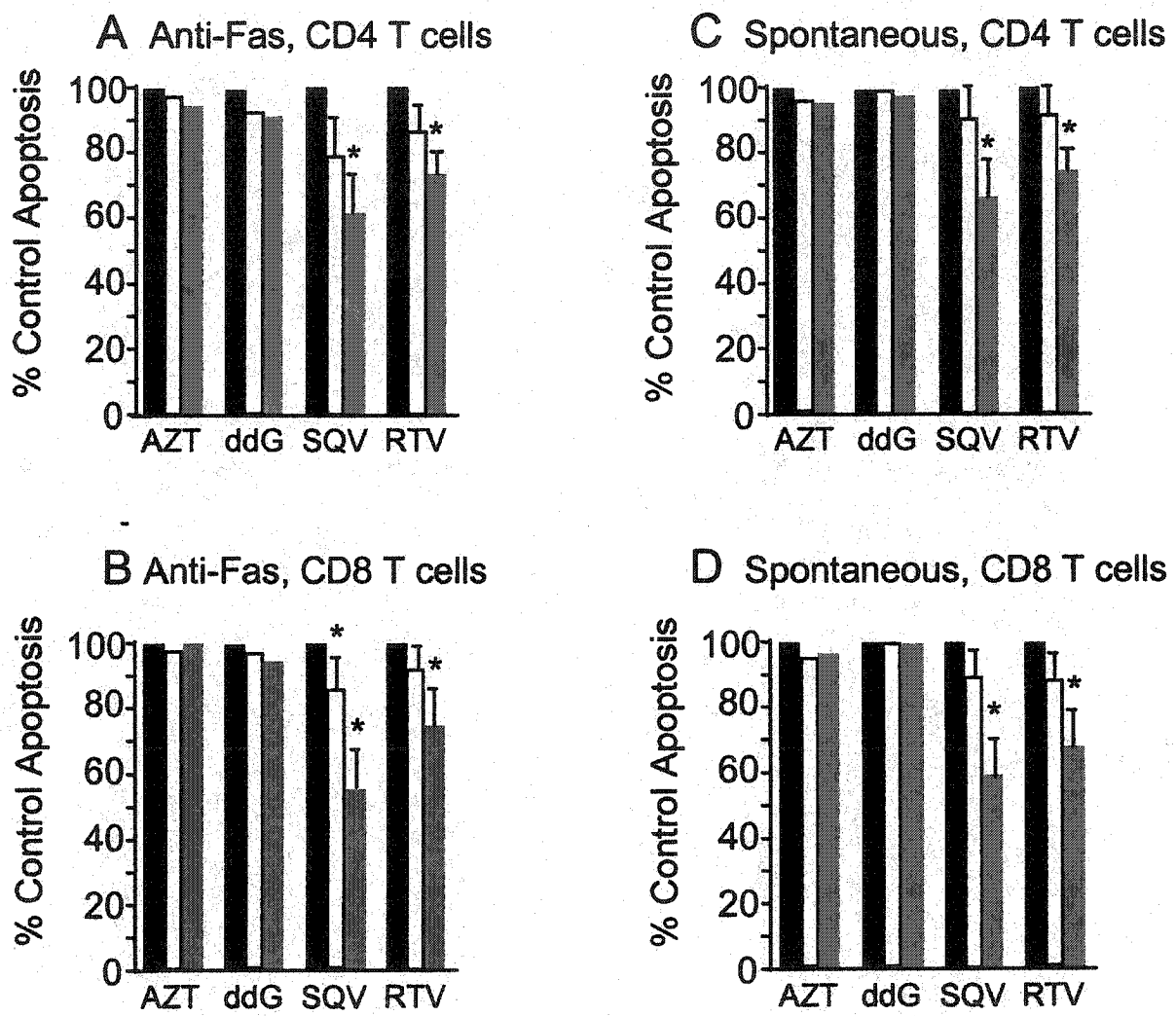
A similar analysis was performed in the anti-Fas treated cells (Figure 4). CD4 T cell apoptosis was reduced in the 10μM RTV-treated cells by 26% (p=0.002), and by 35% (p=0.001) in the 10μM SQV-treated cells but was not altered by AZT, or ddG. In CD8 T cells 10μM RTV reduced anti-Fas-induced apoptosis by 23% (p=0.006) and 10μM SQV reduced apoptosis by 44% (p=0.003). Similar to the observations in the spontaneous apoptosis analysis, anti-Fas stimulated cells exhibited a stepwise – or dose responsive – inhibition of apoptosis with PI therapy.

3.1.2 Effect of PIs on Apoptosis is Not Mediated by Differences in Viral Suppression

To directly assess the possibility that the differences in the observed apoptosis were due to disparity in the magnitude of viral suppression, we analysed viral replication in samples from one patient in the above analysis.

Figure 4. Inhibition of CD4 and CD8 T cell apoptosis in PBLs from HIV-positive patients by PI but not RTI therapy.

PBLs from seven HIV-infected but untreated patients were isolated and incubated with indicated concentrations (black bars 0.1μM, white bars 1.0μM, grey bars 10μM) of AZT, ddG, RTV or SQV. Thereafter cells were stimulated with either an agonistic anti-Fas antibody (0.5μg/mL; A, B) or with an isotype matched antibody (control C, D) and analysed for annexin-V and propidium iodide staining by flow cytometry for CD4 (A, C) and CD8 (B, D) T cell apoptosis. (* $p < 0.02$) Percent of control apoptosis was calculated by (% annexin-V positive cells following drug treatment divided by % annexin-V positive cells without drug treatment) x 100.


A Anti-Fas, CD4 T cells
% Control Apoptosis
100
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AZT
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SQV
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C Spontaneous, CD4 T cells
% Control Apoptosis
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RTV
*
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B Anti-Fas, CD8 T cells
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D Spontaneous, CD8 T cells
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AZT
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RTV
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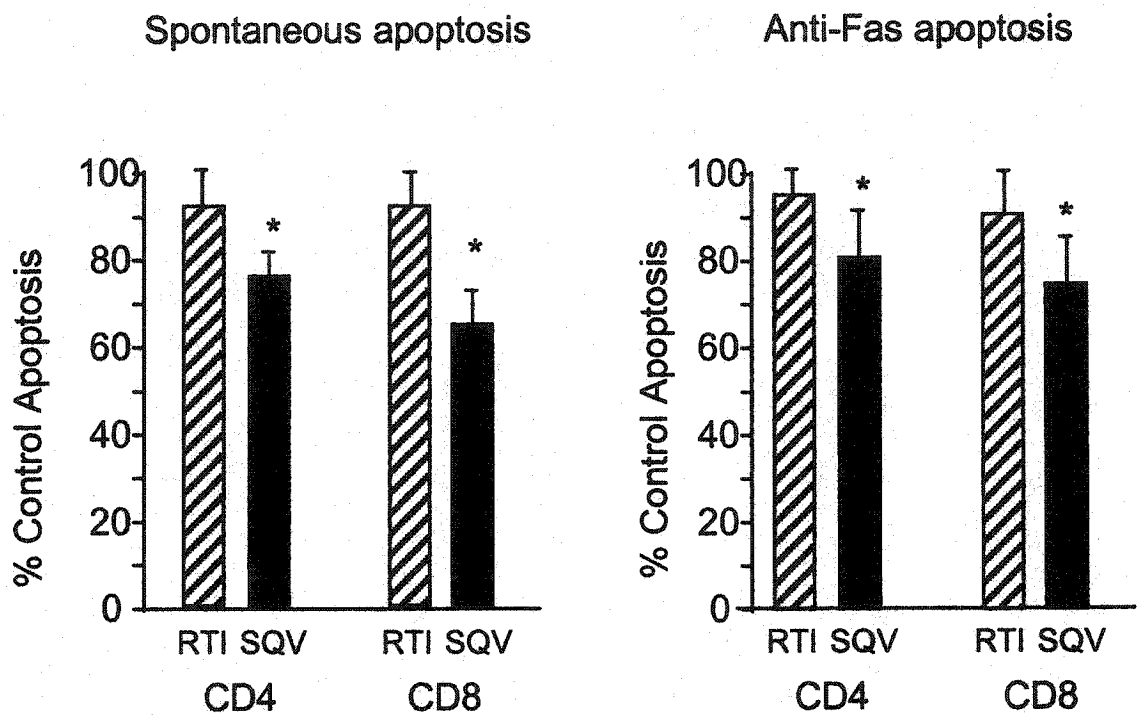
Supernatants from cell cultures treated with RTV, SQV, AZT, or ddG were evaluated by Dr. Kelley Chambers; however the p24 antigen concentration was below the assay limit of detection of 12.5pg/mL. Viral RNA in culture supernatants from the same patient were quantified by Dr. Sharon Cassol, and a mean viral load in RTI-treated cells of 1173 copies/mL (standard deviation 310), and in PI-treated cells of 1830 copies/mL (standard deviation 1280) ($p=0.5$) was found even though apoptosis was inhibited only in the PI-treated, but not the RTI-treated cells.

As we did not detect differences in viral replication, it remained possible that small differences were still present, albeit at levels that are below the sensitivity range of the assays. We therefore performed additional experiments with PBLs from ten HIV-infected but untreated patients (mean CD4 T cell count = 268, standard deviation 77; mean viral load 107,822 copies/mL, standard deviation 58,679) using a combination of RTIs (to maximize antiretroviral effects) in comparison to monotherapy with a PI. Combination therapy using 10μM ddI, 10μM ddC, and 10μM AZT did not abrogate apoptosis, either in anti-Fas stimulated cells, or in isotype treated cells (Figure 5). In contrast, and similar to what was observed above, 10μM SQV inhibited spontaneous CD4 T cell apoptosis ($p=0.02$), CD8 T cell spontaneous apoptosis ($p=0.002$), CD4 T cell Fas-mediated apoptosis ($p=0.03$) and CD8 T cell Fas-mediated apoptosis ($p=0.002$).

Figure 5. SQV but not combination RTI treatment blocks apoptosis in T cells from HIV positive patients.

PBLs from ten HIV-infected but untreated patients were cultured with either 10μM SQV (black bars) or 10μM AZT, 10μM ddC and 10μM ddI (RTI group; hatched bars), and analysed for spontaneous and anti-Fas (CH11) induced (0.5μg/mL) apoptosis in either CD4 or CD8 T cells as indicated. (* $p < 0.02$) Mean viral loads were not significantly different between treatment groups. (n=10). Apoptosis was assessed as in Figure 4, and percent of control apoptosis was calculated as in Figure 4.


Spontaneous apoptosis
Anti-Fas apoptosis
% Control Apoptosis
100
80
60
40
20
0
*
RTI SQV
RTI SQV
CD4
CD8

3.1.3 PIs Inhibit Apoptosis of Uninfected Cells

The effect of PIs on apoptosis in uninfected cells was assessed using a T cell line. Jurkat cells are pseudodiploid human T cells established from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia by Schneider *et al.* [420] Cells were cultured in the presence of escalating doses of NFV (0.1-10μM). After 3 days, cells were stimulated to undergo apoptosis through Fas receptor ligation. As seen in cell cultures from HIV-infected donors, inhibition of apoptosis occured in NFV-treated but not in AZT- or methanol-treated cells. (data not shown)

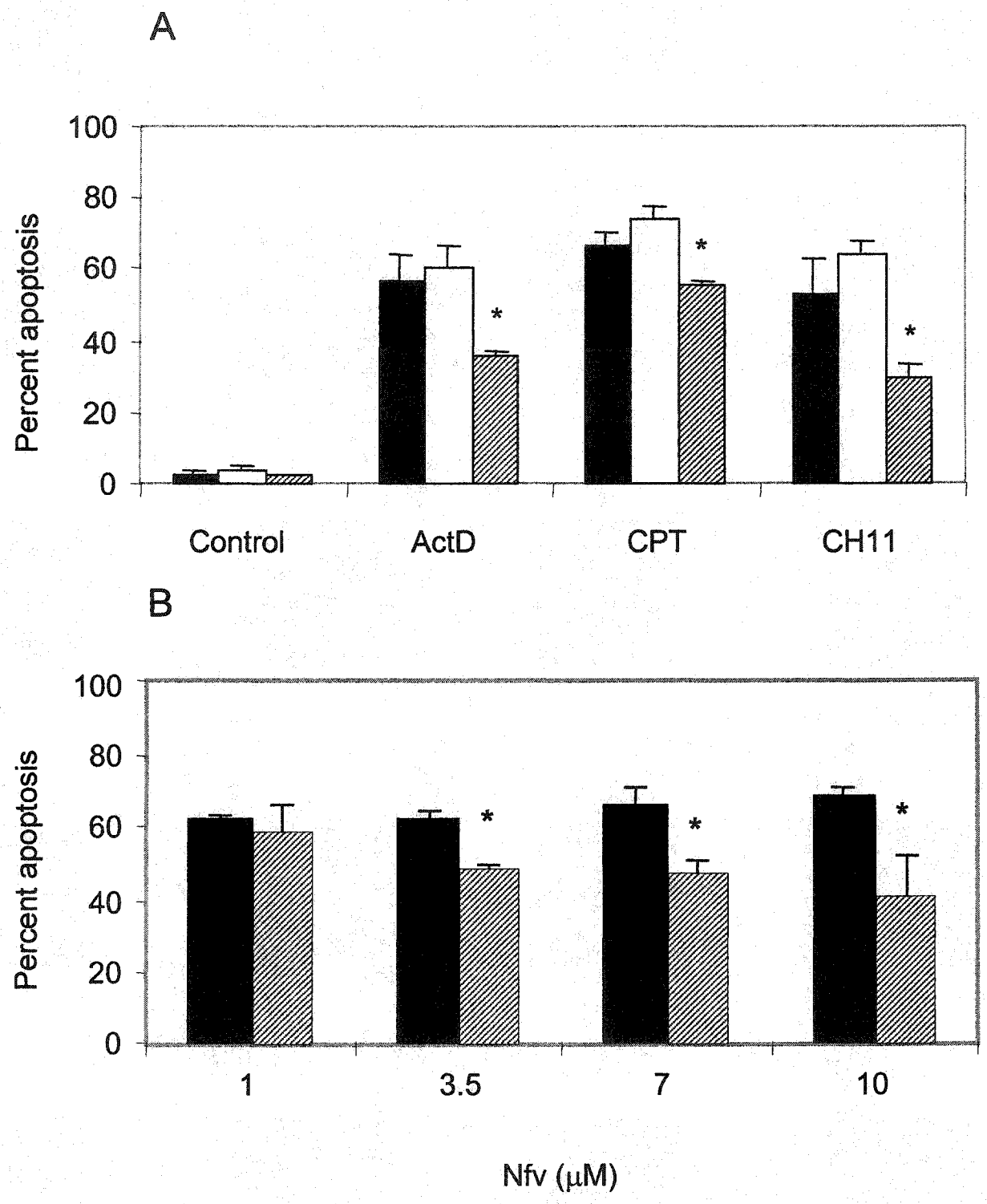
3.1.4 Characterisation of PI-Mediated Inhibition of Apoptosis

Next we analysed the effect of NFV on apoptosis induced by other stimuli. Jurkat T cells were pretreated with methanol, AZT or NFV (7μM) for 3 days and stimulated to undergo apoptosis with ActD, CPT or an agonistic anti-Fas antibody (CH11).

Treatment of cells with the cancer-chemotherapeutic agent ActD [421] leads to single-strand breaks in DNA, [422] and induces apoptosis, [423] whereas CPT, another cancer-chematherapeutic agent, [424] induces apoptosis via a direct effect on mitochondria, resulting in PTPC opening, $\Delta\psi_m$, and subsequent release of cytochrome *c* and AIF resulting in post mitochondrial activation of caspases 9, 3 and 7. [90-92] NFV inhibited apoptosis induced by all three compounds (Figure 6A) at a minimal dose of 3.5μM. (Figure 6B) Cultures treated with 7μM and 10μM also showed significant inhibition of apoptosis.

Figure 6. Characterisation of NFV-mediated inhibition of apoptosis in uninfected cells.

(A) Jurkat T cells were cultured for 3 days with methanol (black), 10μM AZT (white) or 7μM NFV (hatched) prior to being stimulated to undergo ActD- (10μg/mL), CPT- (10μM), and anti-Fas-mediated (0.5μg/mL) apoptosis. Apoptosis was assayed following annexin-V and propidium iodide staining by flow cytometry. NFV inhibited ActD- ($p=0.008$), CPT- ($p=0.005$) and anti-Fas-mediated (CH11; $p=0.001$) apoptosis. (n=3) (B) Dose-response curve. Jurkat T cells were treated with MeOH (black) or increasing doses (1-10μM) of NFV (hatched) overnight and then stimulated with 10μM CPT. Apoptosis was assessed as above. While 1μM NFV did not inhibit apoptosis, 3.5μM ($p=0.006$), 7μM ($p=0.02$) and 10μM ($p=0.03$) all inhibited apoptosis. (n=3)


A
100
80
60
40
20
0
Percent apoptosis
*
*
*
Control
ActD
CPT
CH11
B
100
80
60
40
20
0
Percent apoptosis
*
*
*
1
3.5
7
10
Nfv (μM)

NFV concentrations of 7μM are similar to trough levels achieved in patients receiving treatment. [359, 425] Consequently we chose to use this dose for the remainder of our experiments.

3.1.5 PIs Inhibit Apoptosis in a Time-Dependent Manner

The kinetics of inhibition of apoptosis by PIs were investigated. Jurkat T cells were cultured in the presence of methanol, AZT or NFV for 5 days. Aliquots of cells were removed daily and stimulated to undergo apoptosis with ActD, CPT, or an agonistic anti-Fas antibody (CH11). Inhibition of CPT-induced apoptosis by NFV was observed as early as day 1, and lasted throughout the 5 day treatment period, Neither methanol nor AZT had any effect (Figure 7A). Similar results were observed with ActD and CH11. (data not shown)

Since inhibition of apoptosis was observed as early as day 1 and throughout the 5-day treatment, an additional experiment was designed in order to determine the exact pre-incubation time required in order to achieve inhibition of apoptosis. Methanol, AZT or NFV were added to cells either at time of CPT stimulation (0 hours) or 1-12 hours prior to stimulation. Strikingly, resistance to apoptosis was seen within 1 hour of treatment with NFV. (Figure 7B)

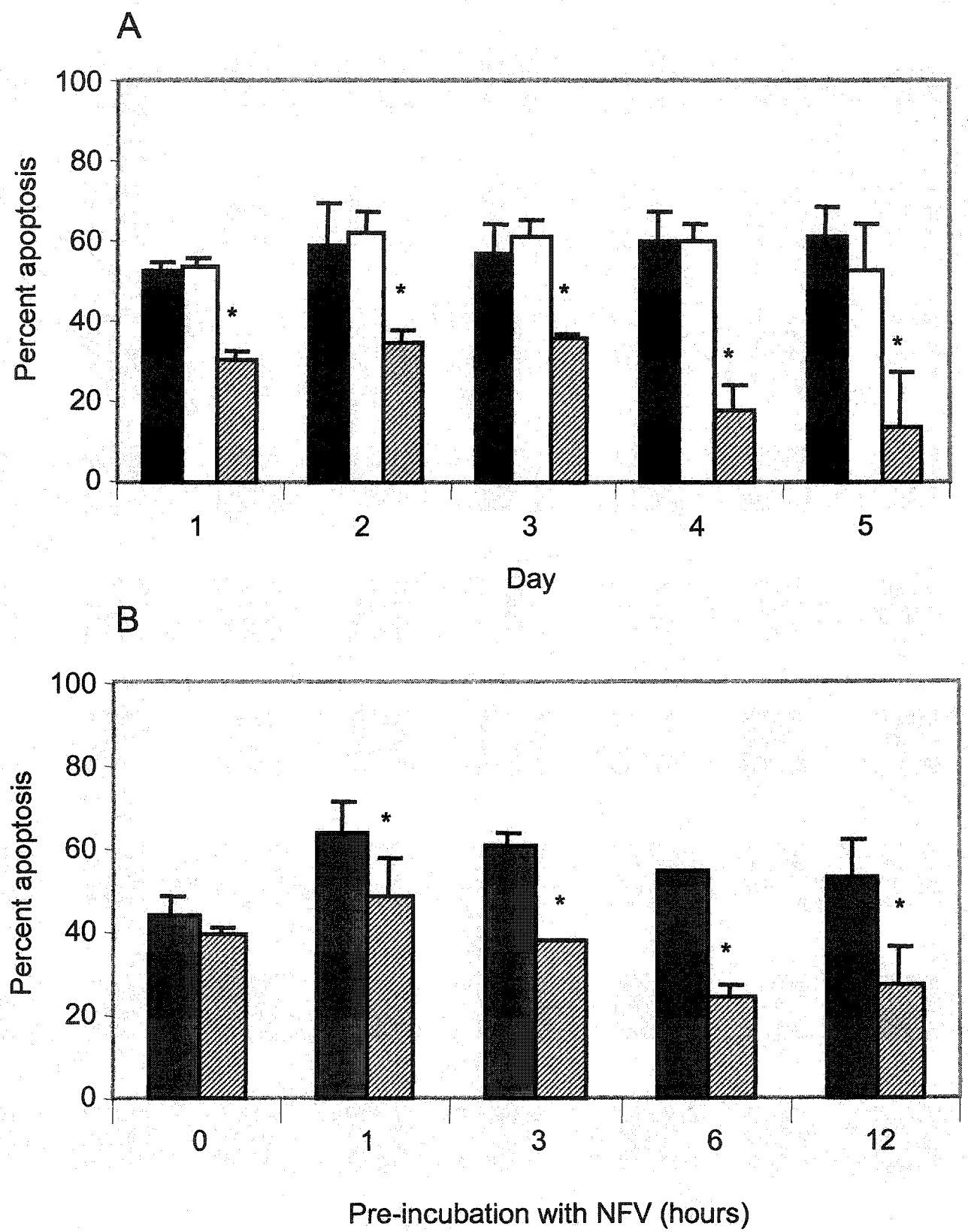
3.2 MECHANISM OF INHIBITION OF APOPTOSIS BY PIs

3.2.1 NFV-Mediated Inhibition of Apoptosis is Independent of Protein and RNA Synthesis

Based on our previous findings, the effect of PIs on transcription and translation was next assessed. First, we determined a dose of CHX (10μg/mL)

Figure 7. Kinetics of inhibition of apoptosis by NFV in Jurkat cells.

(A) Jurkat cells were treated with MeOH (black), 10μM AZT (white) or 7μM NFV (hatched) over a period of 5 days. Apoptosis was induced with 10μM CPT every day and assessed as in Figure 5. Apoptosis is significantly inhibited by NFV as early as day 1 ($p=0.01$). [day 2 ($p=0.04$), day 3 ($p=0.005$), day 4 ($p=0.001$), day 5 ($p=0.03$; $n=3$)]. Similar results were also obtained following stimulation with ActD (10μg/ml), anti-Fas antobody (0.5μg/ml) (data not shown) (B) Jurkat cells were treated with MeOH (black) or 7μM NFV (hatched) for 0, 1, 3, 6, or 12-hours, and stimulated with 10μM CPT. Apoptosis was assessed as in Figure 5. NFV inhibits apoptosis following 1-hour of treatment ($p=0.01$), 3-hours ($p=0.03$), 6-hours ($p=0.02$), 12-hours ($p=0.004$; $n=3$).


A
100
80
60
40
20
0
Percent apoptosis
*
1
2
3
4
5
Day
B
100
80
60
40
20
0
Percent apoptosis
*
0
1
3
6
12
Pre-incubation with NFV (hours)

that inhibited protein synthesis in Jurkat T cells without affecting viability (^{35}S incorporation in NFV-treated cells without CHX 125,554 CPM, ^{35}S incorporation in NFV-treated cells with CHX 64,777 CPM; 51% of control). Cells co-incubated with CHX and NFV showed the same degree of apoptosis inhibition as cells incubated with NFV alone (Figure 8A), demonstrating that NFV-mediated inhibition of apoptosis is independent of protein synthesis.

Next, we assessed two caspase family members, pro-caspase 8 (full length 55KDa) and pro-caspase 3 (full length 32KDa), both of which are necessary for apoptosis following Fas ligation. As shown in Figure 8B, pro-caspase 8 and pro-caspase 3 levels were not altered following treatment with NFV compared to control cells.

Finally, RPAs failed to show differences at the mRNA level for a cross-section of antiapoptotic proteins and pro-apoptotic proteins in cells pre-treated with methanol, AZT, or NFV for 3 days (Figure 8C). Data from three independent experiments (groups 1, 2, 3) fail to show differences between treatments in the amount of mRNA present for caspase-3, -8, -9, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TNFR1, FAP-1, Bid, Bfl-1, XIAP, NAIP, cIAP-1, cIAP -2, Bcl-2, Bcl-x_L, and Bcl-x_S.

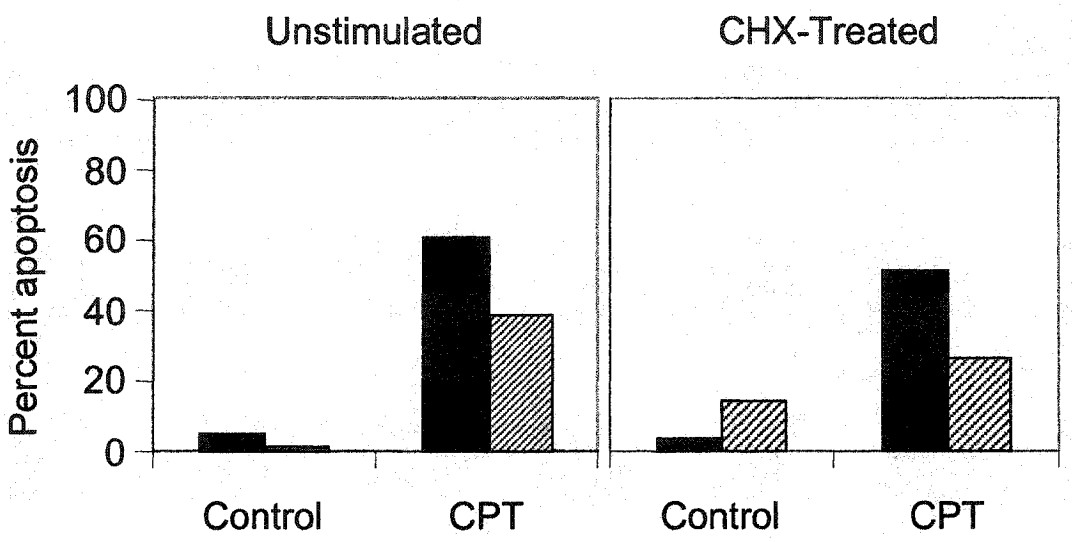
3.2.2 NFV Does Not Inhibit Caspase Activity

Since PIs inhibit the proteolytic activity of the viral protease, certain groups have proposed that PIs might have a similar effect on cellular proteases. [426, 427] As caspases are key regulators of the apoptotic cascade, we chose to assess whether NFV could inhibit caspase activity. Recombinant active caspases

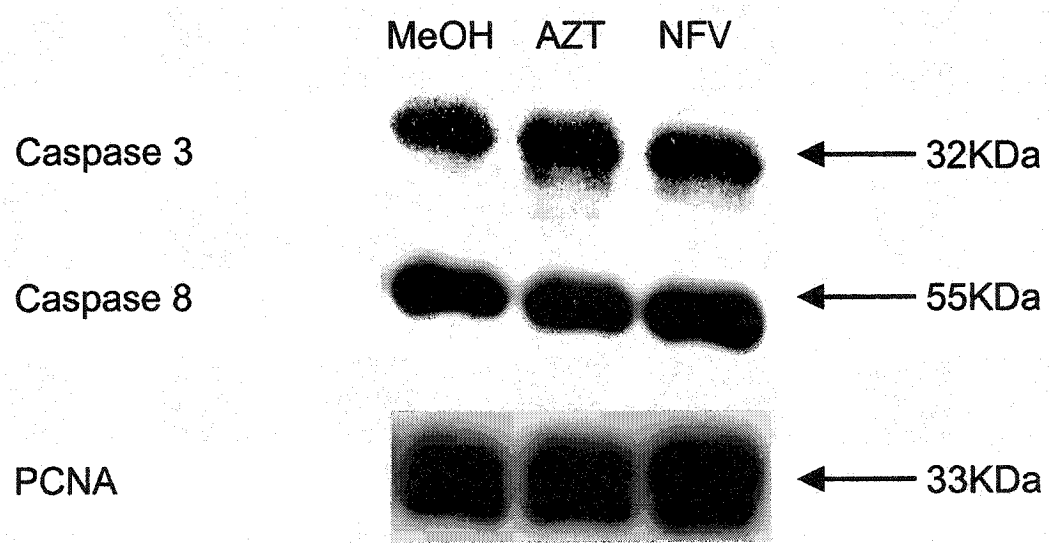
Figure 8. NFV does not alter protein or mRNA expression or caspase activity.

(A) Jurkat control cells (left panel) or cells were pre-treated for 1 hour with 10μg/ml CHX (right panel) prior to overnight culture with MeOH (black) or 7μM NFV (hatched). Apoptosis was induced by stimulation with 10μM CPT, and assessed as in Figure 5. Data representative of three independent experiments. (B) Immunoblots of pro-caspase 3 and 8 from whole cell lysates of Jurkat cells treated overnight with MeOH, 10μM AZT or 7μM NFV. (40μg of protein per lane) The PCNA immunoblot confirms equal loading. (C) Ribonuclease protection assay of Jurkat mRNA (40μg per lane) from three independent experiments (groups 1-3). Cells were treated for 3 days with MeOH, 10μM AZT or 7μM NFV. (P: probe; C: MeOH; A: AZT, N: NFV)

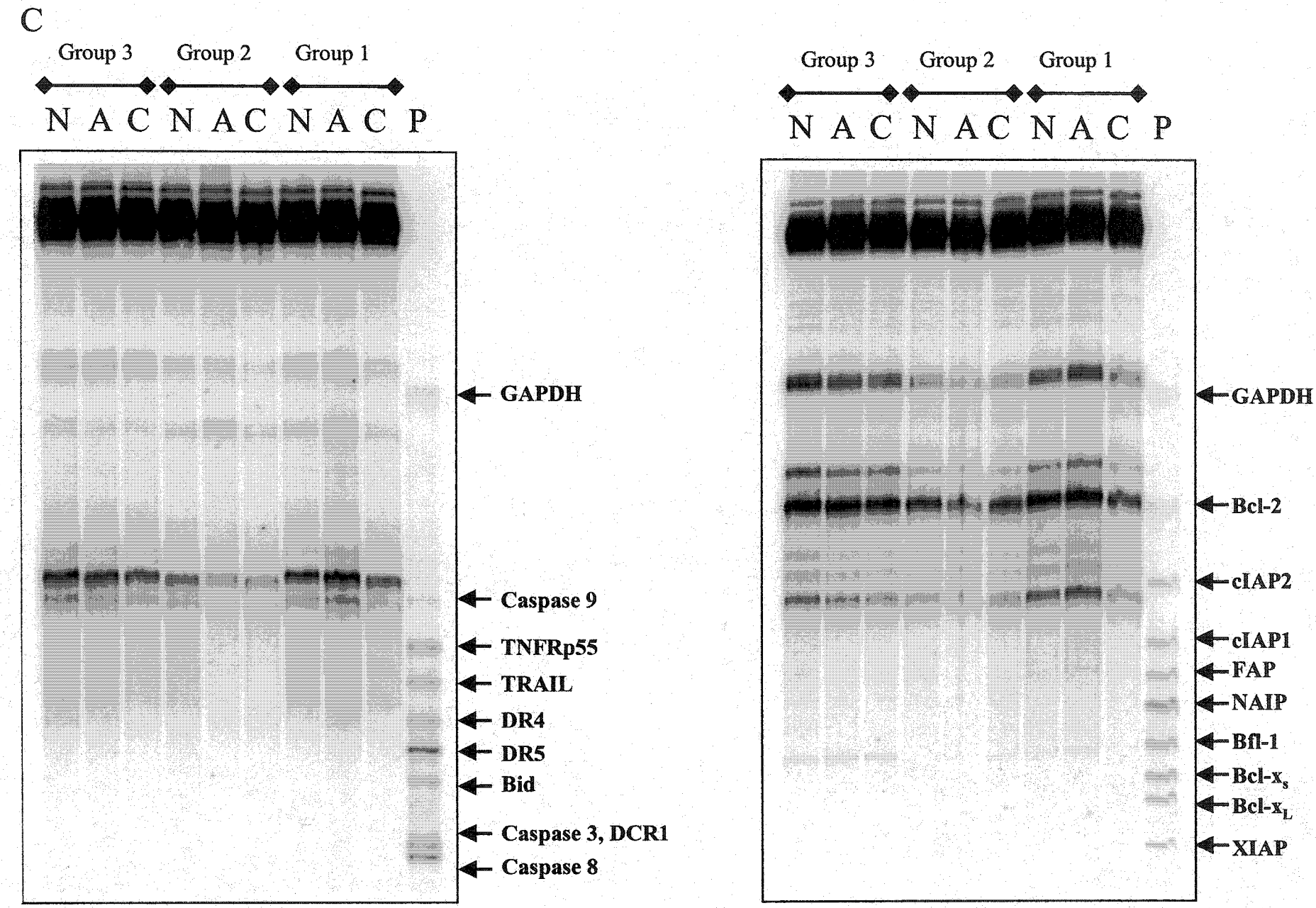
A


Unstimulated
CHX-Treated
Percent apoptosis
100
80
60
40
20
0
Control
CPT
Control
CPT

B


MeOH
AZT
NFV
Caspase 3
32KDa
Caspase 8
55KDa
PCNA
33KDa

C


Group 3
Group 2
Group 1
N A C N A C N A C P
GAPDH
Caspase 9
TNFRp55
TRAIL
DR4
DR5
Bid
Caspase 3, DCR1
Caspase 8
Group 3
Group 2
Group 1
N A C N A C N A C P
GAPDH
Bcl-2
cIAP2
cIAP1
FAP
NAIP
Bfl-1
Bcl-x_s
Bcl-x_L
XIAP

1, 3, 6, 7, or 8 were incubated in the presence or absence of NFV and fluorogenic tetrapeptide substrates specific for each respective caspase. Following cleavage of the fluorogenic substrate by active caspases, fluorescence is released, allowing assessment of caspase activity as a function of time. As shown in Figure 9, NFV does not inhibit caspase 3 activity (Figure 9A); similarly, NFV has no effect on activity of caspases 1, 6, 7, or 8 (data not shown). To confirm the activity of NFV, we demonstrate that NFV inhibits HIV protease cleavage of a fluorogenic gag/pol consensus site (Figure 9B).

3.2.3 NFV Blocks Loss of Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential loss occurs in many forms of apoptosis, and serves to link activation of early initiator caspases with activation of downstream effector proteases and nucleases. We analysed whether NFV-induced inhibition of apoptosis occurred before or after mitochondrial membrane permeability changes.

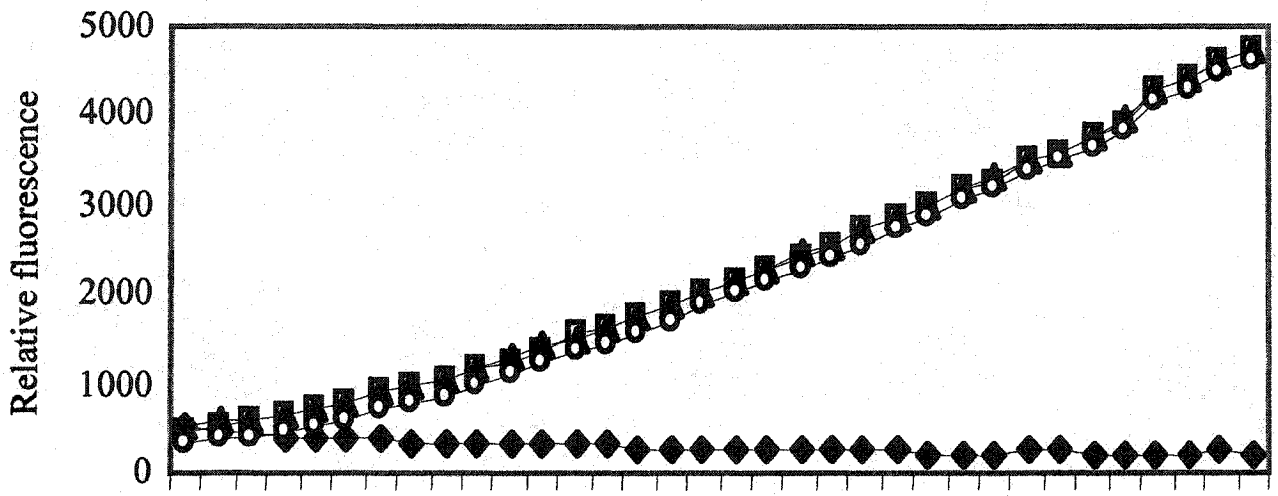
$DiOC_6(3)$ is a cell permanant green-fluorescent lipophillic dye that stains mitochondria with intact mitochondrial transmembrane potential when used in low concentrations. Mitochondria that have lost their transmembrane potential lose the ability to retain the dye, and are stained weakly with $DiOC_6(3)$. We refer to this as $DiOC_6(3)$ low. [428]

Jurkat T cells were incubated in the presence of methanol, AZT, or NFV, and stimulated to undergo apoptosis with ActD, CPT, or an agonistic anti-Fas antibody (CH11). In NFV-treated samples, the apoptotic fraction (annexin-V positive, propidium iodide negative; Figure 10Aiii) had a decreased proportion of

Figure 9. NFV does not modulate recombinant caspase activity.

(A) Active human recombinant caspase 3 was incubated with its fluorogenic substrate (DEVD-AFC) alone (white squares) or in the presence of 100μM DEVD-FMK (black diamonds), 7μM NFV (black triangles) or MeOH (white circles), and the kinetics assessed over 30 min. (top panel) Similar results were also obtained with active human recombinant caspases 1, 6, 7, and 8. (Data not shown) Data representative of at least three independent experiments for each caspase tested. (B) HIV-1 protease cleavage of gag-pol fluorogenic substrate was assessed alone (white squares), with 7μM NFV (black triangles) or methanol (white circles).

A


5000
4000
3000
2000
1000
0
Relative fluorescence

B

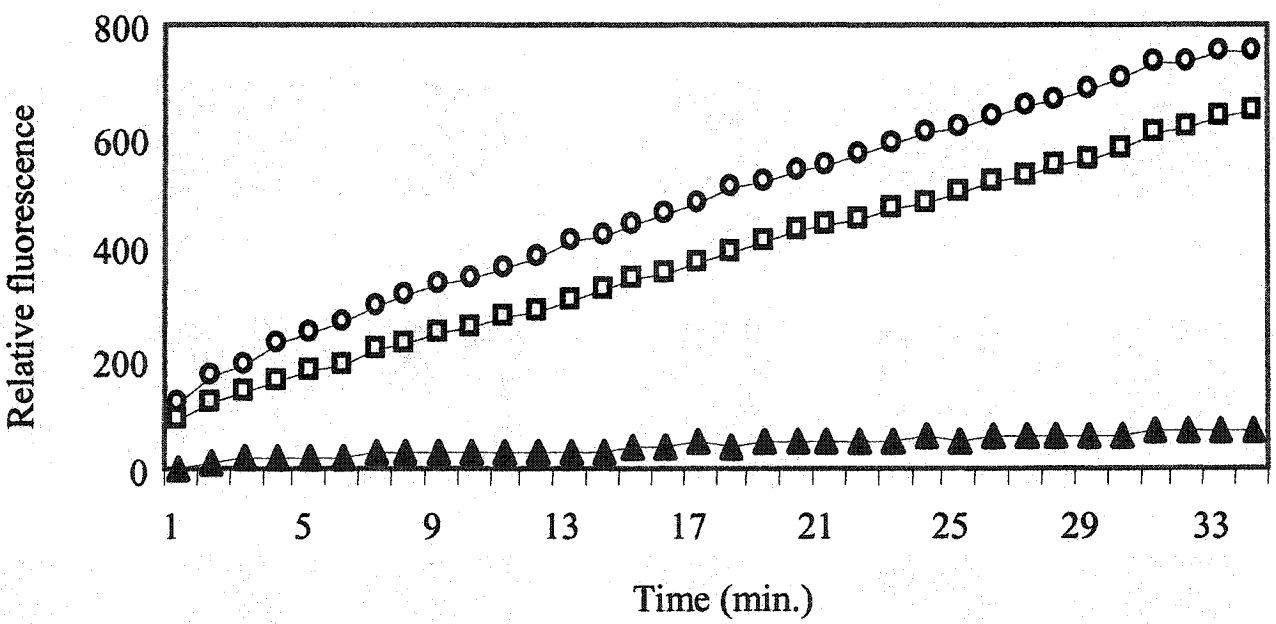
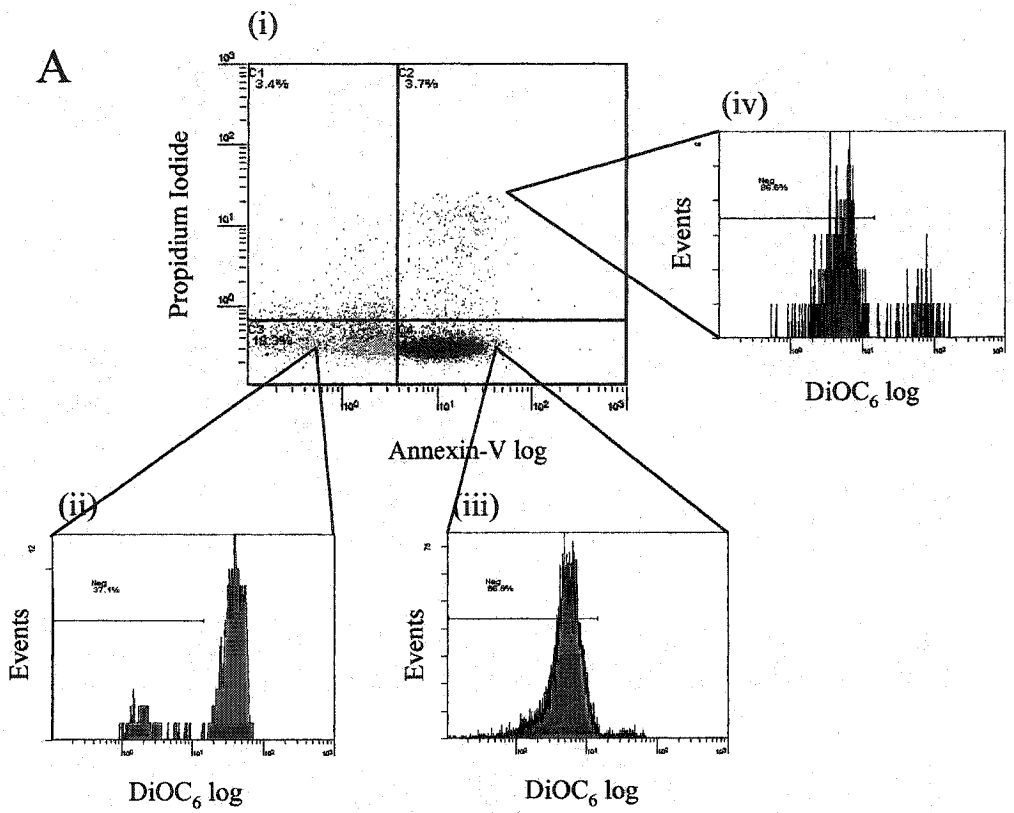
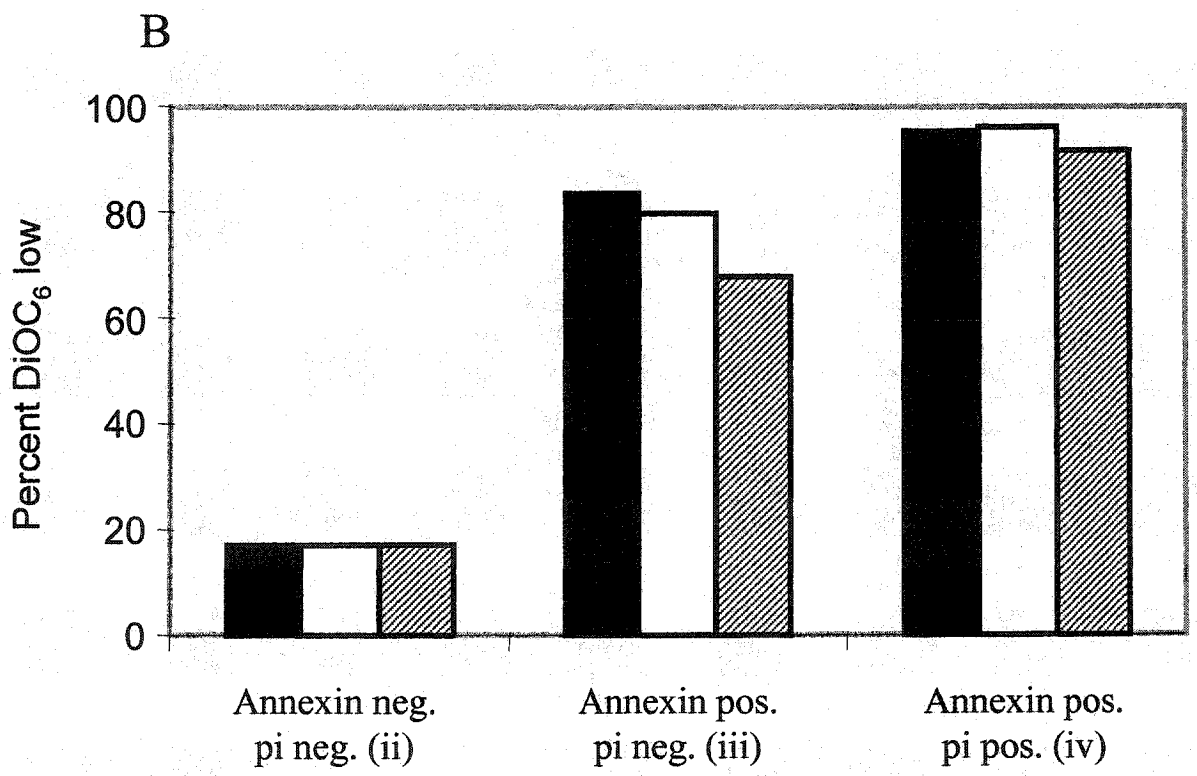

800
600
400
200
0
Relative fluorescence
1
5
9
13
17
21
25
29
33
Time (min.)

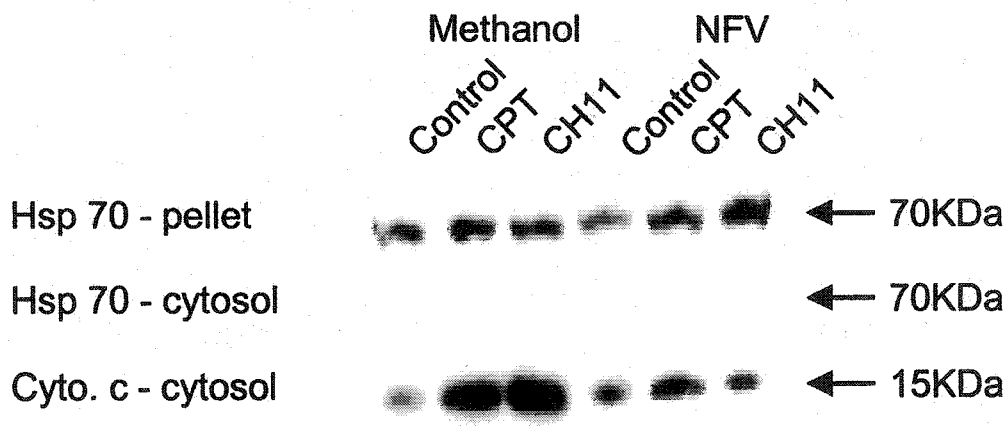
Figure 10. NFV inhibits loss of mitochondrial transmembrane potential as well as cytochrome *c* release.

(A) Representative histogram of flow cytometric analysis of Jurkat cells. The mitochondrial transmembrane potential of healthy (annexin-V negative, propidium iodide negative; Aii) apoptotic (annexin-V positive, propidium iodide negative; Aiii) and dead (annexin-V positive, propidium iodide positive; Aiv) cells is assessed using $DiOC_6(3)$. (B) Cumulative data from A refers to the percentage of cells exhibiting a $DiOC_6(3)$ low phenotype. Cells were treated with MeOH (black), 10μM AZT (white) or 7μM NFV (hatched). Apoptosis was induced by 10μM CPT. Data representative of three independent experiments. (C) NFV treatment inhibits cytochrome *c* release. Jurkat cells were treated overnight with MeOH or 7μM NFV, and apoptosis was induced by stimulation with 10μM CPT or 0.5μg/ml anti-Fas antibody (CH11) for 8 hours as indicated. Immunoblots were then performed of hsp 70 in the mitochondrial pellet (upper), cytosol (middle) and of cytochrome *c* release in cytosol (lower; 40μg of protein per lane). The blots are representative of two independent experiments.


A
(i)
Propidium Iodide
Annexin-V log
(ii)
(iii)
(iv)
Events
DiOC6 log


B
Percent DiOC6 low
100
80
60
40
20
0
Annexin neg.
pi neg. (ii)
Annexin pos.
pi neg. (iii)
Annexin pos.
pi pos. (iv)

C


Methanol
NFV
Control
CPT
CH11
Control
CPT
CH11
Hsp 70 - pellet
Hsp 70 - cytosol
Cyto. c - cytosol
70KDa
70KDa
15KDa

$DiOC_6(3)$ low cells (Figure 10B), indicating that NFV-treated cells which had become apoptotic exhibited less loss of mitochondrial transmembrane potential than untreated cells. Together, our data suggested that NFV might inhibit apoptosis by virtue of inhibiting $\Delta\psi_m$.

3.2.4 NFV Inhibits the Release of Apoptotic Mediators from Mitochondria

To determine whether NFV influenced mitochondrial events of apoptosis, cytochrome *c* release in cells induced to undergo apoptosis, with or without prior NFV treatment, was assessed by Dr. Zilin Nie. Cytosolic and mitochondrial fractions of Jurkat T cells stimulated with CPT were blotted for cytochrome *c*. NFV pretreated cells demonstrated a decrease in cytochrome *c* release from the mitochondria as compared to control cells (Figure 10C - bottom). This suggested that NFV inhibited apoptosis at or above the level of loss of mitochondrial membrane permeability.

3.2.5 NFV Inhibits Mitochondrial PTPC Opening

To confirm the postulate that NFV inhibits apoptosis at the mitochondrial level, the effects of NFV on apoptosis induced by factors that directly open the mitochondrial PTPC was assessed by Dr. Julian Lum. When added to intact cells or isolated mitochondria, the HIV accessory molecule Vpr causes $\Delta\psi_m$ and the consequent release of cytochrome *c* and AIF. This is mediated by binding of Vpr to ANT. [231] Both Bcl-2, and bongkrekic acid (BA), an ANT-specific ligand, inhibit the apoptotic effects of Vpr. [13]

To determine whether NFV inhibits PTPC opening, intact Jurkat cells were incubated with recombinant Vpr (peptides 52-96) in the presence or absence of NFV. Wild-type Vpr at a concentration of 2.5μM induced significant apoptosis, but this effect was inhibited by NFV (Figure 11A). Furthermore, NFV treatment resulted in a decrease in the percentage of cells which exhibited a $DiOC_6(3)$ low phenotype (Figure 11B), indicating that NFV inhibited loss of mitochondrial transmembrane potential in these cells.

Western blot analyses also performed by Dr. Lum also confirmed that cells co-incubated with Vpr and NFV had less release of cytochrome *c* and less pro-caspase 8 cleavage than cells treated with Vpr alone (Figure 11C). Hsp70 blots served as controls for mitochondrial fractionation. (Figure 11D)

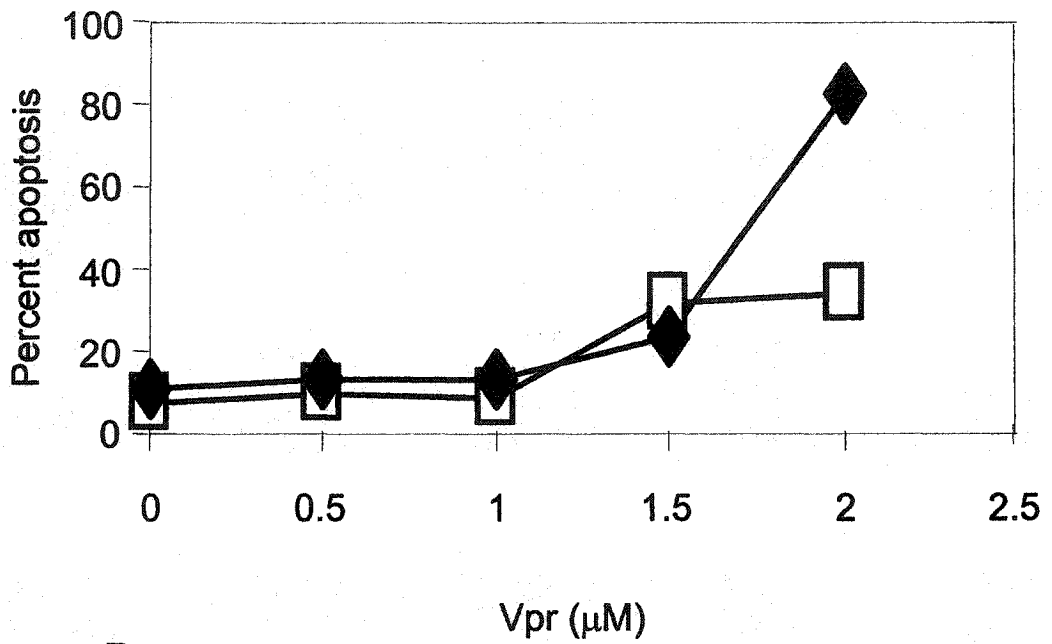
In order to further characterise the effect of NFV on mitochondrial PTPC opening, experiments were performed in isolated mitochondria. Atractyloside (Atr), a diterpenoid glycoside, interacts with ANT and directly causes $\Delta\psi_m$ with the release of AIF and cytochrome *c*. [13]

Mitochondria were isolated from Jurkat T cells and incubated overnight with BA, NFV or methanol. Following this incubation, mitochondria were treated with Atr (5mM) or Vpr (2.5μM), and PTPC opening was assessed by $DiOC_6(3)$. Both BA and NFV inhibited Vpr- and Atr-mediated PTPC opening (Figure 12); surprisingly the inhibition in response to NFV was greater than that observed with BA. Our cumulative data therefore indicated that NFV inhibited apoptosis via a direct effect on PTPC opening.

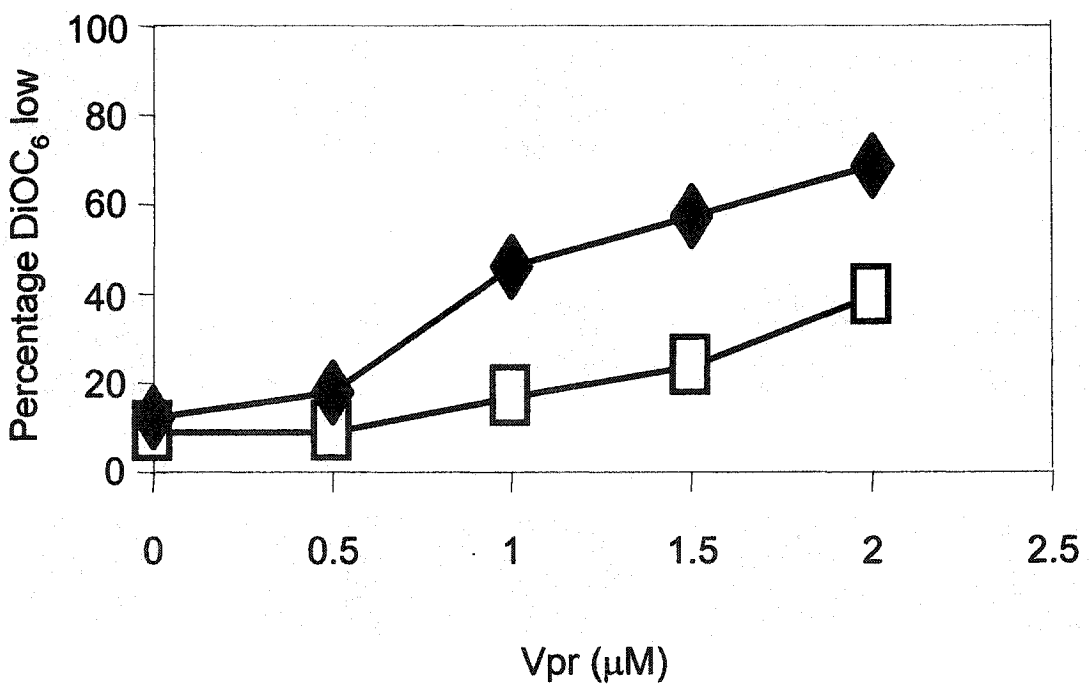
Figure 11. NFV inhibits apoptosis induced by PTPC opening.

(A) Jurkat T cells were treated with MeOH (black diamonds) or 7μM NFV (white squares) for 1-hour prior to overnight stimulation with 2.5μM Vpr. Apoptosis was assessed as in Figure 5. Data are representative of three independent experiments. (B) Mitochondrial $\Delta\psi_m$ was assessed in the above cells using $DiOC_6(3)$. Data are representative of three independent experiments. (C) Jurkats were treated with MeOH or 7μM Nfv for 1-hour prior to overnight stimulation with BSA or 2.5μM Vpr, and immunoblotted for cytochrome *c* release (upper) and pro-caspase 8 cleavage (lower) in the cytosolic fraction. (D) Immunoblot of hsp 70 in pellet and cytosolic fractions confirms mitochondrial localisation.

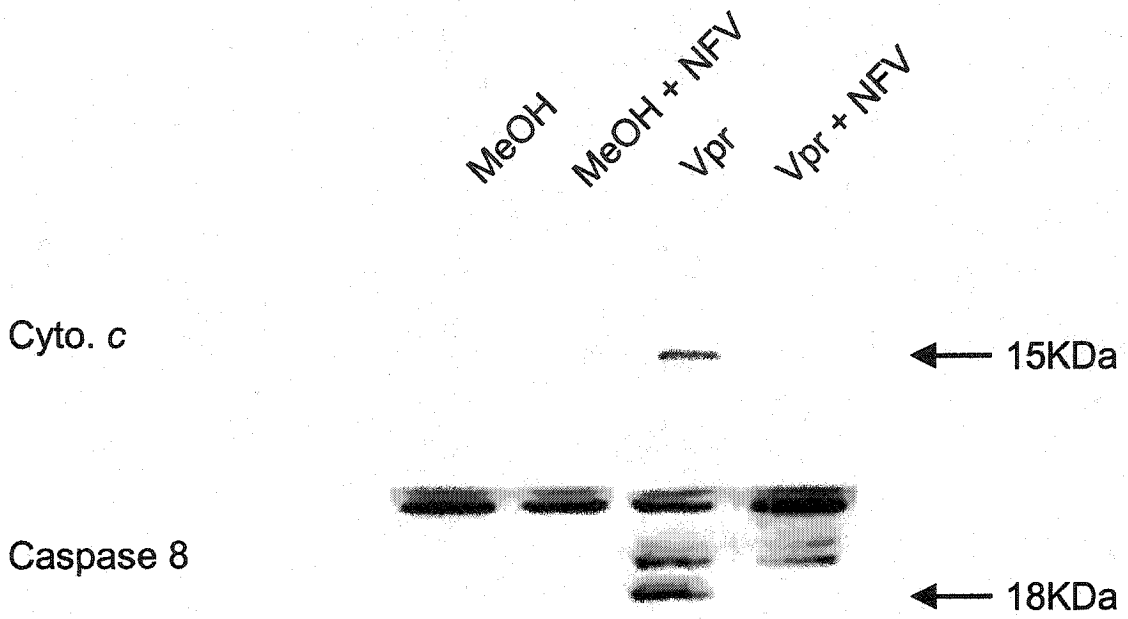
A


Percent apoptosis
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Vpr (μM)

B


Percentage DiOC6 low
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Vpr (μM)

C


MeOH
MeOH + NFV
Vpr
Vpr + NFV
Cyto. c
15KDa
Caspase 8
18KDa

D

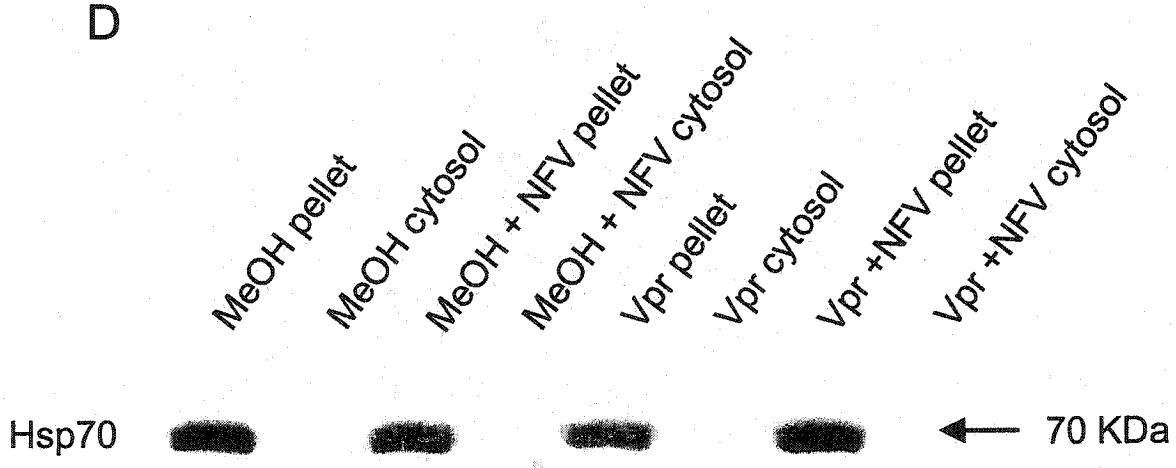
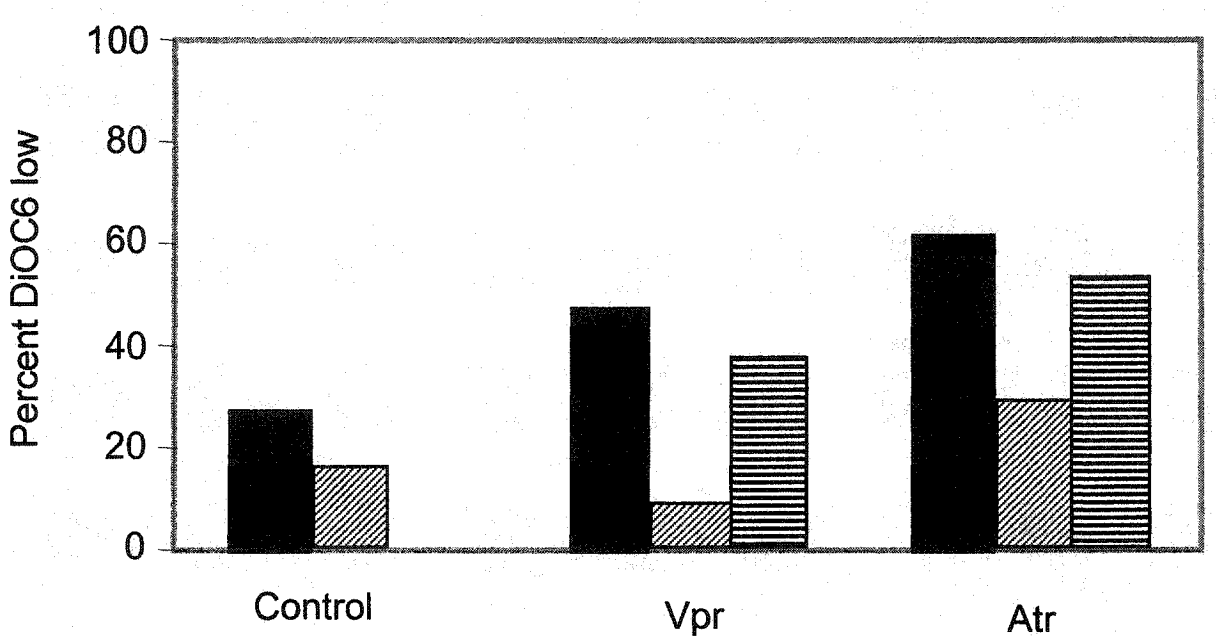

MeOH pellet
MeOH cytosol
MeOH + NFV pellet
MeOH + NFV cytosol
Vpr pellet
Vpr cytosol
Vpr +NFV pellet
Vpr +NFV cytosol
Hsp70
70 KDa

Figure 12. PIs inhibit PTPC opening in isolated mitochondria.

Isolated mitochondria were treated overnight with methanol (black), 7μM NFV (diagonally hatched) or 50μM BA (horizontally hatched) prior to being stimulated with 2.5μM Vpr or 5mM ATR. Loss of $\Delta\psi_m$ was assessed by flow cytometry using $DiOC_6(3)$.


Percent DiOC6 low
100
80
60
40
20
0
Control
Vpr
Atr

3.2.6 Effect of NFV on Type I Fas Signalling

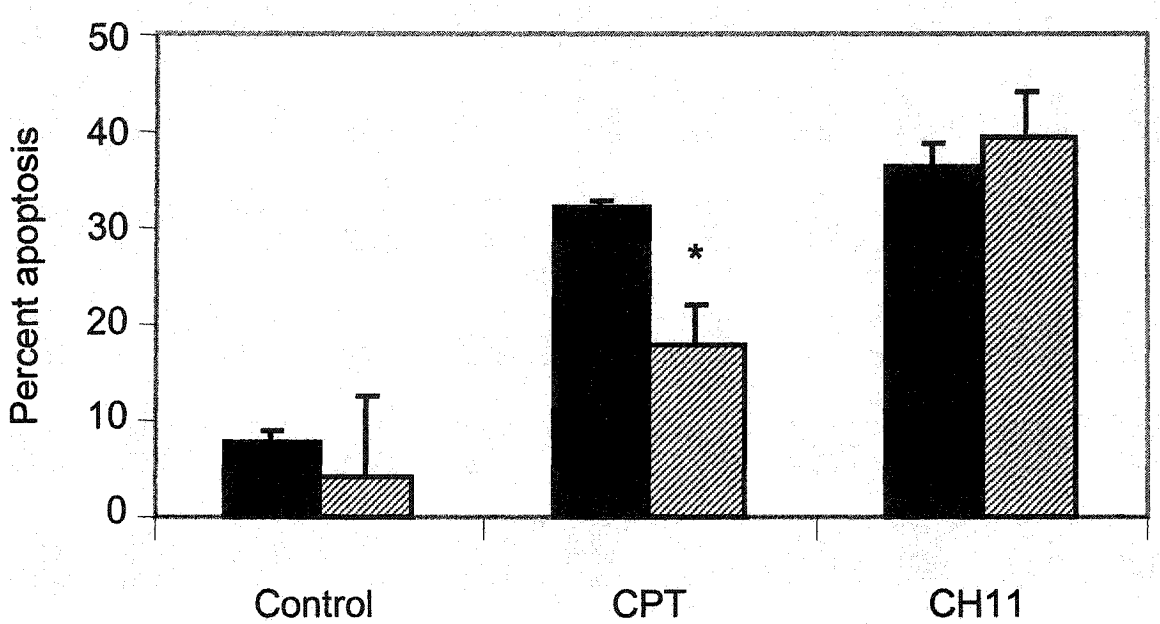
If PIs block apoptosis through inhibition of PTPC opening, then apoptosis that does not depend on mitochondrial activation should be unaffected by PIs. Fas receptor ligation may activate one of two different pathways that lead to apoptosis – one that is mitochondrially dependent and one which is not. [87-89]

Cells with a type II Fas signalling pathway recruit FADD to the trimerised Fas receptor, causing caspase 8 activation and cleavage of Bid. This in turn leads to PTPC opening, $\Delta\psi_m$, release of cytochrome *c*, activation of caspases 9, 7 and 3, DNA fragmentation and cell death. [90-92] Jurkat T cells utilize a type II pathway in response to Fas receptor ligation. In contrast, in cells with a type I Fas signalling pathway, caspase 8 activation immediately causes caspase 3 activation, thereby bypassing the requirement for mitochondrial amplification. H9 T cells, a lymphoblastic cell line, [429] utilize a type I signal transduction pathway in response to Fas ligation. [87-89]

We therefore evaluated the effect of NFV on H9 cells treated with either Fas, which would induce apoptosis independently of changes in mitochondrial membrane permeability, or with CPT, which activates a mitochondrial-dependent pathway. [430] (Figure 13) Whereas NFV-pretreated H9 cells underwent the same amount of apoptosis as control cells in response to Fas ligation, NFV inhibited CPT-induced apoptosis in H9 cells. Thus, NFV inhibited the apoptotic pathway that required mitochondrial PTPC opening, but did not inhibit the pathway that was independent of mitochondria.

Figure 13. NFV inhibits CPT- but not anti-Fas antibody (CH11)-mediated apoptosis in type I cells.

H9 cells were treated overnight with MeOH (black) or 7μM NFV (hatched) and stimulated with 10μM CPT or 0.5μg/ml anti-Fas antibody (CH11). Apoptosis was assessed as in Figure 5. NFV treatment significantly inhibited CPT- ($p=0.03$) but not anti-Fas antibody (CH11)-induced apoptosis. ($n=3$)


Percent apoptosis
50
40
30
20
10
0
*
Control
CPT
CH11

3.3 *IN VIVO* EFFECTS OF PIs ON APOPTOSIS

3.3.1 Establishment of a PI dosing regimen

Animal models have become very useful in the study of the mechanisms involved in apoptotic cell death. In order to make use of these tools and establish whether PIs could inhibit apoptosis in vivo, we first had to determine whether therapeutic plasma levels of PIs could be achieved.

Drug dosing experiments were performed in 20-22g male Balb-c mice which received either 125mg/Kg or 250mg/Kg NFV by gastric lavage every 8 hours for a period of 24 hours. Immediately preceding the fourth dose, and every two hours post-dose for eight hours, plasma samples were collected and assayed to determine drug concentration. At these doses, neither NFV nor its principle metabolite M8 was detectable 6 hours post gastric lavage. Since co-administration of RTV in human subjects significantly increases PI levels as compared to treatment with PIs alone, [431] we treated another group of mice with 125mg/Kg NFV and 13mg/Kg RTV to exploit the inhibitory effect of RTV on the metabolism of NFV.

NFV levels, which were assessed by Mr. Germain Carignan in two NFV/RTV-treated mice at each time-point, were between 8172 and 8914ng/mL at 1 hour, 5999 and 6006ng/mL at 2 hours, 5559 and 6048ng/mL at 3 hours and 4005 to 4217ng/mL at 4 hours. NFV levels of 1199 to 1258ng/mL 8 hours following the administration of the PIs indicated a significant trough level; therefore we opted to assess this dosing regimen for its antiapoptotic effects.

3.3.2 PIs Inhibit Apoptosis in a Mouse Model of Stroke

The transient global forebrain ischæmia model, where transient interruption of all the blood supply to the brain induces a stroke, was used in order to assess whether PIs could inhibit apoptosis following stroke. Five mice in each of four treatment groups received NFV/RTV or control vehicle by gastric lavage every 8 hours. Two groups were pre-treated with PIs, one 24 hours and another 1-hour prior to being subjected to transient global forebrain ischæmia, whereas one group was treated 6 hours following transient global forebrain ischæmia. Another group of mice was given control vehicle. All mice continued treatment for 48 hours after the stroke.

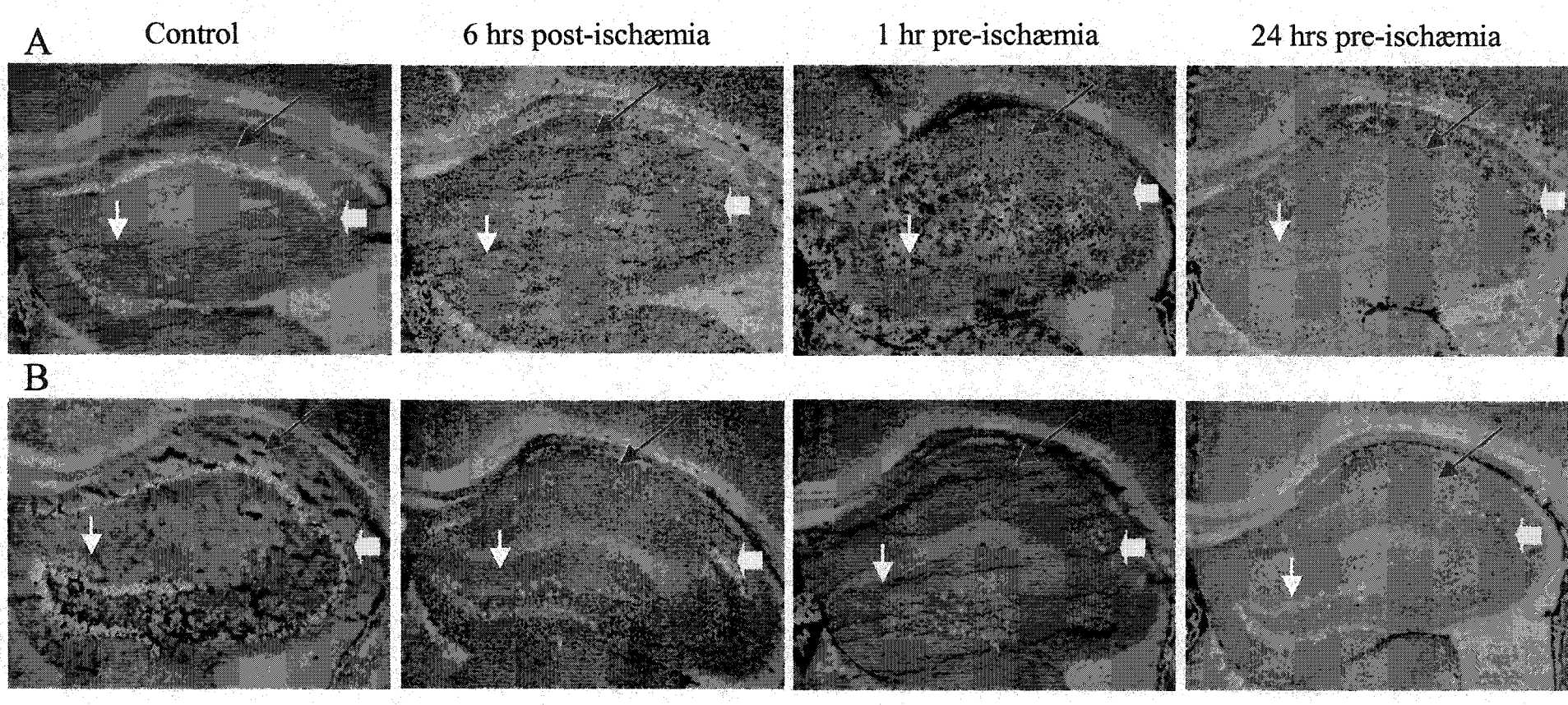
In Figure 14, hippocampal sections corresponding to animals displaying both the least (Panel A) and the most (Panel B) amount of apoptosis in each treatment group are represented. The pertinent regions are (1) CA1 region (red arrow), (2) CA2 region (yellow arrow), and (3) dentate gyrus (white arrow). The most sensitive region to ischæmia is the CA1 region, [432] whereas other regions are a bit more resistant but may also undergo apoptotic injury.

TUNEL staining of sections revealed a significant amount of apoptosis in the brains of control mice. All regions, especially the CA1 region are protected in the sections from mice pre-treated with PIs for 24 hours and 1 hour prior to stroke. Interestingly, protective effects of PIs were also observed in the sections from mice treated with PIs 6 hours after being subjected to ischæmia.

To establish whether PIs prevent or merely delay hippocampal cell death, an additional experiment was performed in which two mice were pre-treated with

Figure 14. PIs inhibit apoptosis in hippocampal neurons following ischæmia.

TUNEL staining of hippocampal sections (5x magnification) of three treatment groups of five mice each were administered NFV/RTV by gastric lavage. The first group was treated 24 hours prior to transient forebrain ischæmia, another was treated 1 hour prior to forebrain ischæmia, and the third group was given PIs 6 hours following transient forebrain ischæmia. An additional group was treated with 1% ethanol/water as control. Mice displaying the least (panel A) and most (Panel B) amount of apoptosis in each treatment groups are represented.


A
Control
6 hrs post-ischæmia
1 hr pre-ischæmia
24 hrs pre-ischæmia
B

PIs for 24-hours prior to transient global forebrain ischæmia and monitored for a period of 30 days. Hippocampal section of these mice showed similar amounts of apoptosis than those pre-treated with PIs and euthanised 48 hours after stroke. (data not shown)

3.3.3 PIs Inhibit Apoptosis of PBMCs in Patients Treated with PEP

Four healthy subjects (two male) without evidence of immune disease were enrolled in a PEP clinical trial. The median age of this cohort was 31.5 years (range: 28 to 37) and median duration of antiretroviral exposure was 5.5 days (range: 4 to 7). We assessed spontaneous, anti-Fas- and CPT-mediated apoptosis of PBMCs prior to, during, and after PEP.

No changes were observed in the rate of spontaneous or Fas-induced apoptosis in the four PEP patients; (Table 1) however, CPT-induced apoptosis was inhibited in all four patients during the course of ART. Apoptotic response to CPT was restored to that observed prior to drug treatment once drug treatment was terminated. (Figure 15)

Table 1. *Ex vivo* apoptosis of PBMCs from patients taking PEP.

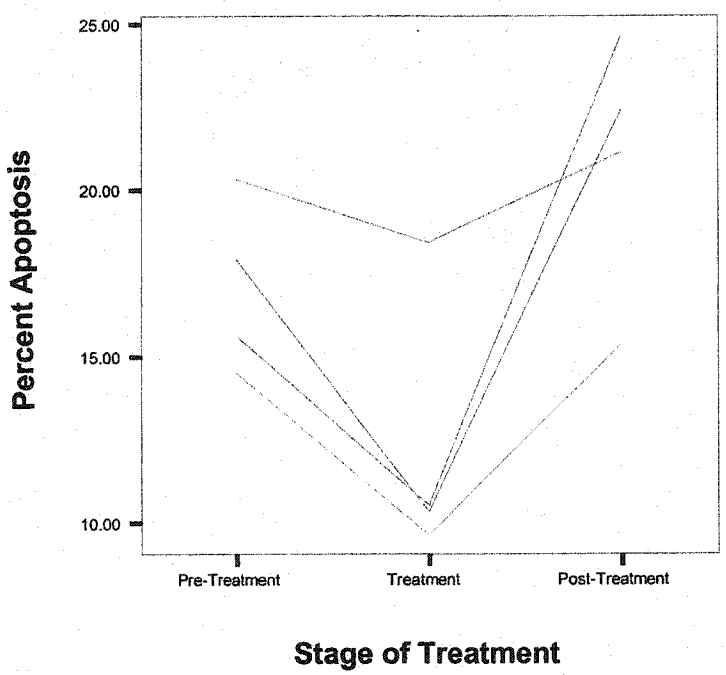
Measure		N	Pre-Antiretroviral	On Therapy	Post-Antiretroviral
Apoptosis Susceptability (%)	Spontaneous*	4	4.6	5.8	7.1
	Camptothecin**	4	16.8	10.4	21.8
	CH11*	4	5.5	5.4	8.9

* Comparisons between Pre-ARV and On therapy, and On therapy and Post-ARV were not statistically significant

** Wilcoxon Signed Rank Test: Pre-ARV vs On therapy, p=0.068; On therapy vs Post-ARV, p=0.068

Figure 15. PIs inhibit CPT-induced apoptosis *ex-vivo*.

PBMCs from patients taking PEP (AZT, 3TC, NFV) were isolated prior to, during, and after ART and stimulated to undergo apoptosis using anti-Fas antibody (CH11), and CPT. (Case a: red, Case b: blue, Case c: green, Case d: purple) Apoptosis was assayed as in Figure 5.


25.00
20.00
15.00
10.00
Percent Apoptosis
Pre-Treatment
Treatment
Post-Treatment
Stage of Treatment

4. DISCUSSION

The main focus of this project was to determine whether HIV PIs could inhibit a cell's ability to undergo apoptosis, as an increasing number of reports suggest that PIs have immunomodulatory effects that are independent of their effects on suppression of viral replication. Our working hypothesis was that HIV PIs would inhibit a cell's ability to undergo apoptosis. As such, we decided to treat PBLs from HIV-infected patients and uninfected Jurkat T cells with HIV PIs to assess the effects of these drugs on apoptosis. The mechanism by which HIV PIs mediate their effects was also investigated, as was the ability of these drugs to influence apoptosis *in vivo*.

4.1 HIV PIs INHIBIT APOPTOSIS IN PBLs FROM HIV-INFECTED PATIENTS AND IN UNINFECTED CELL LINES

Initially, we assessed the effects of both PIs and RTIs on apoptosis in PBLs from antiretroviral naïve HIV-infected patients. The RTIs selected were AZT and ddG, the former because it was the first licensed agent and is widely used, and the latter because it has recently been reported to be a potent new member of the class. [433, 434] The PIs RTV and SQV were also used.

Our findings clearly demonstrated that PIs - but not RTIs - inhibited apoptosis in cells from HIV-infected patients. The observed inhibition was dose-dependent, and occurred in response to both anti-Fas and isotype antibody stimulation. However, the significance of these preliminary results was difficult to interpret, as it was unclear whether HIV PIs directly inhibited apoptosis at the cellular level, or whether inhibition occurred indirectly as the result of suppression

of viral replication. One particular observation suggested that the observed inhibition was in fact the direct result of the PIs: though similar levels of viral suppression were obtained with triple RTI therapy as compared to single PI therapy, inhibition of apoptosis was only observed in PI-treated cultures (in both CD4 and CD8 T cells).

If PIs inhibit the pro-apoptotic activity of any of the requisite mediators of apoptosis, they would predictably inhibit apoptosis in experimental conditions that do not involve HIV. The postulate that PIs directly inhibit apoptosis by a mechanism that is independent of their effects of viral suppression was assessed in an uninfected Jurkat T cell line.

We observed that cell cultures treated with the PI NFV [359, 425] showed a time- and dose-dependent inhibition of apoptosis. This study also demonstrates that NFV inhibited Jurkat T cell apoptosis induced by a variety of different stimuli, including actinomycin D (a chemotherapeutic agent), [435] camptothecin (a topoisomerase I inhibitor), [436] and an agonistic anti-Fas antibody (CH11), [35] suggesting that PIs affect a common component within the different apoptotic pathways tested.

The fact that NFV, an HIV PI whose chemical structure differs from RTV and SQV, [356, 437, 438] inhibited apoptosis in a similar manner to RTV and SQV suggests that inhibition of apoptosis by PIs may be a generalized effect of this class of drugs.

The combined results from experiments performed in cells from HIV-infected patients and in uninfected cell lines demonstrated that some of the

immunologic effects of PIs were related to an effect that was independent of changes in viral replication.

4.2 PROPOSED MECHANISM BY WHICH PIs MODULATE APOPTOSIS

Apoptosis is a complex phenomenon regulated at many different levels, including cell membrane expression of the TNF superfamily of death receptors, intracellular apoptosis modifiers including caspases, cFLIPs, Bcl-2 family members and IAPs, and mitochondrial proteins such as components of the PTPC, cytochrome *c* and AIF. Having identified a new phenomenon by which HIV PIs inhibited apoptosis, we proceeded to characterise the mechanism by which this process transpired.

Inhibition of apoptosis within one hour of NFV treatment suggested that PIs acted through a mechanism that was independent of transcription or protein synthesis. This was confirmed at the mRNA expression level for a variety of pro- and antiapoptotic factors as determined by ribonuclease protection assay. Additionally, experiments with CHX, an inhibitor of protein synthesis, [439] confirmed that inhibition of apoptosis did not depend upon protein synthesis. While others have reported that PIs may affect the generation of active caspase 1 (ICE) or caspase 3, [426, 427] our experiments showed that NFV had no effect on the ability of the recombinant caspases 1, 3, 6, 7 and 8 to cleave their tetrapeptide consensus cleavage sequences.

In trying to establish the level at which the block in the apoptosis cascade occurred following PI treatment, we chose to ascertain whether the block took

place prior to or following mitochondrial involvement. We analysed NFV-treated Jurkat T cells following CPT-mediated apoptosis, and monitored $\Delta\psi_m$ using $DiOC_6(3)$. Our results demonstrated maintenance of mitochondrial transmembrane potential levels in the apoptotic NFV-treated cell population, suggesting that NFV inhibited $\Delta\psi_m$. The fact that NFV pre-treatment also inhibited cytochrome *c* release and pro-caspase 8 activation in CPT-treated Jurkats provided additional support to our data.

The release of cytochrome *c* from mitochondria is an important step in the apoptosis cascade. Normally, cytochrome *c* resides in the space between the outer and inner membranes of mitochondria, but once in the cytosol, cytochrome *c* mediates the assembly of an apoptosome – a large complex composed of Apaf-1 oligomers [23, 440] and pro-caspase 9. The formation of this complex allows for the autocatalytic activation of caspase 9 and subsequent activation of other caspases.

Given that opening of PTPCs results in $\Delta\psi_m$ and correlates with the release of cytochrome *c* and AIF from mitochondria, we assessed whether NFV could inhibit release of cytochrome *c*. Our results showed that cytochrome *c* release from the mitochondria in response to an apoptotic stimulus was inhibited following NFV treatment of cells, which corroborated the above $DiOC_6(3)$ data.

To confirm that NFV inhibited apoptosis by influencing mitochondria, we also assessed the effects of NFV on Vpr-induced apoptosis. Vpr is cell permeable and directly interacts with PTPCs, resulting in their opening and the subsequent release of apoptogenic factors from mitochondria. [231] Over-

expression of Bcl-2 or pre-treatment with cyclosporin A and BA - two inhibitors of PTPC opening - inhibits Vpr-mediated $\Delta\psi_m$. [407, 441]

NFV inhibited Vpr-induced apoptosis, thereby supporting the hypothesis that NFV inhibited apoptosis by preventing PTPC opening. Experiments using isolated mitochondria illustrated that NFV inhibited both Atr- and Vpr-mediated PTPC opening to degrees greater than that seen by BA, thus providing additional support to our data.

Finally, we treated H9 cells using apoptotic stimuli that either required (CPT) or bypassed (Fas ligation) mitochondrial PTPC opening. Whereas NFV inhibited CPT-induced apoptosis in H9 cells, it did not inhibit apoptosis induced by Fas ligation, therefore confirming that NFV inhibited apoptosis via an effect on mitochondrial PTPC opening. Our data contrasts published reports suggesting that HIV PIs inhibit the enzymatic activity of caspases. [426, 427]

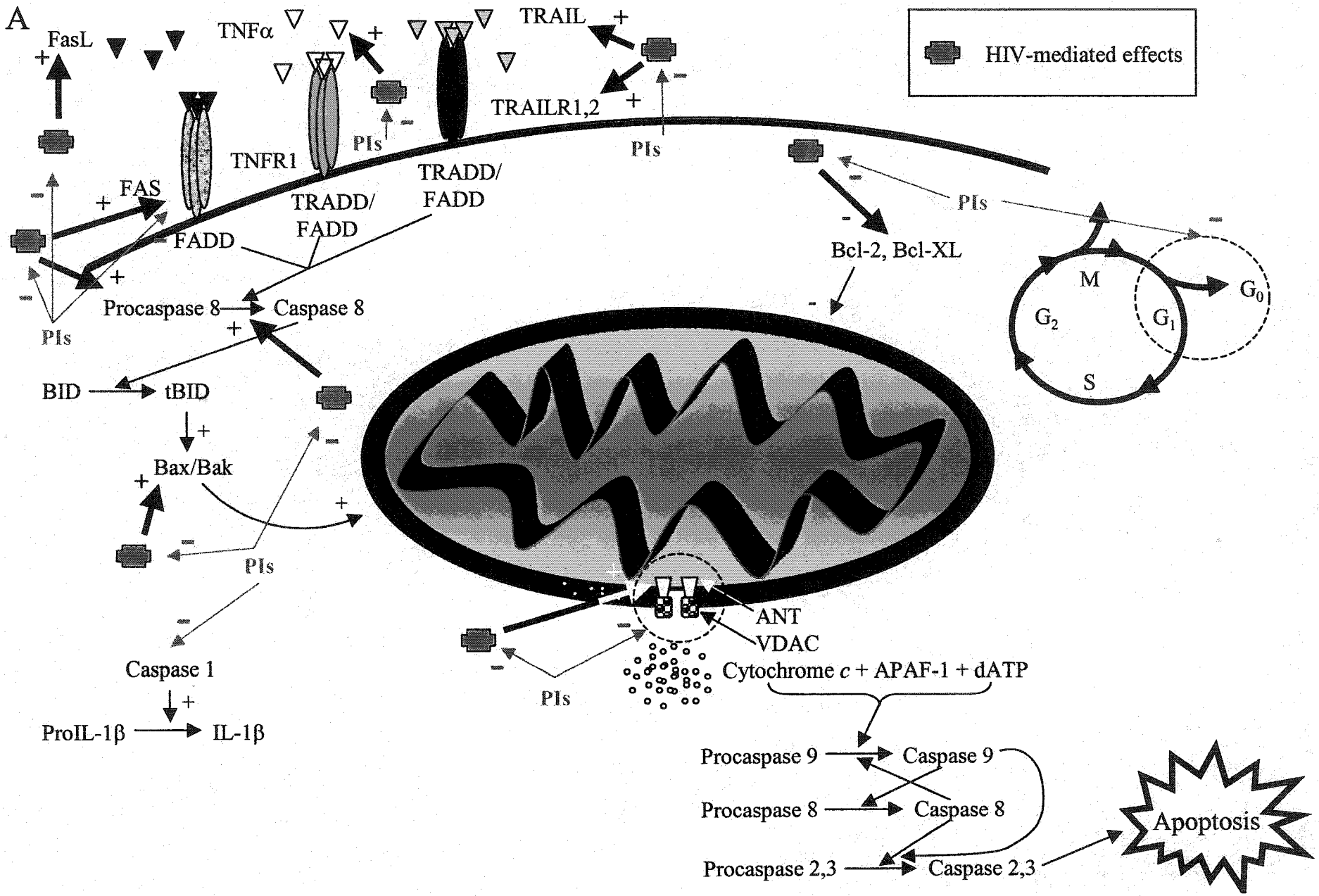
Schematic representations of the pathways by which PIs inhibit apoptosis in infected (Figure 16A) and uninfected (Figure 16B) cells summarise our current state of knowledge.

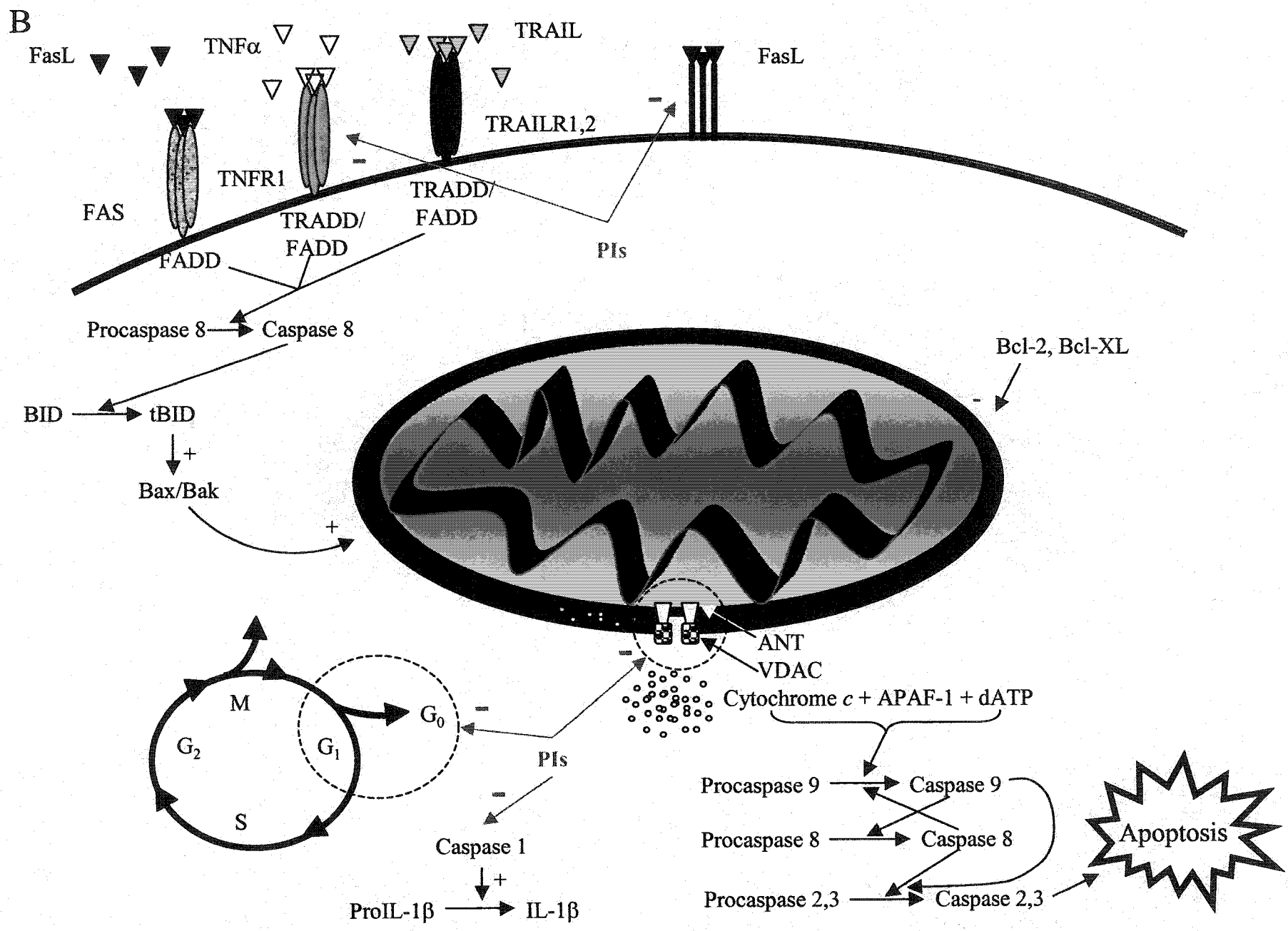
4.3 IMMUNOMODULATORY PROPERTIES OF PIs

Recent investigations into the immunomodulatory properties of the PI class of drugs have shown that these drugs influence numerous aspects of cellular metabolism in ways that would not necessarily be predicted by their substrate specificity. Over the course of the past few years, reports confirming the initial observation of PI-mediated inhibition of P-glycoprotein (P-gp) activity

Figure 16. Effects of PIs on apoptotic pathways in cells.

Schematic representation of the apoptotic pathways that are targeted by PIs. Both the extrinsic and intrinsic apoptotic pathways triggered by HIV viral products are influenced by PIs. (A) HIV-infected cells. (B) Uninfected cells.


A
FasL
TNFα
TRAIL
HIV-mediated effects
TRAILR1,2
PIs
TNFR1
FAS
TRADD/
FADD
FADD
Procaspase 8
Caspase 8
Bcl-2, Bcl-XL
M
G0
G2
G1
S
BID
tBID
Bax/Bak
ANT
VDAC
Cytochrome c + APAF-1 + dATP
Caspase 1
ProIL-1β
IL-1β
Procaspase 9
Caspase 9
Procaspase 8
Caspase 8
Procaspase 2,3
Caspase 2,3
Apoptosis


B
FasL
TNFα
TRAIL
FasL
TRAILR1,2
TNFR1
FAS
TRADD/
FADD
TRADD/
FADD
FADD
PIs
Procaspase 8
Caspase 8
Bcl-2, Bcl-XL
BID
tBID
Bax/Bak
ANT
VDAC
Cytochrome c + APAF-1 + dATP
M
G2
G1
G0
S
PIs
Procaspase 9
Caspase 9
Procaspase 8
Caspase 8
Apoptosis
Caspase 1
ProIL-1β
IL-1β
Procaspase 2,3
Caspase 2,3

have surfaced. [442, 443] The effect of HIV PIs on P-gp activity has multiple ramifications, including reduced IL-2 release from activated T cells. [444] Interestingly, in addition to modulating P-gp activity, PIs also influence other ATP-dependent drug export mechanisms such as multidrug resistance-associated protein 2 (MRP2) transport [442] and MRP1 expression. [445]

The ability of PIs to influence homeostasis has been reported in many different cell types including human myelocytic leukemia and dendritic cells (DCs). [446] Targeted cellular processes include telomerase activation [447, 448] and cell cycle progression. [449] Recent reports of regression of Kaposi sarcoma associated with PI therapy have prompted researchers to investigate the anti-angiogenic properties of PIs. It was determined that PIs inhibit *in vivo* growth and invasion of angiogenic tumour-cell lines, thus rendering PIs potent anti-tumour agents. [450, 451]

As many as 50 percent of patients with HIV/AIDS may experience one or more of the components of the lipodystrophy syndrome, [452] which comprises at least four components: loss of subcutaneous adipose tissue (lipoatrophy), truncal fat accumulation, and/or subcutaneous lipomas (lipoaccumulation), hyperlipidæmia, and insulin resistance. [452] The loss of subcutaneous adipose tissue (lipoatrophy) may occur as the result of PI-mediated modulation of several host-proteins involved in lipid and carbohydrate metabolism. This hypothesis is supported by the demonstration of enhanced subcutaneous adipocyte apoptosis in biopsy samples taken from lipoatrophic areas of patients with PI-associated lipodystrophy [453] and by increased adipocyte death by HIV PIs *in vitro*. [454]

Treatment with PIs may induce apoptosis of adipocytes by binding cytoplasmic retinoic-acid binding protein-1 (CRABP-1), which is involved in adipocyte differentiation. [455] PIs increase lipolysis *in vitro* [456] and impair preadipocyte maturation, [454, 456-459] potentially via altered expression of adipogenic factor sterol-regulatory-element-binding-protein-1 (SREBP1). [460]

Hyperlipidæmia, another potential complication with HIV PIs in AIDS therapy, [452, 455, 461, 462] may also result from modulation of host-proteins by PIs. Increases in synthesis of very low-density lipoprotein (VLDL) [456, 463] have been attributed to at least 2 factors: the increased peripheral adipocyte apoptosis associated with PI-induced lipoatrophy, which results in the release free fatty acids; and the increased intracellular stabilisation of apolipoprotein B (ApoB), the principal protein component of triglyceride and cholesterol-rich plasma lipoproteins. [464] This occurs as the result of inhibition of proteasomal degradation of pre-secretory ApoB in conjunction with a specific block in lipoprotein secretion due to inhibition of neutral-lipid biosynthesis and transfer (which results in "stockpiling" of ApoB within the cell). [464] Previous investigations into the modulation of proteasome activity by PIs indicated that PIs specifically inhibited the chymotryptic activity of the 20 S proteasomes. [465] A compounding factor in PI-mediated hyperlipidæmia may involve the reduction in production of CD36 mRNA and cell-surface expression of CD36 seen with PI treatment. [466, 467] CD36 is a scavenger receptor expressed on macrophages that mediates the uptake of modified lipoproteins and functions as a high-affinity transporter of long-chain fatty acids. [468, 469] Finally, PIs may exert their

influence on lipid metabolism by inhibiting cellular enzymes such as lipoprotein lipase. [470]

New-onset or worsening diabetes was associated with PIs by as early as 1997. [471, 472] PI-mediated changes in cellular signalling in response to insulin may explain the acquired insulin resistance that occurs in some patients who receive PI-based therapies. [452] Potential mechanism(s) by which PIs alter insulin metabolism include: increasing all trans-retinoic acid-dependent alkaline phosphatase activity, resulting in stimulation of retinoic acid signalling, [473] reducing insulin effects on insulin-receptor substrate (IRS)-1-phosphorylation, [474] reducing association of phosphatidylinositol 3-kinase with IRS-1, [474] decreasing phosphorylation of protein kinase B (PKB or Akt), [474] inhibiting insulin-stimulated glucose uptake [470, 475] by selective inhibition of glucose transporter (Glut) 4 function, and [476, 477] increasing retinal dehydrogenase activity and/or gene expression. [477, 478]

Another metabolic complication that ocurs in the context of PI-containing therapy is hyperbilirubinemia. Unconjugated hyperbilirubinemia, which develops in up to 25% of patients taking indinavir, [479] occurs as the result of direct inhibition of the bilirubin-conjugating activity of UDP-glucuronosyltransferase by PIs. [480]

The ability of PIs to inhibit apoptosis in uninfected cells has now been reported by several different groups, [426, 481-484] whereas others have observed that inhibition of apoptosis is limited only to HIV-infected cultures. [485, 486] By contrast, some groups have reported that PIs do not modulate apoptosis,

[449, 485] while still others claim that PIs enhance apoptosis in certain cell types. [453, 454] Poor solubility of HIV PIs may be a factor contributing to these discrepancies. [487]

4.4 *IN VIVO* EFFECTS OF HIV PIs

Disease models have proven to be valuable tools in investigating potential antiapoptotic mediators. An animal model of ischæmia was used in order to facilitate the assessment of the effects of HIV PIs *in vivo*. In addition, the advent of PEP has presented us with the opportunity to evaluate the effects of PI-containing therapy on apoptosis in HIV-negative populations.

Reduction in blood flow to the brain deprives neuronal cells of oxygen and glucose, and results in ischæmic injury. The transient global forebrain ischæmia model, in which a transient interruption of all the blood supply to the brain induces stroke, was used in order to assess whether PIs could inhibit apoptosis following ischæmic injury.

Our data plainly reveal that apoptosis of neurons in hippocampal sections of ischaemic mice pre-treated with PIs was reduced in comparison to controls. The fact that groups of mice treated at the time of stroke and 6 hours post-stroke showed significant decreases in apoptosis levels suggests that PIs may be valuable tools in the struggle encountered in the treatment of stroke.

Investigations into the risk of HIV transmission in health-care workers subsequent to percutaneous and mucus-membrane exposure to HIV-infected body fluids revealed that the use of zidovudine was associated with a 79%

decrease in the risk of HIV transmission. [488] These findings led to the recommendation of PEP for health-care workers exposed to HIV in work-related settings.

Our data reveals that short-term ART in HIV-negative patients has no impact on spontaneous or anti-Fas-mediated apoptosis, but that CPT-induced apoptosis is inhibited during therapy. This suggests that HIV PIs are capable of modulating apoptosis *in vivo* in HIV-negative as well as HIV-positive persons. In contrast to the PI-mediated inhibition of anti-Fas-induced apoptosis observed in Jurkat T cells, no inhibition was observed in the PBMCs of patients on PEP therapy. This observation can be explained by the fact that levels of cellular activation in the patient PBMCs were low, as none of the patients had evidence of any type of infection. As discussed in the context of AICD, activation of T cells is required in order to make them sensitive to Fas-mediated apoptosis. Primary stimulation, whether resulting from infection with HIV or activation by cytokines, results in increased susceptibility to Fas-mediated apoptosis. [190-192]. As the levels of anti-Fas-mediated apoptosis were similar to spontaneous apoptosis levels, no inhibition was observed in patients taking PEP therapy.

In this particular trial, patients received both a PI and an NRTI, thus rendering our efforts to attribute our observations to one or the other drug problematic. Evidence presented by us and others suggests that the observed inhibition in CPT-mediated apoptosis is PI specific, [426, 481-486, 489, 490] whereas the apoptosis-inducing properties of NRTIs [491-494] and NNRTIs [495] have been well documented.

These demonstrations of an antiapoptotic effect *in vivo* validate prior *in vitro* data that demonstrate the antiapoptotic effect of PIs. (For review see [496] and references therein) Further, these findings lend credibility to the suggestion that treatment of HIV with PI-based therapy improves immunologic outcomes in comparison with PI sparing therapies. This point may be of particular importance to patients with advanced immunodeficiency.

To date, candidate apoptosis inhibitors have suffered from unacceptable toxicities; however, the extensive clinical experience with HIV PIs indicates a satisfactory safety profile despite their unwanted effects on lipid and glucose metabolism, and makes them worthy of consideration as novel antiapoptotic therapies for such disease processes. Pharmacologic modification of apoptosis may offer a therapeutic opportunity for patients affected with diseases in which enhanced apoptosis contributes to the underlying pathogenesis. Therefore, therapeutic modification of apoptosis may offer benefit for disease processes including, but not limited to, inflammatory bowel disease, hepatitis, host response to bacterial infection and sepsis.

4.5 IMPLICATIONS FOR THE MANAGEMENT OF HIV

The clinical significance of antiapoptotic effects of PIs remains to be fully defined; however both the antiapoptotic effect on CD4 T cells, [426, 427, 482, 489, 497] and putative antiapoptotic effects on CD34 progenitor cells [482] may serve to increase CD4 cell number in HIV-infected patients independent of the drugs' effects on viral replication. Whether this effect is significant *in vivo* has

been disputed, [498] yet it is supported by several distinct lines of evidence: (1) enhanced CD4 T cell levels in PI-containing versus -sparing regimens that achieve equivalent suppressing of viral replication, [383, 385] (2) increases in CD4 T cell number despite no observable anti-viral effect, [387, 389, 499] (3) greater increases in CD8 T cell number following initiation of PI therapy than non-PI therapy, [500] and (4) dramatic decreases in CD4 T cell number following discontinuation of PI-containing therapy, even when PI therapies were unsuccessful in achieving viral suppression. [389, 395]

Together with *in vitro* observations of antiapoptotic effects, our observations argue in favour of a clinically significant effect of PI-containing therapy on CD4 T cell number compared to PI-sparing therapies. However until results from ongoing prospective trials of PI-containing versus -sparing therapies of equally high potency are available, these effects remain speculative.

Therapeutic modulation of apoptosis in the context of HIV infection remains a controversial topic. For example, inhibitors of T cell apoptosis may offer a unique opportunity to boost CD4 T cell number in patients with advanced immunodeficiency. However, inhibition of HIV infected T cell apoptosis by adenovirus E1B 19K protein [501] or caspase inhibitors [180, 206, 502] may enhance virus production and establish persistent viral infection, however a recent report suggests that inhibition of caspases may not necessarily lead to increased viral replication. [503] Thus, antiapoptotic strategies need to be considered only in addition to effective antiretroviral therapy. One potential concern which may surface in PI-mediated modulation of apoptosis in disease is

the reported hepatotoxicity of these drugs. In one study, PIs caused cholestasis and hepatocyte injury in rats and dogs. [504]

4.6 FUTURE DIRECTIONS

Follow-up experiments to identify the exact site of action of PIs within mitochondria will include the assessment of potential interactions between HIV PIs and components of the PTPC. Direct quantitation of PTPC-regulated $\Delta\psi_m$ has been greatly facilitated by the generation of proteoliposomes. [505] Conceptually, these protocols are based on the isolation and purification of target proteins from mitochondrial-rich tissues, and the subsequent integration of these proteins into lipid vesicles. When reconstituted into liposomes, certain complexes containing the hexokinase/VDAC/ANT complex confer permeability and conductance properties to the system that resembled those of the PTPC. [506] For the purposes of our experiments, ANT or whole PTPCs will be purified from rat mitochondria and inserted into lipid vesicles. The ability of PIs to inhibit vesicle opening in response to various stimuli including Vpr and Atr will be assessed. In addition, two- and three-dimensional gel electrophoresis of mitochondrial lysates incubated in the presence of radiolabeled PI should also permit identification of the PI-binding protein(s).

The findings that HIV PIs inhibit apoptosis in both infected and uninfected cells *in vitro* and *in vivo* lend credibility to the suggestion that treatment of HIV with PI-based therapy improves immunologic outcomes in comparison with PI sparing therapies. Such a finding may be of particular importance to patients with

advanced immunodeficiency. In order to fully address this question, prospective studies comparing virologically equivalent regimens that either contain or do not contain PIs are underway.

Lastly, more studies are indicated to assess the clinical utility of apoptosis modification by PIs in both animal models and in select human disease states. Given the role of apoptosis in the pathogenesis of amyotropic lateral sclerosis (ALS), sepsis and other disorders, these diseases would be candidate models to evaluate possible protective effects of PIs.

4.7 CONCLUSIONS

Recently, a variety of immunomudulatory properties have been attributed to HIV PIs. We assessed the effect of NFV on apoptosis induced by a variety of stimuli and demonstrated inhibition of apoptosis within 1-hour of treatment at doses as low as 3.5μM. The effects of NFV were (1) not protein synthesis dependant as demonstrated by experiments where inhibition was not altered by CHX pre-treatment, (2) not due to altered expression of pro-caspases 3 and 8, (3) not due to differences in transcription of a panel or apoptosis regulatory molecules, and (4) were not due to inhibition of caspase 1,3,6,7 or 8 activity. Rather, NFV inhibited mitochondrial events associated with apoptosis. By incubating cells with or without NFV, then inducing apoptosis and determining $DiOC_6(3)$ loss (as a marker of mitochondrial PTPC opening) within specific fractions (e.g. dead, apoptotic etc), we demonstrated that NFV inhibited apoptosis by virtue of inhibiting loss of mitochondrial transmembrane

permeability. Specifically, NFV (1) decreased mitochondrial cytochrome *c* release, (2) inhibited Vpr- and/or Atr-induced PTPC opening and consequent cytochrome *c* release and caspase activation, and 3) blocked forms of apoptosis that involve mitochondrial signalling (e.g. CPT treatment) but not those forms of apoptosis which do not require mitochondrial signalling (e.g. type 1 Fas pathway).

In addition, our data provide proof of the concept that PIs modify apoptosis *in vivo*, as shown in a mouse model of ischæmia and in patients taking PEP. These results have implications not only for the care of patients infected with HIV, but potentially for the therapy of other diseases associated with enhanced levels of apoptosis.

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and indinavir for previously untreated HIV-1 infection: the OzCombo1 study. Aids, 2000. **14**(9): p. 1171-80.
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APPENDIX I: Sources of chemicals

3, 3 dihexyloxacarbocyanine iodide (Molecular Probes, Eugene, OR)
^{35}S-labelled methionine (ICN Biomedicals, Inc., Irvine, CA)
Actinomycin D (Sigma-Aldrich, Oakville, ON)
Agonistic anti-Fas antibody (Clone CH11; Beckman-Coulter, Burlington, ON)
Annexin-V-FITC (BD Biosciences, Mississauga, ON)
Anti-caspase 3 antibody (Transduction Laboratories, Lexington, KY)
Anti-caspase 8 (gift from Dr. P. Krammer)
Anti-CD4-APC (Beckman-Coulter, Burlington, ON)
Anti-CD8-PerCP (Beckman-Coulter, Burlington, ON)
Anti-cytochrome *c* antibody (BD Biosciences, Mississauga, ON)
Anti-Fas antibody (BD Biosciences, Mississauga, ON)
Anti-hsp-70 antibody (BD Biosciences, Mississauga, ON)
Anti-IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Oakville, ON)
Anti-IgM antibody (Beckman-Coulter, Burlington, ON)
Anti-PCNA antibody (Santa-Cruz Biotechnologies, Santa Cruz, CA)
Aprotinin (Sigma-Aldrich, Oakville, ON)
Azidothymidine (AZT; Sigma-Aldrich, Oakville, ON)
Camptothecin (Sigma-Aldrich, Oakville, ON)
Cell Quest Software (Becton Dickinson, Mississagua, ON)
Cellular DNA Fragmentation ELISA (Roche Molecular Biochemicals, Laval, QC)
Cycloheximide (Sigma-Aldrich, Oakville, ON)
DEVD-AFC (Enzyme System Products, Livermore, CA)
DEVD-FMK (Enzyme System Products, Livermore, CA)
Dideoxycytidine (Sigma-Aldrich, Oakville, ON)
Dideoxyguanosine (Sigma-Aldrich, Oakville, ON)
Dideoxyinosine (Bristol Myers Laboratories, Morrisville, NC)
Dithiothreitol (Sigma-Aldrich, Oakville, ON)
Dounce homogenizer (Kontes Glass, Vineland, NJ)
EDTA (Sigma-Aldrich, Oakville, ON)
Enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Oakville, ON)
Fetal calf serum (GIBCO Laboratories, Burlington, Ontario)
Ficoll Paque Plus (Amersham Pharmacia Biotech, Baie d'Urfé, QC)
Glutamine (Sigma-Aldrich, Oakville, ON)
Glycerol (Sigma-Aldrich, Oakville, ON)
H9 cells (American Type Culture Collection, Rockville, MD)
HEPES (Sigma-Aldrich, Oakville, ON)
HIV PR gag-pol substrate (Bachem, King of Prussia, PA)
Hoechst 33258 (Sigma-Aldrich, Oakville, ON)
IETD-AFC (Enzyme System Products, Livermore, CA)
IETD-FMK (Enzyme System Products, Livermore, CA)
ImageQuant software (Molecular Dynamics, Amersham, Baie d'Urfe, QC)
Jurkat T cells (American Type Culture Collection, Rockville, MD)
KCl (Sigma-Aldrich, Oakville, ON)

Leupeptin (Sigma-Aldrich, Oakville, ON)
Lopinavir (Abbott Laboratories, North Chicago, IL)
Mannitol (Sigma-Aldrich, Oakville, ON)
MCA-VDQVDGW[K-DNP] (Calbiochem, San Diego, CA)
Na_3VO_4 (Sigma-Aldrich, Oakville, ON)
NaCl (Sigma-Aldrich, Oakville, ON)
NaF (Sigma-Aldrich, Oakville, ON)
NaOAc (Sigma-Aldrich, Oakville, ON)
Nelfinavir (Agouron Pharmaceuticals, San Diego, CA)
NP-40 (Sigma-Aldrich, Oakville, ON)
Nucleic acid sequence-based amplication assay (Organon Teknika, Durham, NC)
p24 ELISA assay (Mendel, Guelph, ON)
Paraformaldehyde (Sigma-Aldrich, Oakville, ON)
Penicillin (Sigma-Aldrich, Oakville, ON)
Pepstatin
Percoll (Amersham Pharmacia Biotech, Oakville, ON)
Phenylmethylsulfonyl fluoride (Sigma-Aldrich, Oakville, ON)
Phosphate (Sigma-Aldrich, Oakville, ON)
Phytohemagglutinin (Sigma-Aldrich, Oakville, ON)
Polyvinylidene difluoride membrane (Millipore, Mississauga, ON)
Protein assay (Pierce, Rockford, IL)
Proteinase K (Sigma-Aldrich, Oakville, ON)
Recombinant active caspases 1, 3, 6, and 8 (Biomol, Plymouth Meeting, PA)
Recombinant HIV-1 PR (Bachem, King of Prussia, PA)
Recombinant synthetic Vpr peptide (Genemed Systems, San Francisco, CA)
Ribonucleotide protection assay (BD Biosciences, Mississauga, ON)
Ritonavir (Abbott Laboratories, North Chicago, IL)
RNeasy mini kit (Qiagen, Mississauga, ON)
RPMI 1640 (GIBCO Laboratories, Burlington, ON)
Saquinavir (Roche Laboratories, Nutley, NJ)
Sodium deoxycholate (Sigma-Aldrich, Oakville, ON)
Stainless steel rounded-end biomedical needle (Popper and Sons Inc., New York, NY)
Streptomycin (Sigma-Aldrich, Oakville, ON)
Sucrose (Sigma-Aldrich, Oakville, ON)
Tris (Sigma-Aldrich, Oakville, ON)
Trypan Blue (GIBCO Laboratories, Burlington, ON)
TUNEL Kit (Roche Diagnostics Corp., Montreal, QC)
Tween 20 (Sigma-Aldrich, Oakville, ON)
VEID-AFC (Enzyme System Products, Livermore, CA)
WEHD-AFC (Enzyme System Products, Livermore, CA)
zVAD-FMK (Enzyme System Products, Livermore, CA)
γ-immune globulin (Sigma-Aldrich, Oakville, ON)

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EDUCATION

Ph.D. Degree: Faculty of Medicine, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 1997-2003.
Thesis Title: "A New Role for HIV Protease Inhibitors: Suppression of Apoptosis"

B.Sc. (Hon.): Faculty of Science, Department of Biochemistry, University of Ottawa, 1993-1997.
Thesis Title: "Tumour Necrosis Factor-α (TNFα), Leptin and Apoptosis in Adipocytes"

EMPLOYMENT HISTORY

DEPARTMENT OF NATIONAL DEFENCE, 28 (OTTAWA) MEDICAL COMPANY

Medical Assistant, November 1991- 2002.

- Provide advanced medical care to Canadian Forces members in a variety of clinical and field environments
- Function as a member of an emergency medical response team
- Teach Basic Trauma Life Support (BTLS) and First Aid techniques

NATIONAL RESEARCH COUNCIL OF CANADA, INSTITUTE FOR BIOLOGICAL SCIENCES, DEPARTMENT OF NEUROSCIENCES

Student Research Assistant, Summer 1997.

- Characterisation of calcium channel subtypes using RT-PCR
- Culture of various cell types
- Immunocytochemistry

UNIVERSITY OF OTTAWA, LOEB MEDICAL RESEARCH INSTITUTE, DEPARTMENT OF ENDOCRINOLOGY

Student Research Assistant, Summer 1996.

- DNA and RNA isolation and analysis
- Western analysis

AWARDS

ONTARIO HIV TREATMENT NETWORK STUDENTSHIP, 2001-2002.
UNIVERSITY OF OTTAWA Excellence Award, 2001-2002.
UNIVERSITY OF OTTAWA, Admission Scholarship, 2000-2001.
FRIENDS OF 28 MEDICAL COMPANY EDUCATION GRANT, 2001.
ONTARIO GRADUATE SCHOLARSHIP IN SCIENCES AND TECHNOLOGY, 2000-2001.
UNIVERSITY OF OTTAWA, Excellence Award, 1999-2000.
UNIVERSITY OF OTTAWA, Excellence Award, 1998-1999.
ONTARIO HIV TREATMENT NETWORK STUDENTSHIP, 1998-2000.
UNIVERSITY OF OTTAWA, Admission Scholarship, 1997-1998.

UNIVERSITY OF OTTAWA, Admission Scholarship, 1993-1994.
UNIVERSITY OF OTTAWA, Bourse pour études en français, 1993-1994.

PUBLICATIONS

Phenix, B.N., Badley, A.D., (2002) Influence of mitochondrial control of apoptosis on the pathogenesis, complications and treatment of HIV infection. Biochimie, 84(2-3):251-64.

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Badley, A.D., Parato, K., Cameron, D.W., Kravcik, S., **Phenix, B.N.**, Ashby, D., Kumar, A., Lynch, D.H., Tschopp, J., Angel, J.B., (1999) Dynamic correlation of apoptosis and immune activation during treatment of HIV infection. Cell Death and Differentiation, 6:420-432.

Shillabeer, G., **Phenix, B.N.**, Tibbo, E., Lamprecht, C., Lau, D.C.W., (1997) Rat preadipocytes differentiated in culture and isolated mature adipocytes contain the necessary machinery for apoptosis. Obesity Research, 15(Suppl.1):345-346.

Shillabeer, G., Tibbo, E., Lamprecht, C., **Phenix, B.N.**, Lau, D.C.W., (1997) Interleukin-1β-converting enzyme (ICE) expression and apoptosis in adipocytes. Clinical Investigative Medicine, 20(Suppl.18):278-279.

PRESENTATIONS

Phenix, B.N., Lum, J.J., Nie, Z., Sanchez-Dardon, J., Badley, A.D., HIV protease inhibitors possess antiapoptotic effects that are mediated at the level of mitochondrial transmembrane potential loss. Oral presentation at the Tenth Annual Canadian Conference of HIV/AIDS Research, Toronto, Ontario, June 2001.

Pilon, A.A., Lum, J.J., Sanchez-Dardon, J., **Phenix, B.N.**, Douglas, R., Badley, A.D., Induction of apoptosis by an HIV-1 non-nucleoside reverse transcriptase inhibitor. Poster presentation at the Keystone Symposia: HIV pathogenesis, Keystone, Colorado, April 2001.

Lum, J.J., Pilon, A.A., Sanchez-Dardon, J., **Phenix, B.N.**, Kim, J.E., Mihowich, J., Jamison, K., Hawley-Foss, N., Lynch, D.H., Badley, A.D., Induction of cell death in HIV infected macrophages and latently infected memory CD4 T cells by TRAIL/APO2L. Poster presentation at the Eighth Conference of Retroviruses and Opportunistic Infections, Chicago, Illinois, February 2001.

Phenix, B.N., Mbisa, G., Lynch, D.H., Badley, A.D., Characterisation of monocyte-derived macrophage resistance to apoptosis. Oral presentation at the Ninth Annual Canadian Conference of HIV/AIDS Research, Montreal, Québec, May 2000.

Phenix, B.N., Beckett, B., Alam, A., Sekaly, R.P., Krammer, P.H., Lynch, D.H., Badley, A.D., HIV protease induces apoptosis of HIV infected T cells through activation of caspase 8. Oral presentation at the Eighth Annual Canadian Conference of HIV/AIDS Research, Victoria, British Columbia, May 1999.

Phenix, B.N., Angel, J.B., Mandy, F., Kravcik, S., Parato, K., Chambers, K.A., Gallicano, K., Hawley-Foss, N., Cassol, S., Cameron, D.W., Badley, A.D., Decreased HIV-associated T cell apoptosis by HIV protease inhibitors. Oral presentation at the Sixth Conference on Retroviruses and Opportunistic Infections, Chicago, Illinois, February 1999.

Shillabeer, G., **Phenix, B.N.**, Tibbo, E., Lamprecht, C., Lau, D.C.W., Rat preadipocytes differentiated in culture and isolated mature adipocytes contain the necessary machinery for apoptosis. Poster presentation at the Canadian Diabetes Association Annual Meeting, 1997.

PROFESSIONAL MEMBERSHIPS

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Cell Death Society
Let's Talk Science

CONTRIBUTION OF COLLABORATORS

Measurement of p24 antigen concentration in culture supernatants (Section 3.1.2) was done by Dr. Kelley Chambers.

The levels of HIV-1 RNA in tissue culture supernatants (Section 3.1.2) were quantified by Dr. Sharon Cassol.

Cytochrome *c* release assays and Western analyses (Section 3.2.4) were performed by Dr. Zilin Nie.

Vpr-mediated Jurkat T cell apoptosis experiments and Western analyses (Section 3.2.5) were performed by Dr. Julian Lum.

PI concentration in plasma and brain tissue (Section 3.3.1) was determined by Mr. Germain Carignan in collaboration with Dr. Rolf Van Heejwick.

Transient forebrain ischæmia experiments (Section 3.3.2) were performed in collaboration with Mrs. Li Dong and Dr. André Douen.

Experiments with samples from patients undergoing PEP therapy (Section 3.3.3) were performed in collaboration with Dr. Curtis Cooper.