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Christina Gavegnano Date

Multi-mechanism antiretroviral approach targeting macrophage derived viral sanctuaries

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James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
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Molecular Systems Pharmacology
April 2012

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Abstract

Multi-mechanism antiretroviral approach targeting macrophage derived viral sanctuaries

By Christina Gavegnano

Macrophages are a significant viral reservoir for HIV-1, and eradication cannot occur without elimination of virus from these cells. Current HAART cannot achieve eradication, and understanding how HIV-1 ensconces current therapies and hides in viral reservoirs is necessary to achieve systemic eradication. The **goal** of this thesis is to understand these mechanisms by assessing the following parameters in activated and resting macrophages *versus* lymphocytes: 1) antiviral potency and intracellular concentrations of nucleoside analogs, 2) potency of clinically relevant nucleoside analogs, protease inhibitors (PI), and Raltegravir (RAL; integrase inhibitor), 3) levels/ratios of dNTP/rNTP, 4) targeted inhibition of pro-HIV pathways. Additionally, drug-based methods to systemically eliminate macrophages will be explored, with the goal of employing the optimized therapy in rhesus macaques to better understand the role of macrophages in HIV-1 infection. The **hypothesis** of this thesis is 1) nucleoside analogs, PI, and RAL may not efficiently eliminate HIV-1 in macrophages, 2) different levels/ratios of dNTP/rNTP in macrophages *versus* lymphocytes could alter potency of nucleoside analogs, and 3) targeted inhibition of pro-HIV pathways will markedly reduce HIV-1 replication in macrophages and lymphocytes. AZT, TDF, and 3TC display the most favorable intracellular concentration/potency profile, and should be considered for all cocktails in treatment of HIV-1 infection. Potency of HIV-1 PI is not altered by activation state of lymphocytes or macrophages, and should be considered for cocktails aimed at targeting HIV-1 replication across both cell types. Jak inhibitors Tofacitinib (RS-1294) and Jakafi (RS-1374) are potent, safe, sub-micromolar inhibitors of HIV-1 replication and should be considered for combination studies in both lymphocytes and macrophages. Ratios of rNTP:dNTP are similar in macrophages, but not lymphocytes, conferring preferential incorporation of rNTP into the growing viral DNA strand in macrophages only, and ribonucleosides represent a novel class of antiretroviral agents that specifically targets HIV-1 replication in macrophages. Fosamax and Boniva were identified as specific, potent, sub-micromolar inducers of cell death in macrophages. These data demonstrate that cocktails containing a PI + AZT, TDF, or 3TC, + ribonucleoside inhibitor + Jak inhibitor + Fosamax or Boniva may represent a multifaceted approach to target HIV-1 replication not only in lymphocytes, but also macrophages.

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Abbreviations

HIV-1 (Human Immunodeficiency Virus Type 1)

WHO (World Health Organization)

NRTI (nucleoside reverse transcriptase inhibitor)

PR (HIV-1 protease)

PI (protease inhibitor)

IN (integrase)

RT (reverse transcriptase)

Env (HIV-1 envelope)

HAART (highly active antiretroviral therapy)

AZT (zidovidine)

ABC (Abacavir)

CBV (Carbavir)

(-)-FTC (emtricitabine)

3TC (lamivudine)

TDF (tenofovir disoproxil fumarate)

TFV (tenofovir)

DXG [(-)-beta-D-dioxolane-guanine; Amdoxovir]

ATZ (Atazanavir)

APV (Amprenavir)

DRV (Darunavir)

LPV (Lopinavir)

NFV (Nelfinavir)

SQV (Saquinavir)

TPV (Tipranavir)

RAL (Raltegravir)

TBI (total body irradiation)

ATG (anti-thymocyte globulin)

RS-1294 (Pfizer's Tofacitinib)

RS-1374 (Incyte's Jakafi)

RS-1531 (Astra Zeneca's AZD1480)

RS-1532 (Eli Lilly's LY2784544)

RS-1533 (YM Bioscience's CYT387)

Jak (Janus activating kinase)

STAT (signal transducer and activator of transcription)

Chapter 1: General Introduction

Overall goal of thesis

The overall goal of this thesis is to identify mechanisms to eliminate HIV-1 from macrophages. Each subsection of this thesis will detail why eliminating virus from macrophages is sentinel in the global goal of systemic eradication of HIV-1.

Human immunodeficiency virus type 1 (HIV-1)

HIV-1 is a retrovirus belonging to the genus Lentivirus. Infections by lentiviruses are characterized by remarkably complex interactions with the host and a chronic course of disease. Common features of disease include long and variable incubation periods, persistent viral replication, neurocognitive dysfunction, and destruction of CD4 expressing cells of both the myeloid and lymphoid lineage. All lentiviruses exhibit a common morphogenesis and morphology, a tropism for macrophages, extensive genetic and antigenic variability, and the presence of additional regulatory genes not found in other groups of retroviruses. Lentiviruses have been isolated from several animal species including sheep, goats, horses, cattle, cats, monkeys, and humans. HIV-1 and HIV-2 are related viruses with different pathogenesis in humans. Since its identification in 1981, HIV, the causative agent of Acquired Immune Deficiency Syndrome (AIDS), has escalated to a global pandemic. An estimated 34 million people are living with HIV-1, and in 2010, 2.7 million new infections were counted, along with 1.8 million deaths from AIDS-related illnesses (Table 1.1) [1, 2]. Of these infected individuals, nearly 3.4 million are children under the age of 18. There are more than 1.2 million people infected living with HIV in the US, and 39 % of new infections in the US are within the pediatric population. Persons with HIV are at increased risk for TB disease, and those with TB have a high risk for death. The same applies to persons co-infected with hepatitis C virus (HCV).

Number of people living with HIV-1/AIDS in 2010	Total: 34.0 million (31.6-35.2 million) Adults: 31.4 million (28.4-31.5 million) Women: 16.8 million (15.8-17.6 million) Children (< 15 years): 3.4 million (3.0-3.8 million)
People newly infected with HIV-1 in 2010	Total: 2.7 million (2.4-2.9 million) Adults: 2.3 million (2.102.5 million) Children (< 15 years): 390,000 (340,000-450,000)
AIDS deaths in 2010	Total: 1.8 million (1.6-1.9 million) Adults: 1.5 million (1.4-1.6 million) Children (< 15 years): 250,000 (220,000-290,000)

Table 1.1: Global summary of the AIDS epidemic in 2010. Groups and subtypes are classified according to geographical location. HIV-1. Group M (major) contains subtypes A-K, and is most prevalent worldwide. The most common subtypes in group M are subtype B, which predominates in North America, Europe, parts of South America and India, subtype C, which predominates in sub-Saharan Africa, and subtype E which predominates in Southeastern Asia, while groups N (new) and O (outliner), have been identified in Africa and Eastern Europe [1, 2].

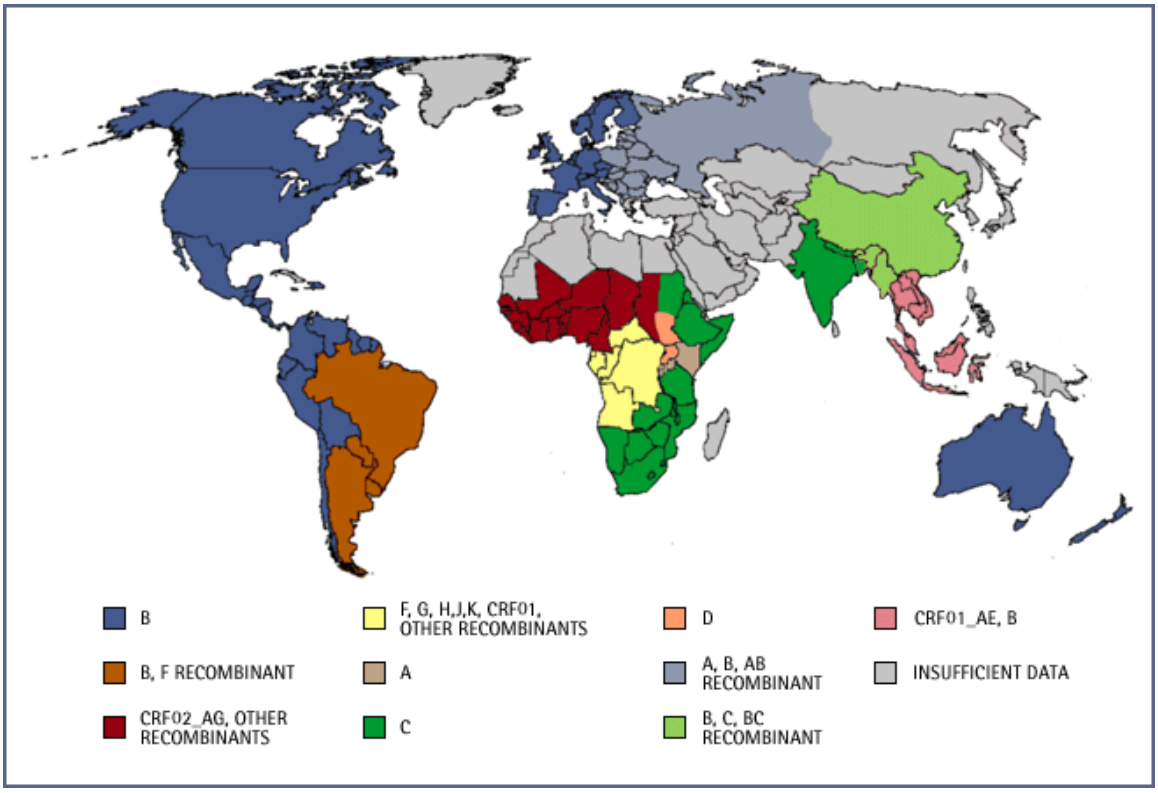


Figure 1.1: Global distribution of HIV-1 clades and subtypes. [1, 2].

HIV-1 genomic organization

HIV-1 is a sense strand RNA virus which packages 2 copies of a positive-sense RNA strand genome of approximately 10,000 nucleotides (10 kb) into each virion. The genome is flanked by two identical long terminal repeats (LTR) and contains the three major genes *gag, pol,* and *env* as well as accessory proteins Vif, Vpr, Vpu, Tat, Rev, and Nef. The structural proteins matrix (p17MA), capsid (p24CA), nucleocapsid (p7NC), and p6, are encoded in *gag*. Enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) are encoded in *pol*. The *env* gene encodes the envelope glycoproteins gp120 and gp41, which are required for viral attachment and entry [3] (Figure 1.2).

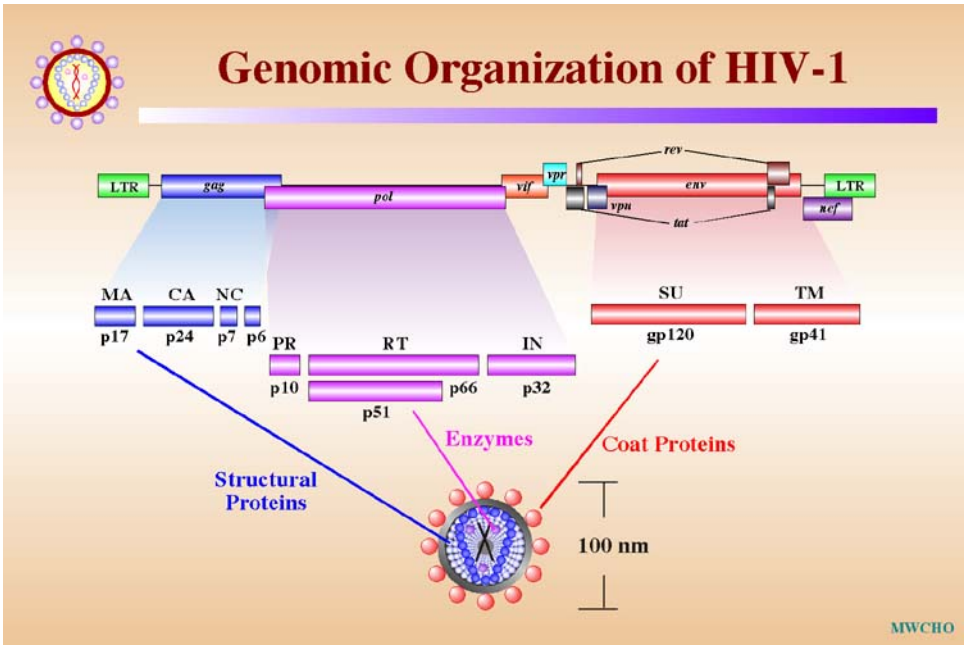


Figure 1.2: HIV-1 genomic organization. Two identical long terminal repeats flank the viral genome, which includes the main genes *gag*, *pol*, and *env* and accessory genes *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* [4].

HIV-1 replication cycle

The first step in the HIV-1 replication cycle is binding of HIV-1 gp120 envelope glycoprotein to the CD4 receptor on the surface of the host cell, which confers a conformation change in gp120, exposing coreceptor-binding sites, which allows for engagement of cellular chemokine coreceptors CXCR4 or CCR5 by gp120.

The next step is fusion, wherein the viral envelope fuses with the lipid membrane of the target cell, releasing the viral core into cytoplasm of the host cell. Viral RNA is then reverse transcribed into double-stranded DNA via virally encoded HIV-1 RT enzyme, and the DNA translocates to the nucleus and integrates into the host genome via virally encoded IN. This is followed by transcription of the RNA and translation of gene products, assembly at the plasma membrane, budding of the newly formed virion, and protease processing resulting in a mature, infectious virion [3] (Figure 1.3).

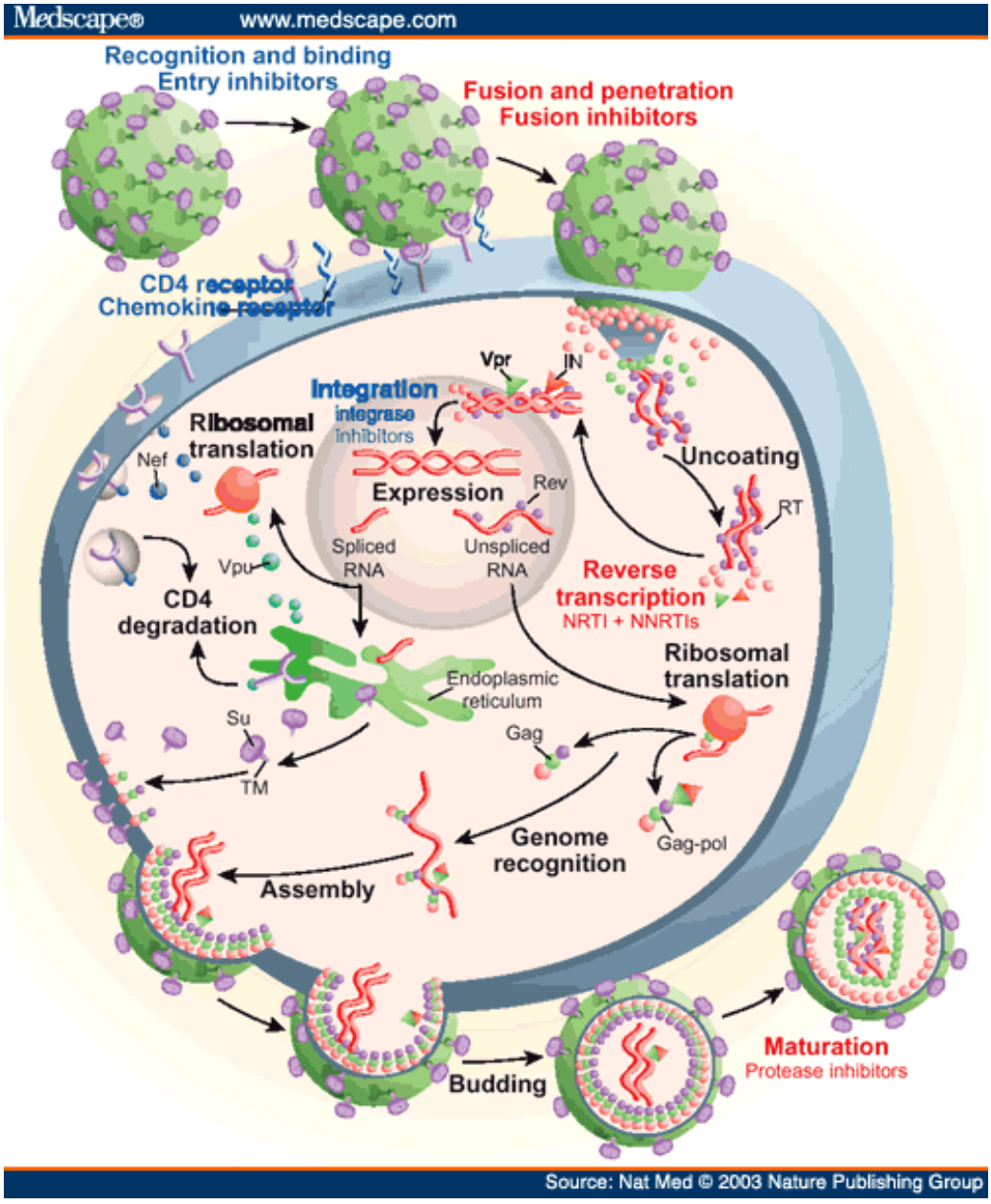


Figure 1.3: HIV-1 replication cycle. HIV-1 binds to the CD4 receptor, followed by binding to coreceptor CXCR4 or CCR5, fusion of virus with the host cell membrane, and entry of the virus into the host cell. Viral RNA is then reverse transcribed by virally encoded HIV-1 RT, and viral DNA is integrated into the host cell genome by HIV-1 IN. This is followed by transcription and translation of viral gene products, processing by HIV PR to form a mature, fully replication competent virus, budding, and release. Targets of existing therapies are indicated in red or blue (entry/fusion, reverse transcription, integration, and protease processing) [5].

Targets of HIV-1 Infection

HIV-1 permissive cells express CD4 and one or both of the appropriate chemokine coreceptor, primarily CXCR4 or CCR5. $CD4^+/CCR5^+$ cells include macrophages (t ½ weeks-years) and a small subset of activated memory T lymphocytes ($t_{1/2}$ upon primary exposure to antigen = years, $t_{1/2}$ upon secondary exposure to antigen = days) while CXCR4 is expressed by most $CD4^+$ T lymphocytes, transformed lymphocytic or monocytic lineages, and macrophages. R5 viruses are consistently macrophage tropic (M-tropic) and display M-R5 phenotype, while X4 viruses display heterogeneous phenotypes. The majority of primary X4 viruses replicate in both macrophages and T cell lines, use CXCR4 either alone or in combination with CCR5 and are dual (D)-tropic, whereas X4 viruses which do not exhibit tropism for macrophages, but maintain ability to replicate in T cell lines, are T-X4 phenotypic (Table 1.2). R5 viruses predominate early in infection, whereas in ~ 50 % of patients, X4 viruses emerge and are associated with rapid progression to AIDS [6-8].

Tropism and coreceptor designation	Target cell coreceptor use	Target cell tropism
M-R5	CCR5	Macrophages $CD4^+$ T lymphocytes
D-R5X4	CXCR4, CCR5	Macrophages $CD4^+$ T lymphocytes
DX4	CXCR4	Macrophages $CD4^+$ T lymphocytes
TX4	CXCR4	$CD4^+$ T lymphocytes

Table 1.2: Coreceptor usage and target cell tropism of HIV-1. [6, 9]

Antiretroviral therapy

Entry inhibitors

Currently, only two anti-HIV drugs are approved by the US Federal Drug Administration (FDA) that inhibit entry of HIV-1 into host cells. Enfuvirtide (Fuzeon®; ENF, T-20) is a peptide derived from a repeat sequence of the transmembrane portion of HIV-1 envelope, gp41, and inhibits the hairpin formation necessary for virus–host cell fusion to occur. Maraviroc (Selzentry®/Celsentri®; UK-427, 857) is a small molecule that inhibits viral entry by binding to the CCR5 coreceptor and inhibiting the receptor–coreceptor viral envelope interaction required for HIV-1 entry into the host cell. [10].

Reverse transcriptase inhibitors

Two classes of reverse transcriptase (RT) inhibitors exist for treatment of HIV-1 infection: nucleoside RT inhibitors (NRTI) and nonnucleoside RT inhibitors (NNRTIs). Nucleoside analogs have a well-established regulatory pathway, with eight FDA approved drugs for the treatment of HIV-1 infection and multiple drugs in various stages of clinical development. NNRTIs also have a long history of FDA approval, with four drugs currently approved. Nucleoside analogs present distinct clinical advantages: low plasma protein binding, sustained antiviral response when a dose is missed (because of their long intracellular half-life) and relative ease of chemical manufacture [11, 12]. By 2011, the eight approved HIV-1 nucleoside analogs were zidovudine (Retrovir®; ZDV, AZT), didanosine (Videx®; ddI), zalcitabine (Hivid®; ddC), stavudine (Zerit®; d4T), abacavir sulfate (Ziagen®; ABC), lamivudine (Epivir®; 3TC), emtricitabine (Emtriva®; (-)-FTC) and tenofovir disoproxil fumarate (Viread®; TDF).

The target of nucleoside analogs in HIV-1 infection is the action of virally encoded RT. This enzyme is active early in the viral replication cycle and converts the

genetic information of the virus, which is stored as RNA, into DNA by reverse transcription, a process necessary for continued viral replication [11, 12]. nucleoside analogs are chiral small molecules that mimic natural nucleotides and require intracellular phosphorylation to become functionally active against HIV-1 RT. In the triphosphate form (Figure 1.4), nucleoside analogs compete with one of the four naturally occurring dNTP, namely, dCTP, dTTP, dATP or dGTP, for binding and DNA chain elongation near the active site of HIV-1 RT [12]. As most nucleoside analogs lack a 3'-hydroxyl terminus, incorporation of the analogue into the growing DNA strand results in termination of the DNA strand and the next phosphodiester bond is not formed. Because of these factors, both the concentration of cellular triphosphorylated nucleoside analog and the levels of cellular dNTP pools play a key role in the efficacy of the nucleoside analogs [13, 14].

By 2011, four NNRTIs were approved by the FDA: etravirine (Intelence®; TMC 125), delavirdine (Rescriptor®, DLV), efavirenz (Sustiva®, Stocrin; EFV) and nevirapine (Viramune®; NVP). NNRTIs are chemically distinct from nucleoside analogs and inhibit HIV-1 RT by interacting with binding pockets of HIV-1 RT, inhibiting its enzymatic activity by causing conformational changes at or near the active site [15].

Integrase inhibitors

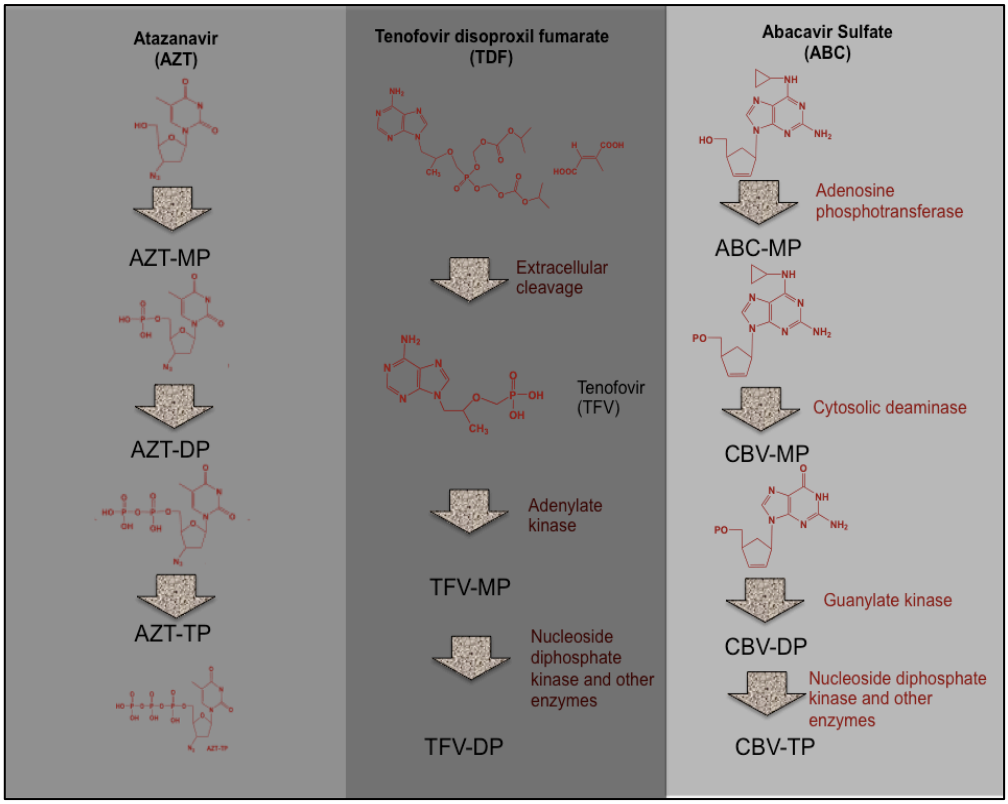
HIV-1 integrase (IN) presents a novel and highly selective target for anti-HIV therapeutics since there is no cellular counterpart. RAL (Isentress®; MK-0518,) received FDA approval in October 2007 and acts to inhibit integration of HIV-1 proviral genome into host cell DNA [16]. It is widely used with Truvada, a fixed dose combination of (-)-FTC and TDF.

Protease inhibitors

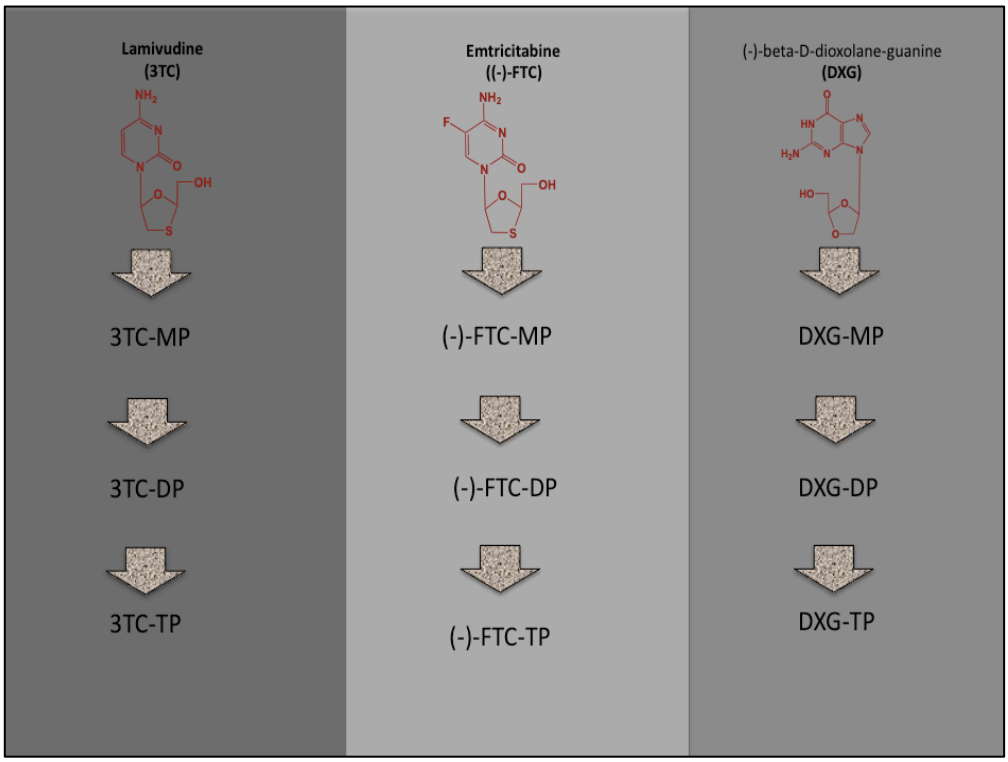
HIV-1 protease inhibitors (PIs) represent a class of ART with long-standing FDA approval. To date, 10 PIs have received FDA approval: Amprenavir (Agenearase®; APV), Tipranavir (Aptivus®; TPV), Indinavir (Crixivan®; IDV), Saquinavir (Invirase®; SQV), Lopinavir/ritonavir (Kaletra®, Aluvia; LPV/r), fosamprenavir (Lexiva®, Telzir; FPV), rRtonavir (Norvir®; RTV), Darunavir (Prezista®; DRV), Atazanavir (Reyataz®; ATZ) and Nelfinavir (Viracept®; NFV). PR cleaves HIV-1 Gag and Gag–Pol (usually inside the virion extracellularly), resulting in a mature, infectious virion. PIs compete for binding in the active site with the natural substrate. Once bound, PIs cannot usually be cleaved, resulting in inactivation of the enzyme [17]. Protease inhibitors are also highly plasma protein-bound [18, 19], primarily to α-1-acid glycoprotein, and once protein bound, PI are no longer functionally available to inhibit HIV-1 replication. Due to this caveat, it is possible that differing levels of plasma proteins across HIV-infected subjects could in theory alter antiviral potency of PI, although to date data clearly defining this factor is not well understood.

The hallmark of a PI is its ability to inhibit HIV-1 replication in macrophages (and lymphocytes), and their ability to inhibit replication in chronically infected lymphocytes. The EC_{50} of multiple PIs have been observed against both acute and chronic HIV-1 infection in lymphocytes, but had only been partially defined in macrophages [20, 21], and the relative impact of activation state of macrophages on potency of PI in macrophages was previously undefined. Understanding the potency of PI against HIV-1 infection in macrophages at various states of activation, all of which represent multiple microenvironments *in vivo*, could provide key information about the ability of PI to inhibit viral replication in humans.

A.


Atazanavir
(AZT)
AZT-MP
AZT-DP
AZT-TP
Tenofovir disoproxil fumarate
(TDF)
Extracellular
cleavage
Tenofovir
(TFV)
Adenylate
kinase
TFV-MP
Nucleoside
diphosphate
kinase and other
enzymes
TFV-DP
Abacavir Sulfate
(ABC)
Adenosine
phosphotransferase
ABC-MP
Cytosolic deaminase
CBV-MP
Guanylate kinase
CBV-DP
Nucleoside diphosphate
kinase and other
enzymes
CBV-TP

B.


Lamivudine
(3TC)
3TC-MP
3TC-DP
3TC-TP
Emtricitabine
((-)-FTC)
(-)-FTC-MP
(-)-FTC-DP
(-)-FTC-TP
(-)-beta-D-dioxolane-guanine
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C.

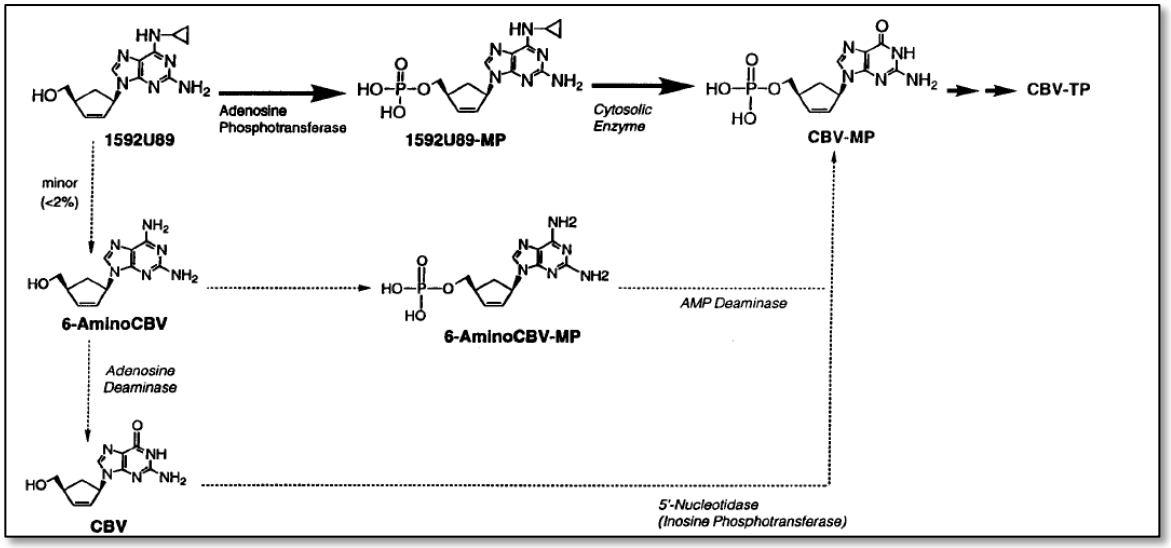


Figure 1.4: Intracellular metabolism of HIV-1 RT inhibitors. Nucleoside analogs are triphosphorylated to their functionally active form by various intracellular kinases (A, B). Abacavir (ABC) undergoes a complex metabolism to its active metabolite, carbovir triphosphate (CBV-TP) (C).

Obstacles in achieving eradication

Eradication of HIV-1 has currently not been achieved, although specific obstacles have been identified as sentinel in achieving systemic eradication. To date, these obstacles are multi-faceted, and occur concomitantly within various microenvironments in various tissues and organs. More specifically, current therapy cannot eliminate virus from all tissues and reservoirs, and is not capable of achieving either systemic eradication or a functional cure to HIV-1 infection. To achieve eradication, the following factors, which are currently unmet, must be satiated: 1) render uninfected HIV-1 target cells "non-susceptible" to HIV-1 infection, 2) mechanism to prevent "re-establishment" of infection in long-lived, latently infected "memory" lymphocytes, 3) mechanism to eliminate virus from long lived cells that harbor virus for years (macrophages, memory lymphocytes), 4) reduce or eliminate emergence of drug-resistant HIV-1.

Mechanisms to render uninfected HIV-1 target cells "non-susceptible to HIV-1 infection

HIV-1 infection cannot occur in resting lymphocytes, and cells must be activated to support productive viral replication. A delicate balance exists wherein both lymphocytes and macrophages must be activated to some extent in order to confer functional adaptive and innate immunity, therefore complete aberration of systemic activation does not represent a feasible *in vivo* approach to inhibit HIV-1 replication. Interestingly, chronic HIV-1 infection is hallmarked by a pro-inflammatory cytokine milieu that orchestrates a global state of hyperactivation, which serves to render uninfected cells supportive of productive viral replication while concomitantly conferring an increase in productive viral replication in already infected cells. Therefore, it follows that an "in-between" state of activation could represent a potential clinical target wherein cells are rendered "pseudo-resting", such that they are activated enough to perform normal

immunologic function, but resting enough such that they are not supportive of productive viral replication. To date, this "middle ground" is not only understudied, but highly elusive, as it requires an exquisitely delicate balance in order to satiate both immunologic function and inhibition of viral replication.

Latency

Establishment of latent HIV-1 occurs early in infection, and can be found in two major cell types: memory lymphocytes and macrophages. Both cell types have long half lives ($t_{1/2}$ = years), and harbor integrated proviral genome for years, with either absent or ongoing low-level replication, eventually undergoing reactivation, which repopulates the systemic periphery with virus [22, 23].

With respect to macrophages, suboptimal levels of drug delivered to these cells could confer repopulation of the periphery with drug resistant HIV-1, while simultaneously reseeding macrophage and memory lymphocyte derived latent viral reservoirs. The role of macrophages relative to latency and viral reservoirs is problematic in strategies aimed at eradication, as macrophages are ubiquitous systemically (Figure 1.7), and drug delivery to adequate levels to eliminate viral replication in macrophages across the CNS, gut, genital tract, lymph nodes, and various other tissues is significantly more difficult than delivery to the periphery, which is the primary mechanism to target virus in lymphocytes. Additionally, multiple reports have demonstrated the *in vivo* relevance of macrophages to HIV infection [22, 24, 25], with recent work from Silvestri, *et al,* confirming the presence of SIV in macrophages in the gut of rhesus macaques systemically depleted of $CD4^+$ T lymphocytes [25]. These data clearly define macrophages as a target that must be considered for therapies aimed at systemic eradication, and underscore the fact that eradication of HIV-1 cannot occur without elimination of virus from latent viral reservoirs, such as macrophages (reviewed

in [26]).

Resistance

Resistance to HIV-1 occurs as a function of selective pressure from the drug, allowing for emergence of drug resistant variants. Emergence of drug resistant HIV-1 often confers cross-resistance to other drugs, reducing the pool of available therapies to treat infected subjects, eventually severely limiting treatment options and available treatment regimens.

Suboptimal levels of drug delivered to various HIV-1 target cells, most notably macrophages [27], may also provide an environment for emergence of drug resistant HIV-1. As macrophages are ubiquitous systemically, are long-lived cells ($t_{1/2}$ = years) [22, 23], and are located in every tissue and organ including the brain and CNS, ability to deliver adequate levels of drug to eliminate infection in these cells is critical to achieve eradication of HIV-1.

Pro-HIV events during HIV-1 infection

Dysregulation of the inflammatory response and activation state of HIV-1 target cells

HIV-infected cells produce a pro-inflammatory cytokine milieu, dominated by production of TNF-α, IL-1β, IL-10, IL-12, IL-15, and IL-18. Together, these cytokines function in an autocrine and paracrine manner to render infected cells capable of producing significantly more robust viral replication, while concomitantly conferring activation of uninfected bystander cells, rendering them significantly more susceptible to HIV-1 infection. A switch to a pro-inflammatory cytokine milieu is associated with an increase in viral loads, decrease in CD4 T cell counts, and rapid progression to AIDS, underscoring the importance of modulation of cytokines in systemic progression to AIDS

and death [28-31]. Therefore, a therapy aimed at viral eradication must consider reduction of the hyper-inflammatory state that is conferred as a function of HIV-1 infection systemically, which could serve to reduce the virus produced per infected cell, while simultaneously reducing the ability of proinflammatory cytokines to prime uninfected cells for infection by HIV-1.

Pro-HIV pathways

A major reason that current therapy cannot satiate these unmet goals is that all approved therapies target steps in the viral replication cycle, which preludes their mechanism of action from targeting virus in latently infected cells, or from rendering uninfected bystander cells non supportive of productive viral replication. These shortcomings, and the mechanism by which they could be addressed, necessitates design of therapies with novel mechanisms of actions which target various cellular factors that may be key to allowing cells to support HIV-1 replication. Various signaling cascades have been identified as supportive or "pro-HIV" pathways, which are upregulated in HIV-infected but not uninfected cells, and which orchestrate a complex series of intracellular signaling cascades that render both HIV-infected cells supportive of viral replication while concomitantly promoting a systemic "pro-HIV" environment wherein bystander cells are more susceptible to infection. Targeted inhibition of these pathways could lead to significantly diminished viral replication kinetics within infected cells, while simultaneously rendering uninfected bystander cells "non supportive" of productive viral replication by down-regulating essential pathways that confer this susceptibility.

The most notable "pro-HIV" pathways that have been identified include NFκB, p38 MAPK, NFAT, PI3K/Akt, and lck (Figure 1.5). Additionally, as a pro-inflammatory, hyper-activated state confers a pro-HIV environment, and the Janus activating kinase

signal transducer and activator of transcription (Jak-STAT) pathway is a known modulator of this state, most notably in cancers and in autoimmune inflammatory disorders such as rheumatoid arthritis, inhibitors of the Jak-STAT signaling pathway were also selected for potency and toxicity studies.

The Jak-STAT pathway is activated by multiple triggers, including binding of pro-inflammatory cytokines to chemokine receptors, or by engagement of CD4, CCR5, or CXCR4 [32]. In mammals, there are four Jaks (Jak1, Jak2, Jak3, and Tyk2), and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5, STAT5b, and STAT6), each of which are separated by slight differences in homology. Upon engagement of CD4 or chemokine coreceptor, Jaks, which are localized to the internalized, cytoplasmic portion of the receptor, autophosphorylate at the JH1 domain located at the C terminus of the protein (Figure 1.6 B). Upon Jak phosphorylation, STATs, which also remain cytoplasmic until activation via phosphorylation, are recruited to the phosphorylated Jaks, which in turn phosphorylate STATs at the SH2 domain (Figure 1.6 A, C). Activated, phosphorylated STATs then translocate to the nucleus where they bind to DNA and promote transcription of a variety of pro-HIV gene products (Figure 1.5, 1.6 A). Inhibition of the Jak-STAT pathway by Jak inhibitors is conferred by inhibition of Jak phosphorylation at the JH1 domain (Figure 1.6 C) [32].

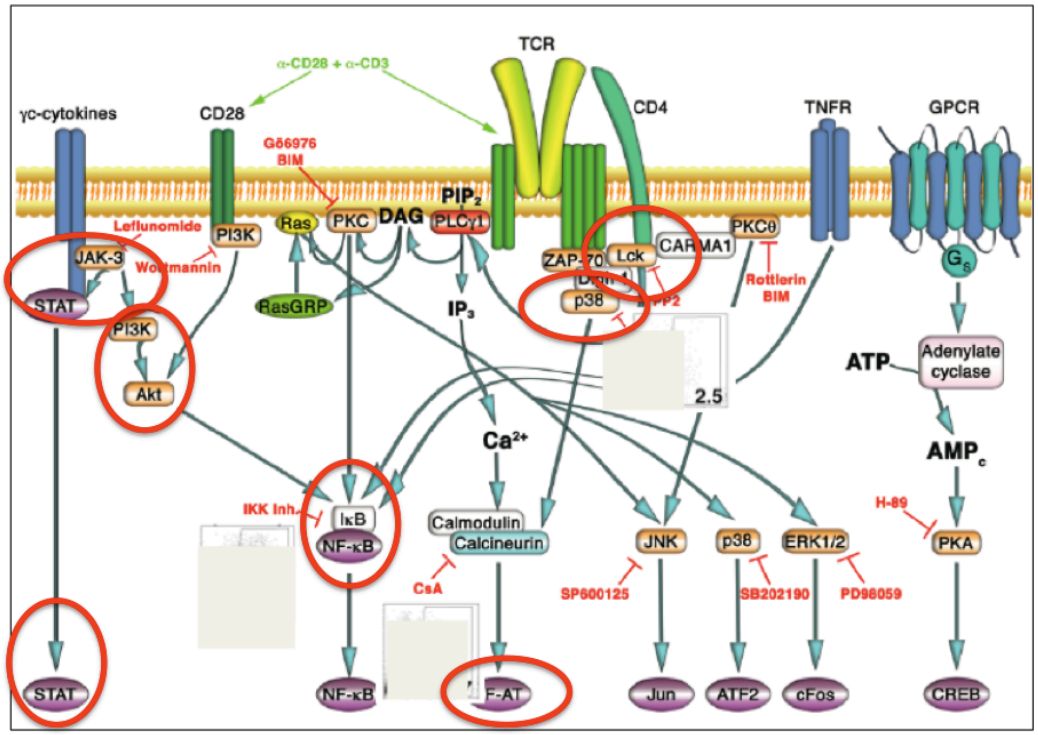


Figure 1.5: Pro-HIV signaling cascades. Various components of the Jak-STAT signaling pathway, as well as Akt, NF-κB, NFAT, p38 MAPK, and lck have all been implicated as modulators of a hyper-activated, pro-HIV environment, and are up-regulated or activated upon HIV infection (red circles). Figure kindly provided by Dr. Vicente Planelles, University of Utah.

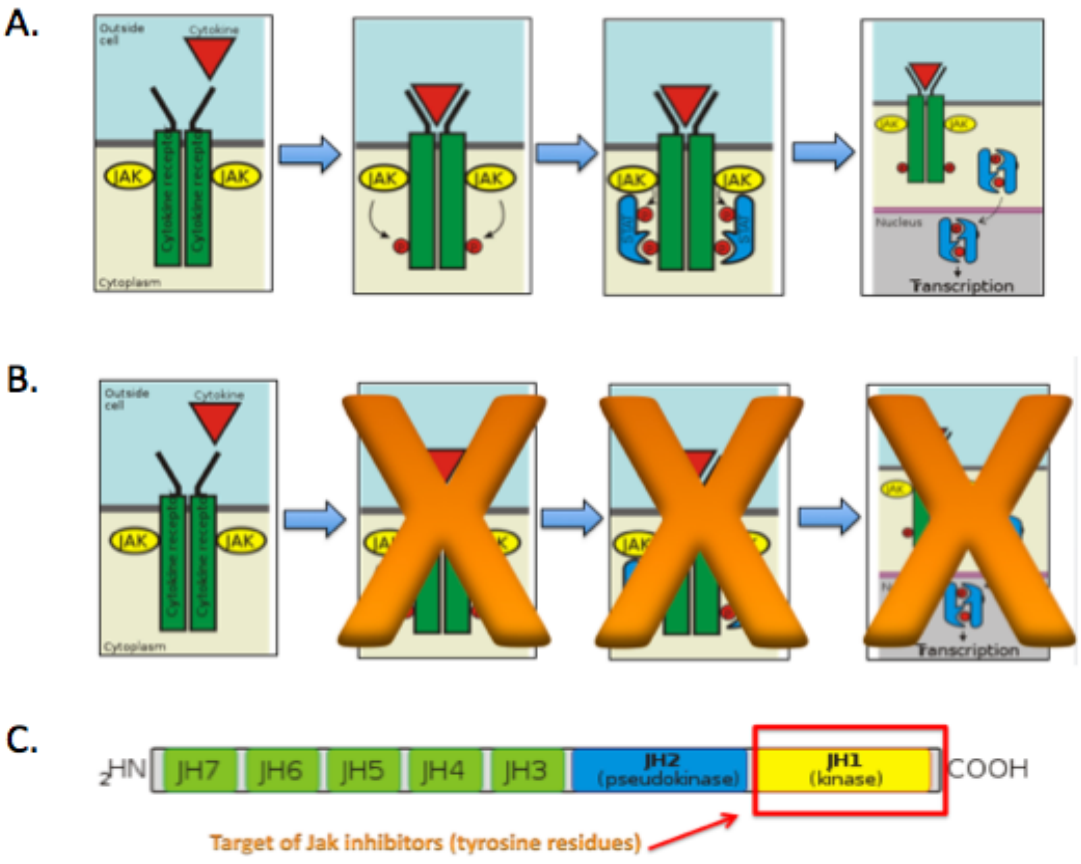


Figure 1.6: The Jak-STAT pathway, mechanism of Jak inhibition, and Jak genomic organization. Upon engagement of CD4 or chemokine coreceptor, Jaks autophosphorylate. Upon Jak phosphorylation, STATs are recruited to the phosphorylated Jaks, which in turn phosphorylate STATs. Activated, phosphorylated STATs translocate to the nucleus where they bind to DNA and promote transcription of a variety of pro-HIV gene products (A). Inhibition of Jak-STAT signaling by Jak inhibitors Tofacitinib or Jakafi occurs by inhibition of Jak phosphorylation at the C terminus JH1 domain (C), which in turn prevents activation of the Jak-STAT signaling cascade (B).

Activation state of macrophages and relationship to HIV-1 infection

The ubiquitous nature of macrophages systemically dictates that they comprise multiple and often independent microenvironments, all of which are subject to a variety of stimuli that can confer a state of resting, classical activation, or alternative activation. Resting macrophages are found in tissues and organs wherein no active inflammatory state, pathogen, or disease is present, and where little active phagocytosis is required. As a function of this resting state, there is little or absent expression of autocrine and paracrine cytokines, which in turn prevents the cell from auto-activation via receptor engagement of pro-inflammatory cytokines or m-CSF, a pro-HIV and pro-activation macrophage produced factor (reviewed in [26]).

Activated macrophages can be activated to result in a “classical” or “alternative” activation. Classical activation of macrophages is conferred by exposure to Type 1 or “Th1” type cytokine responses, which are hallmarked by increased levels or interferon gamma (IFN-γ), or by bacterial epitopes, such as that found on lipopolysaccharide. This results in “classical” activation of the macrophage, which results in production of ligands for endogenous receptors, including MIP-1α, MIP-1β, and RANTES (ligands for CCR5), and pro-inflammatory cytokines such as TNF-α and IL-1β. These factors orchestrate a multi-pronged approach wherein macrophages auto-activate and activate bystander macrophages to confer a pro-apoptotic and pro-phagocytic environment, recruit macrophages and other phagocytic cells, and prime systemic immunity for the adaptive immune response [33].

Alternatively activated macrophages are conferred by exposure to IL-4, IL-10, and IL-13, which are Type 2 or “th2” cytokines. Exposure to these cytokines results in production of leukocyte chemo-attractants such as CCL22 and CCL18, as well as counteractors to hyper inflammation including TGF-β and IL-10. In short, alternatively

activated macrophages function to inhibit inflammation via cytokine based means, as opposed to classically activated macrophages, which secrete pro-inflammatory factors for means of priming, recruitment, and increased phagocytosis/apoptosis [33].

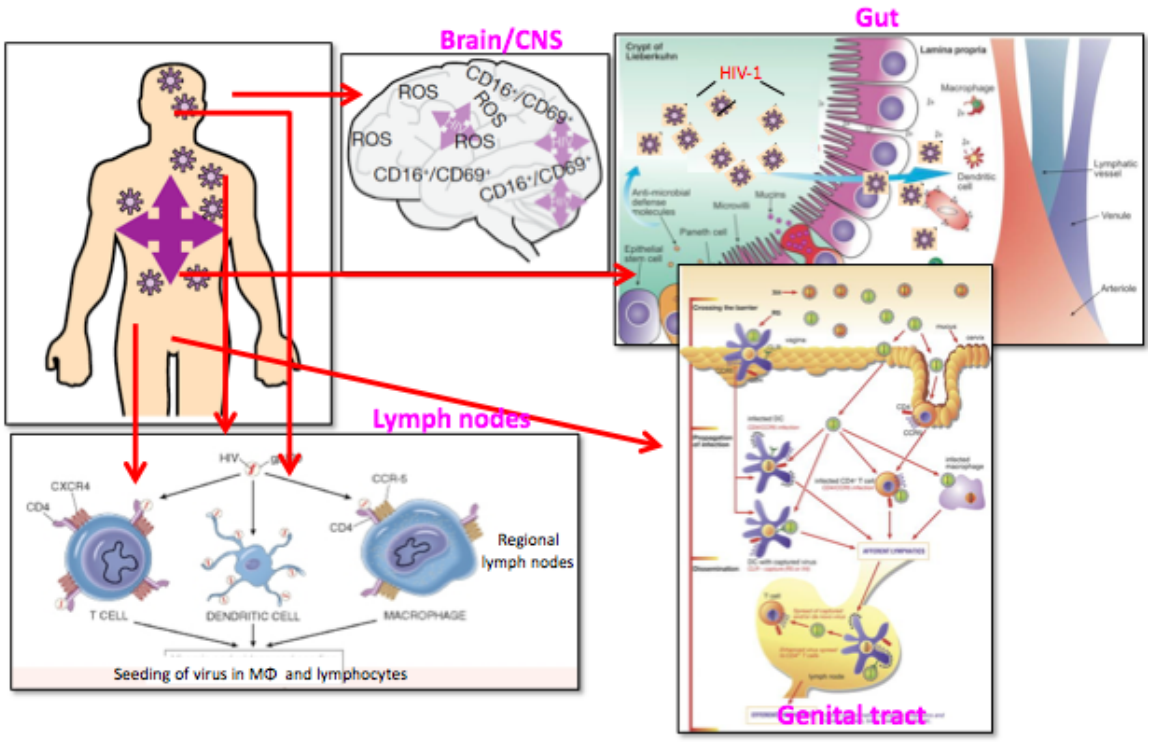


Figure 1.7: Ubiquitous location of macrophages *in vivo*. Macrophages are located in every tissue and organ including the gut, lymph nodes, CNS, and genital tract (reviewed in [26].

Functional cure *versus* systemic eradication

A functional cure is one wherein a therapy or therapies is administered, allowing for a person to remain asymptomatic relative to decline in CD4 T cell counts, increase in viral loads, or ay AIDS-related complications, without actual elimination of all virus from the patient. Virus could be maintained in a latent state indefinitely or in various compartments with low-level ongoing viral replication, but elimination of virus from the patient systemically would not be achieved. Systemic eradication is one wherein a patient is administered a therapy or therapies, allowing for elimination of all latent viral reservoirs, viral sanctuaries, and ongoing replication, across all tissues and organs systemically. This therapy would result in a cure for HIV-1 infection.

To date, neither a functional cure or systemic eradication has been achieved, although recent work by Dr. Gero Huetter on the "Berlin patient" demonstrated what may be the first "functionally cured" patient [34], through a combination of Atripla (600 mg of efavirenz, 200 mg of emtricitabine, and 300 mg of tenofovir), with concomitant stem cell transplant with a D32 deletion mutant, and treatment for a non AIDS related leukemia with amsacrine 100 mg/m^2, fludarabine 30 mg/m^2, cytarabine 2 g/m^2, cyclophosphamide 60 mg/kg, ATG (rabbit anti thymocyte globulin) 5.5mg/kg, and TBI (total body irradiation) 400 cGy (d-5). Although HAART was withdrawn for over 7 years without detectable viremia in either the plasma or tissue, recent samples have demonstrated the presence of low-level virus across various tissues including the gut. Whether this treatment will become the first "functional cure" or not can only be defined by time, although independent of the outcome, enormous hope for a cure, was defined through work with the "Berlin patient".

Macrophage depleting agents: Mechanism and potential therapeutic agent for treatment of HIV-1 infection

Clodronate has a well-established history as that of a macrophage-depleting

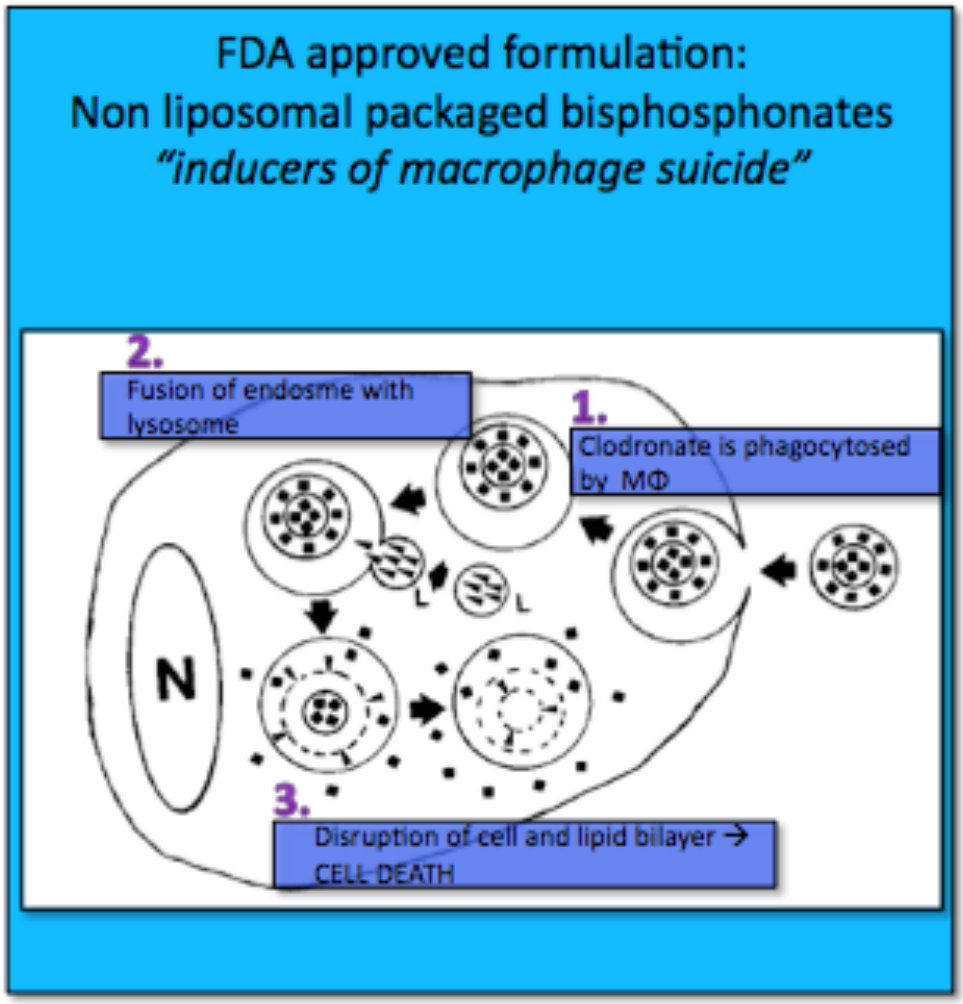
agent [35-37], although therapeutic application has primarily been used to treat rare hyper-proliferative disorders and has not been considered for treatment of HIV-1 infection. Clodronate is a macrophage/phagocytic cell specific inducer of cell death, which is facilitated by phagocytic uptake, precluding non-phagocytic cells from undergoing apoptosis as a function of exposure to clodronate. Clodronate is endocytosed or phagocytosed by macrophage or macrophage precursor, and subsequent intracellular metabolism of the clodronate to the toxic ATP 5'-(β,γ-dichloromethylene) triphosphate) in macrophages and monocytes, conferring selective apoptosis of phagocytic cells only (Figure 1.8 A, B). Recent reports have demonstrated that addition of a nitrogen-containing side chain to the clodronate backbone can significantly increase the potency of the compound. Cross reference of FDA approved drugs with a clodronate backbone with a nitrogen-containing side chain revealed two drugs, Fosamax and Boniva, as potential macrophage-depleting agents, each of which were explored for their macrophage-specific ability to induce cell death in these cells.

Summary: Why elimination of HIV-1 from macrophages is sentinel to achieving eradication

There are multiple reasons why eradication cannot be achieved unless virus can be eliminated from macrophages. Macrophages 1) are ubiquitous, 2) are infected early upon transmission, 3) harbor latent infection for years, 4) can repopulate the periphery with virus upon reactivation, 5) can transmit virus to $CD4^+$ T cells during antigen presentation and stimulation of adaptive immunity, and 6) upon infection can orchestrate a pro-inflammatory cytokine milieu across multiple compartments that renders uninfected cells more susceptible to infection and activates already infected cells further, to produce more virus per unit cell. The complex series of events concomitantly governed by macrophages that favor HIV-1 establishment and disease progression dictate that

eradication cannot occur without elimination of virus from macrophages. To this end, the focus of this thesis is to eliminate virus from macrophages via a multi-faceted approach.

A.


FDA approved formulation:
Non liposomal packaged bisphosphonates
"inducers of macrophage suicide"
2.
Fusion of endosme with lysosome
1.
Clodronate is phagocytosed by MΦ
N
3.
Disruption of cell and lipid bilayer → CELL DEATH

B.

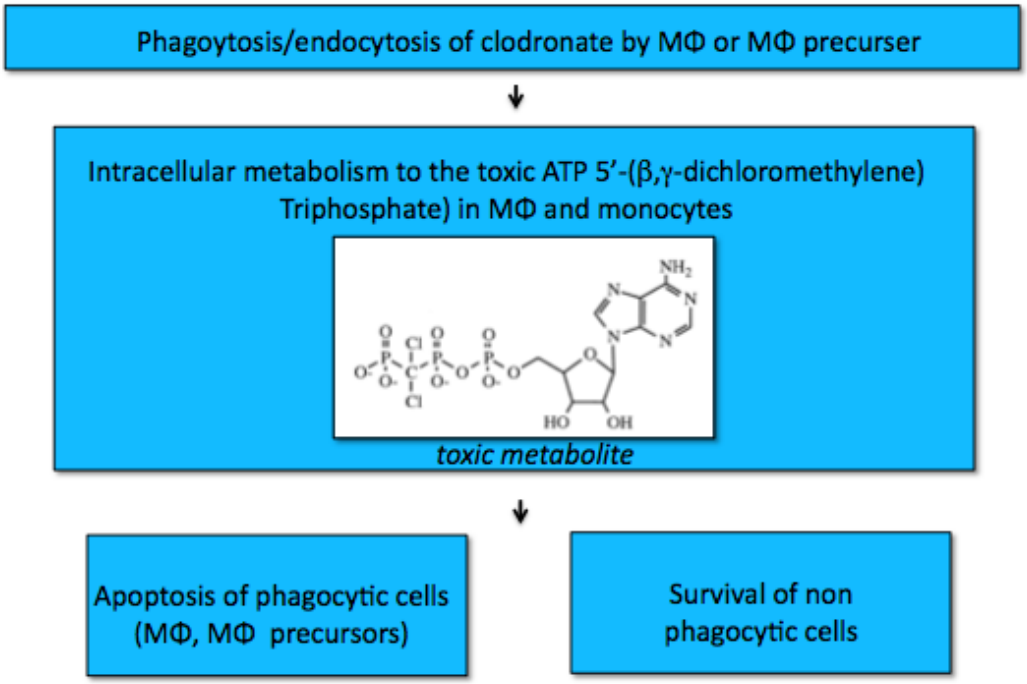


Figure 1.8: Mechanism of action of clodronate. Clodronate is endocytosed or phagocytosed by macrophage or macrophage precursor, and subsequent intracellular metabolism of the clodronate to the toxic ATP 5'-(β,γ-dichloromethylene) triphosphate) in macrophages and monocytes, conferring selective apoptosis of phagocytic cells only (A, B) [36-38].

Chapter 2: Methodology

Preparation of macrophages for cellular pharmacology:

Monocytes were isolated from buffy coats of HIV-1 negative, HBV/HCV-negative donors with density gradient centrifugation coupled with enrichment for $CD14^+$ monocytes with Rosette Sep antibody cocktail (Stem Cell Technologies, Vancouver, British Columbia). Cells were seeded at a concentration of 1.0×10^6 cells/well for 1 hr at 37^oC and 5 % CO_2 to confer plastic adherence prior to repeated washes with 1 x PBS.

Culture of activated or resting macrophages:

Activated macrophages were maintained in medium containing 100 U/ml macrophage colony-stimulating factor (m-CSF, R&D Systems, Minneapolis, MN), supplemented with 2.0 % fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) and 1 % penicillin/streptomyocin (Invitrogen, Carlsbad, CA) for 7 days (37^oC, 5 % CO_2) prior to testing. For resting macrophages, cells were maintained in medium containing m-CSF for 18 hrs prior to two washes with 1 x PBS (to remove m-CSF) and subsequent culture in m-CSF free medium supplemented with 2.0 % fetal calf serum and 1 % penicillin/streptomyocin for 6 more days prior to cellular pharmacology studies. For all conditions, macrophages were stained with CD11b-APC (Miltenyi Biotec, Auburn, CA) and subjected to FACS to determine purity of > 99 %.

Cellular Pharmacology studies:

Resting and activated lymphocytes and macrophages were seeded in 12 well plates at a density of 8.0×10^5 cells/well (viability by trypan blue assay > 95 %), for 7 days. The medium was then replaced with medium containing 10 μM AZT, ABC, CBV,

(-)-FTC, TDF, TFV, DXG, DXG plus AZT (1:1 ratio) or RAL at 37^{o} C, 5 % CO_2. Incubation times of 4 hr were selected in accordance with a typical time of maximal plasma nucleoside analog concentration of < 4 hr following oral administration. (results for the 4 hr incubation are summarized in Table 2.1 and 2.2) [39]. Extracellular medium was removed following these incubation periods and the cells were washed twice with ice-cold 1 x PBS to remove any residual nucleoside analogs. Cells were resuspended in 60 % CH_3OH overnight, and extracts were centrifuged at 13,000 rpm for 10 min. Supernatants were dried under a flow of air, re-dissolved in mobile phase for LC/LC-MS analysis as previously described [40].

Preparation of macrophages for antiviral studies:

Resting or activated macrophages were cultured as described above for 7 days. For acute infection, resting or activated cells were serum starved for 8 hrs prior to infection and cultured for 2 hrs in medium containing various concentrations of test compound for 2 hr prior to removal of drug-containing medium and 4 hr infection with HIV-1_{BaL} at 0.1 MOI in the absence of drug. 4 hrs after infection, virus was removed and drug-containing medium was returned to the cultures. Supernatants were collected on day 7 post-infection and HIV-1 p24 was quantified via ELISA (Zeptometrix Corporation, Buffalo, NY). EC_{50} analysis was performed using CalcuSyn software (BioSoft Corporation, Cambridge, UK). For constitutively activated macrophages, cells were maintained in medium containing m-CSF for 7 days prior to infection, and were constitutively exposed to m-CSF prior to and after infection with HIV-1_{BaL}. Supernatants were collected on day 7 post-infection and HIV-1 p24 was quantified via ELISA as described above. Chronic infections were conducted analogously to acute infections, except that cells were not pre treated with drug prior to infections, and were exposed to drug containing medium after infections were completed as described above. For serum

potency studies in PI, cells were infected and treated as described above, but were maintained in 2.0, 5.0, or 10.0 % serum-containing medium for the duration of the experiment.

Preparation and culture of lymphocytes:

Lymphocytes were isolated from buffy coats derived from healthy donors obtained from Life South Laboratories (Dunwoody, GA). Resting lymphocytes were maintained in PHA-free RPMI media supplemented with 20 % fetal calf serum, 1 % penicillin/streptomyocin and 2 % L-glutamine (Sigma Aldrich, San Jose, CA) for 72 hrs prior to cellular pharmacology studies. Activated lymphocytes were maintained analogously with the exception that medium was supplemented with 6 µg/ml phytohemagglutinin (PHA) (Cape Cod associates, East Falmouth, MA).

Cellular pharmacology studies in lymphocytes:

Cells were exposed to 10 µM AZT, ABC, CBV, (-)-FTC, TDF, TFV, DXG, RAL, or DXG plus AZT for 4 hrs. Drugs and their phosphorylated derivatives were extracted from cells as described above and quantified with LC-MS/MS [40].

Antiviral potency studies in lymphocytes:

Testing was performed in duplicate with at least 3 independent assays. Cells were incubated in RPMI medium (HyClone, Logan, Utah) containing HR-IL2 (26.5 units/ml) and 20 % Fetal Calf Serum. Infections were performed by adding HIV-1_{LAI} followed by a further incubation at 37° C, 5 % CO_2, 1 hr prior to addition of drugs. Assays were performed in 24 well plates (BD Biosciences, Franklin Lakes, New Jersey). One ml of supernatant was collected after 5 days in culture and then centrifuged at 12,000 rpm for 2 hr at 4^{o}C in a Jouan Br43i (Thermo Electron Corp., Marietta, OH). The

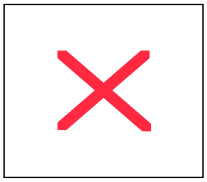
product of the RT assay was quantified using a Packard harvester and direct beta counter and the data were analyzed as previously described [41].

Statistical Methods:

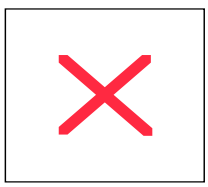
Means, standard deviations, statistical comparisons using unpaired Student t-test, and linear regression calculations were performed using the statistical routines in Microsoft Excel 2007. A p-value of < 0.05 was considered statistically significant.

Assessment of proportionality

Assessment of proportionality between extracellular nucleoside analog concentrations ([NRT]) and intracellular nucleoside analog-TP per 10^6 cells, and estimation of the apparent pharmacodynamic volumes of distribution relative to the site HIV-1 RT, were assessed using linear regression. Briefly: the primary mechanism of action of nucleoside analog-TP involves competitive inhibition of HIV-1 reverse transcriptase enzyme RT, and is typically modeled using the Michaelis-Menton relationship [42].

Fraction HIV-1 RT inhibited (F) =  Equation 1.

Assuming proportionality between extracellular [nucleoside analog] and intracellular [nucleoside analog-TP], Eq. 1 was written in terms of extracellular [nucleoside analog] and the concentration required for 50 % inhibition (EC_{50}):

F=  = $\frac{\frac{[NRTI]}{EC_{50}}}{\frac{[NRTI}{EC_{50}}+1}$ Eq. 2 ,

Therefore, $\frac{[NRTI]}{[NRTI-TP]} = \frac{EC_{50}}{K_i}$ Eq. 3.

The apparent volume of distribution of nucleoside analog-TP (V_{cell}) is unknown since the nucleoside analog-TP was measured per 10^6 cells. Therefore, concentrations listed in tables xx were based on literature derived estimates of cell volume, which are not necessarily indicative of the apparent volume of distribution of the cell compartment containing the viral RT. The apparent pharmacodynamic volume of distribution of the respective cellular compartments containing HIV-1 RT, V_{cell} was estimated noting that [nucleoside analog-TP] = nucleoside analog-TP per 10^6 cells divided by V_{cell}, using a log-transformation of Eq. 3:

$$\log_{10}\left(\frac{EC_{50}}{K_i}\right) = \log_{10}\left(\frac{[NRTI]}{NRTI-TP}\right) + \log_{10}(V_{cell}) \quad \text{Eq. 4.}$$

Thus, a plot of $\log_{10}(EC_{50}/K_i$ *versus* $\log_{10}$([nucleoside analog]/nucleoside analog-TP per 10^6 cells) should be linear with a slope = 1, and an intercept = $\log_{10}(V_{cell})$ if nucleoside analog remains proportional to [nucleoside analog-TP]. However, if [nucleoside analog] is markedly non-proportional to [nucleoside analog-TP] the slope of the line would differ from unity. EC_{50}, [nucleoside analog], and nucleoside analog-TP data were measured in our laboratory, while estimates of enzyme inhibition in the presence of competing dNTP's (K_i), were obtained from the literature. Plots for the respective cell types are contained in Fig. 2.2.

Toxicity studies:

IC_{50} (toxicity) was determined by 5-(3-carboxymethoxyphenyl-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl tetrazolium salt (MTT reduction test using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA),

according to the manufacturer's protocol. Cytotoxicity was considered when the concentrations of the test compounds alone inhibited growth by 50 %).

Viability and proliferation assays in lymphocytes:

Viability and proliferation were measured using the ViaCell Trypan blue system for lymphocytes stimulated for 72 hr with 6 µg/ml PHA or 6 µg/ml PHA + IL-2 prior to addition of either Tofacitinib or Jakafi at various concentrations for 5 days, in duplicates, with each well containing 1.0 x 10^6 cells. ViaCell determines viability and total cell number, which is reported as percent viable cells in each well, and total cell number per well. Numbers are normalized to wells containing media and cells without drug.

Viability assays in macrophages:

Viability was measured using the Viacell Trypan blue system for macrophages maintained in various concentrations of either Tofacitinib or Jakafi for 5 days, in duplicates, with each well containing 1.0 x 10^6 cells. ViaCell determines viability and total cell number, which is reported as percent viable cells in each well, and total cell number per well. Numbers are normalized to wells containing media and cells without drug.

Assessment of inhibition of Jak or Tyk2 by kinase inhibitors:

Enzyme assays for human JAKs were performed using recombinant catalytic domains of the respective kinases expressed in the baculovirus system, with ATP concentrations at 1 mM. Enzyme activities were assayed by measuring the phosphorylation of the peptide biotin-EQEDEPEGDYFEWLE. The phosphorylated peptide was detected by a time-resolved fluorescence resonance energy transfer method. Reactions included purified JAKs, the specified amount of ATP, and 500 nmol/L

peptide in 40 µL assay buffer containing 50 mmol/L Tris-HCl (pH 7.8), 100 mmol/L NaCl, 5 mmol/L DTT, and 0.1 mg/ml bovine serum albumin. Reactions were incubated at room temperature for 1 h and then stopped on addition of 20 µL of 45 mmol/L EDTA, 300 nmol/L streptavidin-allophycocyanin, and 6 nmol/L europium-labeled anti-phosphotyrosine antibody Py20 in assay buffer (Perkin-Elmer). Streptavidin-allophycocyanin and Eu-Py20 were allowed to bind for 40 min before fluorescence was measured using a Fusion plate reader (Perkin-Elmer).

Combination studies assessing antiviral potency:

To evaluate whether combination of Tofacitinib+Jakafi is synergistic, additive, or antagonistic, concentrations of drug at a ratio of EC_{50}:EC_{50}, which is 1:4, were added at various concentrations to lymphocytes, and infections and antiviral potency was performed as described above. Drug interactions were analyzed using Calcucyn (Biosoft, Ferguson, MO, USA), which allows automated simulation of synergism or antagonism.

Antiviral potency in rhesus macaque lymphocytes and macrophages:

Antiviral potency for rhesus macaque lymphocytes and macrophages were performed with cells isolated as described above for human isolations. Antiviral assays were performed as described above for human assays.

Reactivation of latent HIV-1 in primary human lymphocytes:

Latently infected cultured central memory T cells were prepared from primary naïve cells and reactivation of latent HIV-1 infection as previously described [43] were then triggered by antibody-mediated CD3/CD28 co-stimulation in the presence of 0.1, 1.0, 10, and 100 µM of Jak inhibitors. Production of HIV-1 was monitored by intracellular

p24 via flow cytometry.

Extraction of intracellular nucleotide fraction and LC-MS/MS analysis:

For both macrophages and lymphocytes, the isolated cells were washed twice with ice-cold 1 X PBS to remove any residual medium. Cells were resuspended in 70 % CH_3OH overnight, and extracts were centrifuged at 13,000 x *g* for 10 min (Thermo Electron Corp., Marietta, OH). Supernatants were subsequently dried, and the resulting samples were reconstituted in HPLC mobile phase for LC-MS/MS analysis as described previously [40]. The rNTP level measurements were performed using a similar approach as described above for dNTP. The stable isotopes, [^{13}C,^{15}N]ATP, [^{13}C,^{15}N]GTP, [^{13}C,^{15}N]CTP, and [^{13}C,^{15}N]TTP were used for the measurement of ATP, GTP, CTP, and UTP. The m/z parent → product MS/MS transitions 523 → 146, 539 → 162, 496 → 119, and 495 → 81 were applied for the standard stable labeled isotopes and 508 → 136, 524 → 152, 484 → 112, and 485 → 81, for the corresponding sample nucleotides, respectively.

RT purification:

The HXB2 HIV-1 RT gene was previously cloned into pET28a (Novagen), and the N-terminal hexahistidine-tagged-p66/p66 homodimer HIV-1 RT was subsequently expressed in E. coli BL21 (DE3) and purified with Ni2"-NTA chromatography followed by DEAE and SP anion exchange RT of simian immunodeficiency virus (SIV_{agm} Sab) was also previously cloned and purified These RT proteins were quantified and stored in 10 % glycerol dialysis buffer as described previously [44].

Assay for rNTP incorporation during DNA synthesis of HIV-1 RT using ^{32}P-UTP:

Ext-T DNA 23-mer primer (Primer A, 5'-TCGCCCTTAAGCCGCGC- 3'; Ext T

primer, 5'-CTTATAACGATCGCCCTTAAGCC; Ext-G primer, 5'-GAATTCCCGCTAGCAATATTCT-3'; Ext-C primer, 5'-TATAACGATCGCCCTTAAGCCG-3') annealed to the RNA 40-mer template (10 nM complex) was extended by 200 nM HIV-1 RT for 45 min in the 1 X Reaction Buffer with the macrophage and lymphocyte dNTP or NTP concentrations described in Table 1 using the identical non-radioactive UTP and radioactive ^{32}PUTP ratio (690:1). Following a quench with 10 mM EDTA, the reaction products were further purified with a Qiagen nucleotide removal column. The reaction products with ^{32}PUTP were normalized with a 5'-end 32P-labeled 17-mer loading control primer, which was added in an equal amount after the reactions were terminated as described [14]. To monitor the entire DNA polymerization under the conditions described in this experiment, the identical reactions were conducted with the 5'-end ^{32}P-labeled 23-mer Ext-T DNA template annealed to the 40-mer RNA template with non-radioactive dNTPs in both negative (no enzyme) and positive (250 "M dNTP substrate) controls, generating no primer extension and full primer extension, respectively (Figure 3.10).

Chapter 3: Results

Cellular Pharmacology:

The ratio of nucleoside analog-TP in activated and resting macrophages relative to were significantly lower in macrophages *versus* activated lymphocytes independent of activation state ($p < 0.05$), with the exception of TFV, (Figure 3.1, Table 3.1). The intracellular concentration of AZT-TP (using cell volumes of 0.32 pL and 2.66 pL for lymphocytes and macrophages, respectfully, [13] was 18-fold and 35-fold lower in resting macrophages than in resting and activated lymphocytes, respectively (Panel A, Figure 3.1). The intracellular concentration of AZT-TP was 11.6-fold lower in activated macrophages *versus* lymphocytes. AZT-TP levels were significantly higher (3.5-fold) in activated lymphocytes *versus* resting lymphocytes ($p < 0.05$) (Panel A, Figure 3.1, Table 3.1).

For resting cells treated with ABC, the intracellular concentration of CBV-TP, the active metabolite of ABC, was 27.5-fold and 7.5-fold lower in resting macrophages *versus* activated and resting lymphocytes, respectively (Panel B, Figure 3.1, Table 3.1). The intracellular concentration of CBV-TP was 10-fold lower in activated macrophages *versus* lymphocytes. For both ABC and CBV treated cells, the intracellular concentration of CBV-TP was 2-fold lower ($p < 0.05$) in resting *versus* activated lymphocytes (Panel B, C, Figure 3.3, Table 3.3). Resting cells treated with CBV demonstrated an intracellular concentration of CBV-TP that was 12.2-fold and 4.5-fold lower in resting macrophages *versus* resting and activated lymphocytes, respectively. The intracellular concentration of CBV-TP was 28-fold lower in activated macrophages *versus* lymphocytes (Panel C, Figure 3.1, Table 3.1).

In resting cells treated with (-)-FTC, the intracellular concentration of (-)-FTC-TP TP was 113-fold and 49.2-fold lower in resting macrophages *versus* resting and activated lymphocytes, respectively. For activated cells treated with (-)-FTC, the

intracellular concentration of (-)-FTC-TP was 22-fold lower in activated macrophages versus lymphocytes (Panel F, Figure 3.1, Table 3.1).

For resting cells treated with TDF, the intracellular concentration of TFV-DP, the active metabolite of TFV, cells treated with TDF, the intracellular concentration of TFV-DP, the active metabolite of TFV, was 28-fold and 11.7-fold lower in resting macrophages *versus* resting and activated lymphocytes, respectively. For activated cells treated with TDF, the intracellular concentration of TFV-DP was 11.7-fold lower in activated macrophages *versus* lymphocytes (Panel D, Figure 3.1, Table 3.1). Contrarily, resting cells treated with TFV, the intracellular concentration of TFV-DP was not significantly different in resting macrophages *versus* lymphocytes. For activated cells treated with TFV, the intracellular concentration of TFV-DP was not significantly different in activated macrophages *versus* lymphocytes (Panel E, Figure 3.1, Table 3.1).

3TC-treated resting cells demonstrated an intracellular concentration of 3TC-TP that TP was 140-fold and 70-fold lower in resting macrophages *versus* resting and activated lymphocytes, respectfully. For activated cells treated with 3TC, the intracellular concentration of 3TC-TP was 93-fold lower in activated macrophages *versus* lymphocytes (Panel G, Figure 3.3, Table 3.3). Similarly, resting cells treated with DXG displayed intracellular concentration of DXG-TP was 10-fold and 5–fold lower in resting macrophages *versus* resting and activated lymphocytes, respectfully. For activated cells treated with DXG, the intracellular concentration of DXG-TP was 4-fold lower in activated macrophages *versus* lymphocytes (Panel H, Figure 3.1, Table 3.1).

When analysis of co-incubation of resting cells treated with 10 μM DXG+AZT (ratio 1:1), the intracellular concentration of AZT-TP was 11-fold lower in resting macrophages *versus* lymphocytes. For activated cells treated with 10 μM DXG+AZT (ratio 1:1), the intracellular concentration of AZT-TP was 30-fold lower in activated macrophages *versus* lymphocytes (Table 3.2). Quantification of DXG-TP in resting cells treated with 10 μM

DXG+AZT (ratio 1:1) demonstrated that the intracellular concentration of DXG-TP was 31-fold lower in resting macrophages *versus* lymphocytes. For activated cells treated with 10 μM DXG+AZT (ratio 1:1), the intracellular concentration of DXG-TP was 16-fold lower in activated macrophages *versus* lymphocytes (Table 3.2).

RAL-treated resting cells conferred an intracellular concentration of RAL that was 8-fold lower in resting macrophages *versus* lymphocytes. For activated cells treated with RAL, the intracellular concentration of RAL was 93-fold lower in activated macrophages *versus* lymphocytes (Panel I, Figure 3.3, Table 3.1).

Antiviral potency:

As expected, for the EC_{50} of all nucleoside analogs were > 50 μM, since their primary mechanism of action is to inhibit reverse transcription during the infection process. During acute-infection of resting macrophages EC_{50} of nucleoside analogs ranged from 0.4 - 9.42 μM, compared to 0.03 - 0.40 μM in constitutively activated macrophages (Figure 3.1, Table 3.1). ATZ, displayed potency against both acute resting, acute activated, and chronic HIV-1 infection in macrophages (0.03, 0.03, and 0.09 μM, respectively), in agreement with the primary mechanism of action of PI which involves inhibition of viral maturation in already infected cells. RAL displayed similar potency in acutely-infected resting and activated macrophages (0.02 and 0.02 μM, respectively), and displayed an EC_{50} of > 50 μM against chronic HIV-1 infection in macrophages. For comparison, the antiviral potency of nucleoside analogs in activated lymphocytes during acute infection ranged from 0.001-0.3 μM. (Table 3.1).

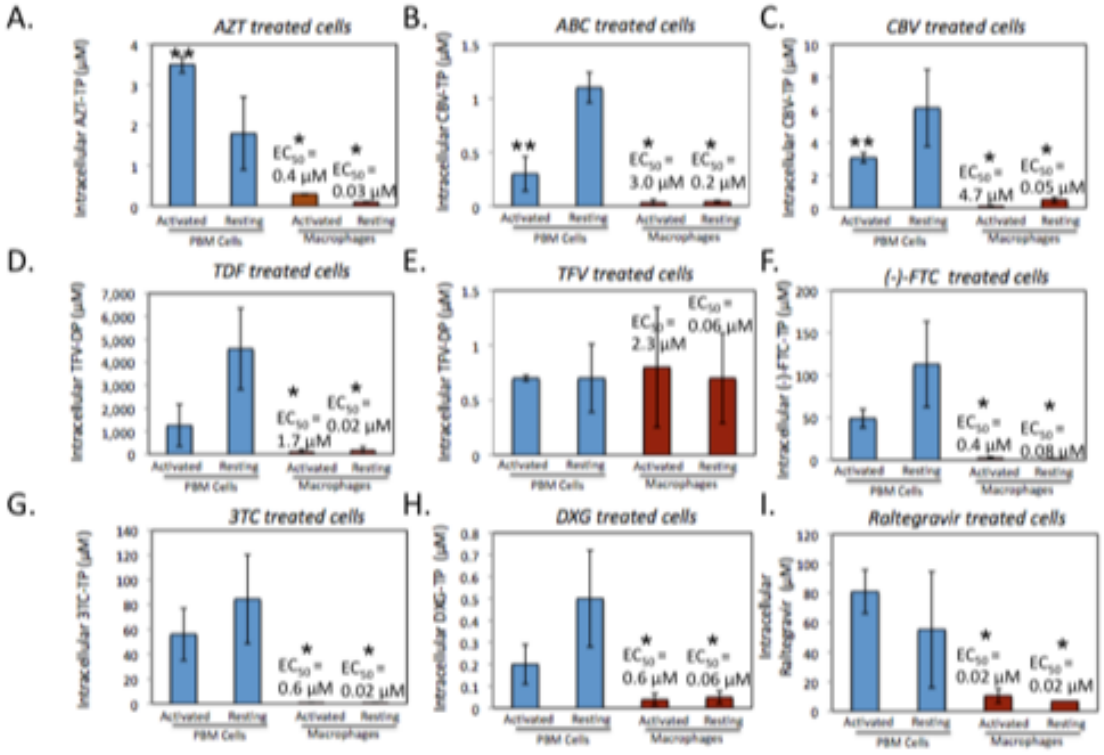


Figure 3.1: Intracellular concentrations and antiviral potency of HAART in resting or activated lymphocytes and macrophages. Error bars indicate standard deviation. * indicates significant difference relative to lymphocytes ($p < 0.01$). ** indicates significant difference relative to resting lymphocytes ($p < 0.05$).

Drug Tested	State of Cells	Active metabolite measured	Intracellular NTP or drug in lymphocytes (μM)	EC_{50} in acutely infected lymphocytes (μM)	Intracellular NTP or drug in MΦ (μM)	EC_{50} in acutely infected MΦ (μM)	EC_{50} In chronically infected MΦ (μM)
AZT	Activated	AZT-TP	3.5 ± 0.9**	0.004 ± .0022	0.3 ± 0.3*	0.4 ± 0.04***	> 50
AZT	Resting	AZT-TP	1.8 ± 0.4	N/A	0.1 ± 0.1*	0.03 ± 0.007	> 50
ABC	Activated	CBV-TP	0.3 ± 0.2**	0.3 ± 0.2	0.03 ± 0.03*	3.0 ± 1.1***	> 50
ABC	Resting	CBV-TP	1.1 ± 0.1	N/A	0.04 ± 0.01*	0.2 ± 0.3	> 50
CBV	Activated	CBV-TP	3.1 ± 0.3**	0.08 ± 0.08	0.1 ± 0.1*	4.7 ± 3.2***	> 50
CBV	Resting	CBV-TP	7.7 ± 3.7	N/A	0.7 ± 0.3*	0.05 ± 0.02	> 50
TDF	Activated	TFV-DP	1,252 ± 896	0.01 ± 0.01	160 ± 123*	1.7 ± 1.2***	> 50
TDF	Resting	TFV-DP	4,589 ± 1,764	N/A	107 ± 144*	0.02 ± 0.02	> 50
TFV	Activated	TFV-DP	0.7 ± 0.3	1.6 ± 1.2	0.7 ± 0.6*	2.3 ± 0.9***	> 50
TFV	Resting	TFV-DP	0.7 ± 0.3	N/A	0.8 ± 0.4*	0.06 ± 0.03	> 50
(-)-FTC	Activated	(-)-FTC-TP	49.2 ± 41.8	0.008 ± 0.007	2.2 ± 2.0*	0.4 ± 0.2***	> 50
(-)-FTC	Resting	(-)-FTC-TP	113 ± 18.8	N/A	1.0 ± 0.6*	0.08 ± 0.02	> 50
3TC	Activated	3TC-TP	56 ± 21	0.06 ± 0.04	0.6 ± 0.4*	0.6 ± 0.3 ***	> 50
3TC	Resting	3TC-TP	84.5 ± 36	N/A	0.8 ± 0.3*	0.02 ± 0.01	> 50
DXG	Activated	DXG-TP	0.2 ± 0.1	0.3 ± 0.2	0.05 ± 0.04*	0.6 ± 0.2***	> 50
DXG	Resting	DXG-TP	0.5 ± 0.2	N/A	0.04 ± 0.03*	0.06 ± 0.02	> 50
RAL	Activated	RAL	80.9 ± 14.7	0.001 ± 0.002	10.5 ± 4.1*	0.02 ± 0.02	> 50
RAL	Resting	RAL	55.3 ± 24.1	N/A	7.0 ± 0.2*	0.02 ± 0.03	> 50
ATV	Activated	ATZ	ND	0.007 ± 0.004	ND	0.03 ± 0.03	0.09

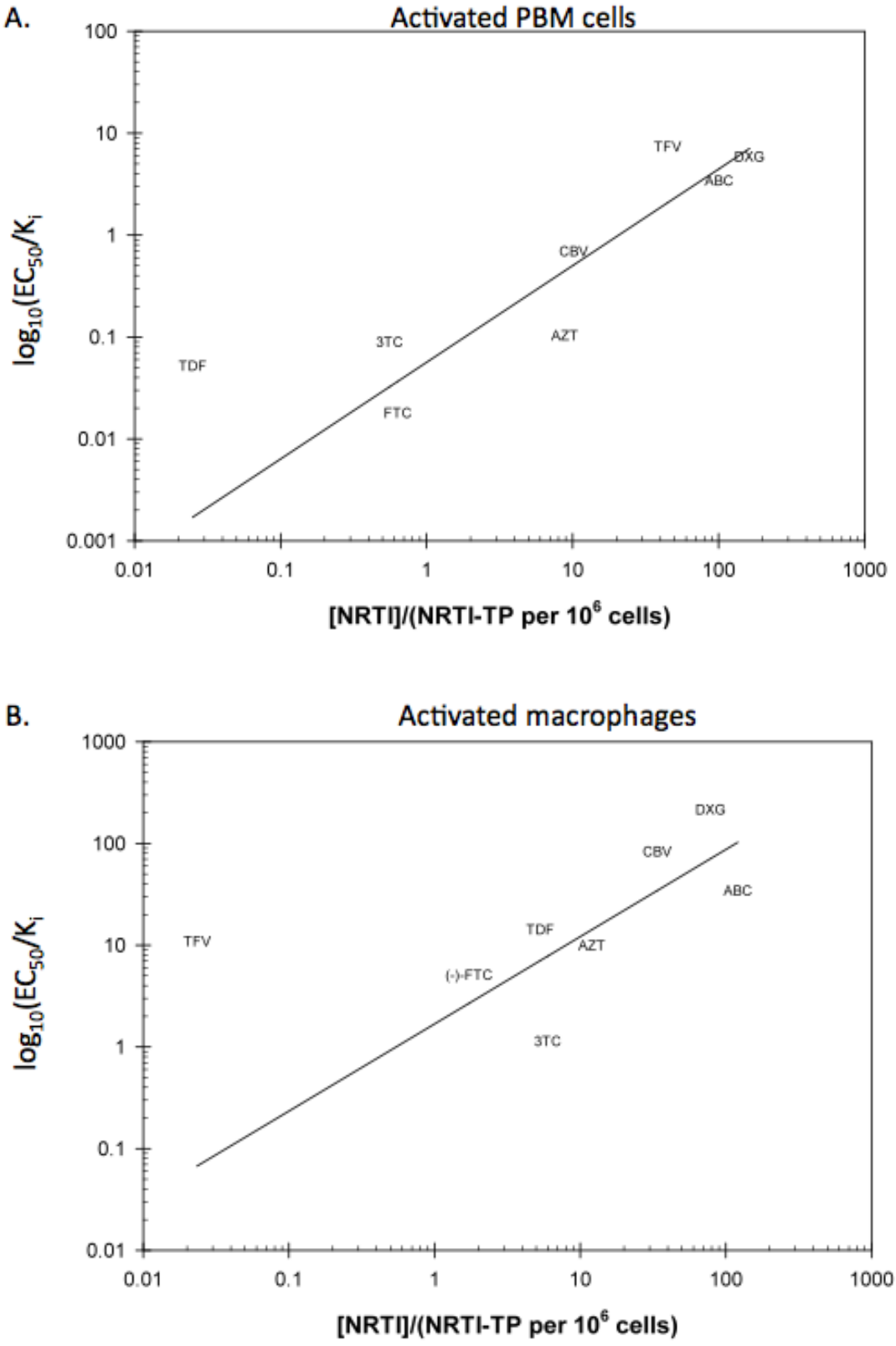
Table 3.1: Intracellular concentrations and antiviral potency of HAART in resting or activated lymphocytes and macrophages. Error bars indicate standard deviation. * indicates significant difference relative to lymphocytes ($p < 0.01$). ** indicates significant difference relative to resting lymphocytes ($p < 0.05$). *** indicates significant difference relative to resting macrophages ($p < 0.05$).

Drug Tested	**Cell Type**	**State of cells**	**Active metabolite measured**	nucleoside analog**-TP (μM)**
AZT alone	lymphocytes	Activated	AZT-TP	3.5 ± 0.9
AZT+DXG (1:1)	lymphocytes	Activated	AZT-TP	2.7 ± 0.6
DXG alone	lymphocytes	Activated	DXG-TP	0.2 ± 0.1
AZT+DXG (1:1)	lymphocytes	Activated	DXG-TP	0.25 ± 0.01
AZT alone	lymphocytes	Resting	AZT-TP	1.8 ± 0.4
AZT+DXG (1:1)	lymphocytes	Resting	AZT-TP	1.4 ± 0.3
DXG alone	lymphocytes	Resting	DXG-TP	0.5 ± 0.2
AZT+DXG (1:1)	lymphocytes	Resting	DXG-TP	0.54 ± 0.12
AZT alone	macrophages	Activated	AZT-TP	0.3 ± 0.3
AZT+DXG (1:1)	macrophages	Activated	AZT-TP	0.09 ± 0.05
DXG alone	macrophages	Activated	DXG-TP	0.05 ± 0.04
AZT+DXG (1:1)	macrophages	Activated	DXG-TP	0.016 ± 0.008
AZT alone	macrophages	Resting	AZT-TP	0.1 ± 0.1
AZT+DXG (1:1)	macrophages	Resting	AZT-TP	0.13 ± 0.01
DXG alone	macrophages	Resting	DXG-TP	0.04 ± 0.03
AZT+DXG (1:1)	macrophages	Resting	DXG-TP	0.02 ± 0.01

Table 3.2: Intracellular concentrations of AZT-TP or DXG-TP in activated or resting lymphocytes or macrophages, either alone or in a 1:1 ratio. DXG or AZT administered in a 1:1 ratio in either lymphocytes or macrophages did not significantly alter intracellular concentrations of either DXG-TP or AZT-TP, independent of activation state.

*Proportionality between extracellular [*nucleoside analog*] and* nucleoside analog-*TP per 10^6 cells*

Linear regression of $\log_{10}(EC_{50}/K_i$ versus $\log_{10}$([nucleoside analog]/nucleoside analog-TP per 10^6 cells) (Eq. 4 cited above, and Figure 3.2), for the activated lymphocytes and macrophages produced slopes = 0.948 (95 % CI: 0.467 – 1.49) and 0.857 (95 % CI: 0.003 – 1.71), which were not significantly different than unity (Figure 3.2). The corresponding estimates of V_{cell} (antilogarithm of the Y- intercepts) for the activated lymphocytes and macrophages were 0.06 (95 % CI: 0.01 – 0.26) compared to 1.68 (95 % CI: 0.12 – 23.6) μl per per 10^6 cells, respectively. However the regression slope for the resting macrophages was 0.341 (95 % CI: 0.058 – 0.737), suggesting non-proportionality between $\log_{10}(EC_{50}/K_i$ versus $\log_{10}$([nucleoside analog]/nucleoside analog-TP per 10^6 cells). Therefore, it was not feasible to estimate V_{cell} for resting macrophages using this method.


A.
Activated PBM cells
100
10
1
0.1
0.01
0.001
0.01
0.1
1
10
100
1000
TFV
DXG
ABC
CBV
AZT
3TC
TDF
FTC
log10(EC50/Ki)
[NRTI]/(NRTI-TP per 10^6 cells)
B.
Activated macrophages
1000
100
10
1
0.1
0.01
0.01
0.1
1
10
100
1000
DXG
CBV
ABC
TDF
AZT
TFV
(-)-FTC
3TC
log10(EC50/Ki)
[NRTI]/(NRTI-TP per 10^6 cells)

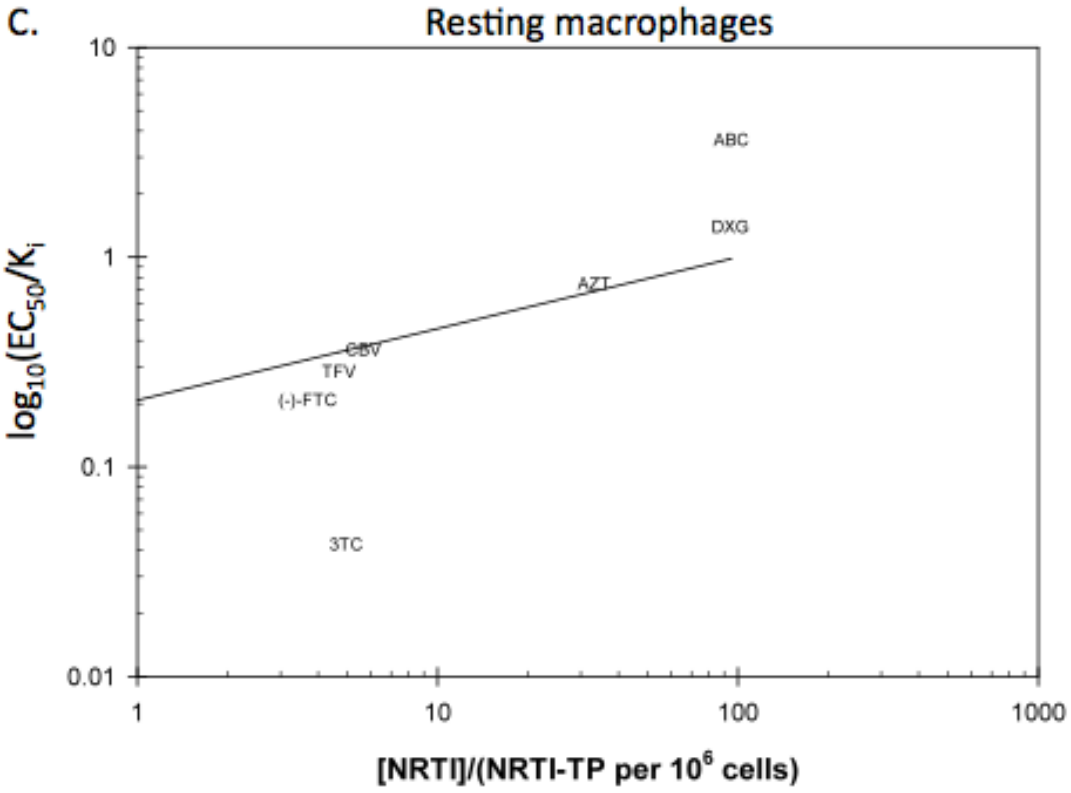


Figure 3.2: **Assessment of proportionality between extracellular [**nucleoside analog**] and** nucleoside analog**-TP/10^6 cells.** $\log_{10}(EC_{50}/K_i$ *versus* $\log_{10}$([nucleoside analog]/ nucleoside analog-TP per 10^6 cells) were plotted and the data fitted using linear regression. A. For activated lymphocytes cells, the fitted curve (excluding the prodrug TDF) produced r^2 = 0.837, slope = 0.948 (similar to unity), and a $V10^6$ (antilogorithm of y-intercept) = 0.056 pl per cell. B. For activated macrophages, the fitted curve produced r^2 = 0.57, slope = 0.857 (similar to unity), and a $V10^6$ (antilogorithm of y-intercept) = 1.68 pl per cell. C. For resting macrophages, the fitted curve produced r^2 = 0.403 and a slope = 0.347 (significantly different from unity). Therefore, it was inappropriate to assume proportionality between [nucleoside analog] and nucleoside analog-TP/10^6 cells in these cells, so that $V10^6$ was not estimated for resting macrophages.

Antiviral potency and toxicity of HIV-1 PI in macrophages and lymphocytes:

The EC_{50} for ATZ, APV, DRV, LPV, NFV, and SQV was not significantly different for activated *versus* resting macrophages (0.001 - 0.006 μM), and was not significantly diminished in macrophages *versus* lymphocytes (Table 3.3, Figure 3.3). TPV was significantly more potent in macrophages (EC_{50} 0.005 ± 0.003 μM and 0.007 ± 0.002 μM) in resting and activated cells, respectively, *versus* 0.05 ± 0.04 μM in lymphocytes ($p < 0.05$). APV and DRV were the least toxic ($IC_{50} > 100$ μM), whereas ATZ, LPV, NFV, SQV, and TPV were more toxic (IC_{50} 4.2 - 67.1 μM) (Table 3.1).

Antiviral potency of HIV-1 PI in macrophages with varying extracellular serum concentrations:

Four PI (APV, LPV, SQV, TPV) were selected as representatives to determine the impact of extracellular serum concentrations upon antiviral potency in acutely infected resting primary human macrophages. The potency was approximately one log higher for macrophages maintained in 2.0 % serum *versus* that observed for 5.0 and 10.0 % (0.005-0.006 μM *versus* 0.02-0.06 μM, respectively). There was no significant difference in potency for macrophages maintained in 5.0 or 10.0 % serum (Table 3.2).

PI tested	EC_{50} Acutely infected Resting MΦ (μM)	EC_{50} Acutely infected Activated MΦ (μM)	EC_{50} in Chronically infected MΦ (μM)	EC_{50} in lymphocytes (μM)	IC_{50} in VERO cells (μM)	IC_{50} in CEM cells (μM)	IC_{50} in lymphocytes (μM)
ATZ	0.002 ± 0.001	0.002 ± 0.002	0.002 ± 0.003	0.007	25.6	17.1	18.9
APV	0.02	0.01	0.03	0.005 ± 0.001	> 100 (4.8)	> 100 (25.6)	> 100 (5.8)
DRV	0.003 ± 0.0007	0.004 ± 0.008	0.007 ± 0.008	0.007	> 100 (10.7)	Ø100 Ø(3.1)	> 100 (30.3)
LPV	0.006 ± 0.001	0.002 ± 0.001	0.005 ± 0.003	0.006 ± 0.0004	28.3	13.2	34.4
NFV	0.003 ± 0.001	0.003 ± 0.0007	0.008 ± 0.0006	0.003 ± 0.003	11.8	4.2	29.1
SQV	0.006 ± 0.001	0.004 ± 0.001	0.009 ± 0.003	0.005	> 100 (15.5)	11.6	15.4
TPV	0.005 ± 0.003	0.007 ± 0.002	0.01 ± 0.009	0.05 ± 0.04	96.7	12.2	67.1

Table 3.3: Antiviral potency of HIV-1 PI in lymphocytes and macrophages, and toxicity in lymphocytes, Vero, and CEM cells. Antiviral potency ranged from 0.002-0.007 μM in acutely infected resting or activated macrophages. Antiviral potency in chronically infected macrophages ranged from 0.002-0.01 μM. Antiviral potency in lymphocytes ranged from 0.003-0.05 μM. IC_{50} (toxicity(ranged from 4.2 to > 100 μM depending on the PI tested.

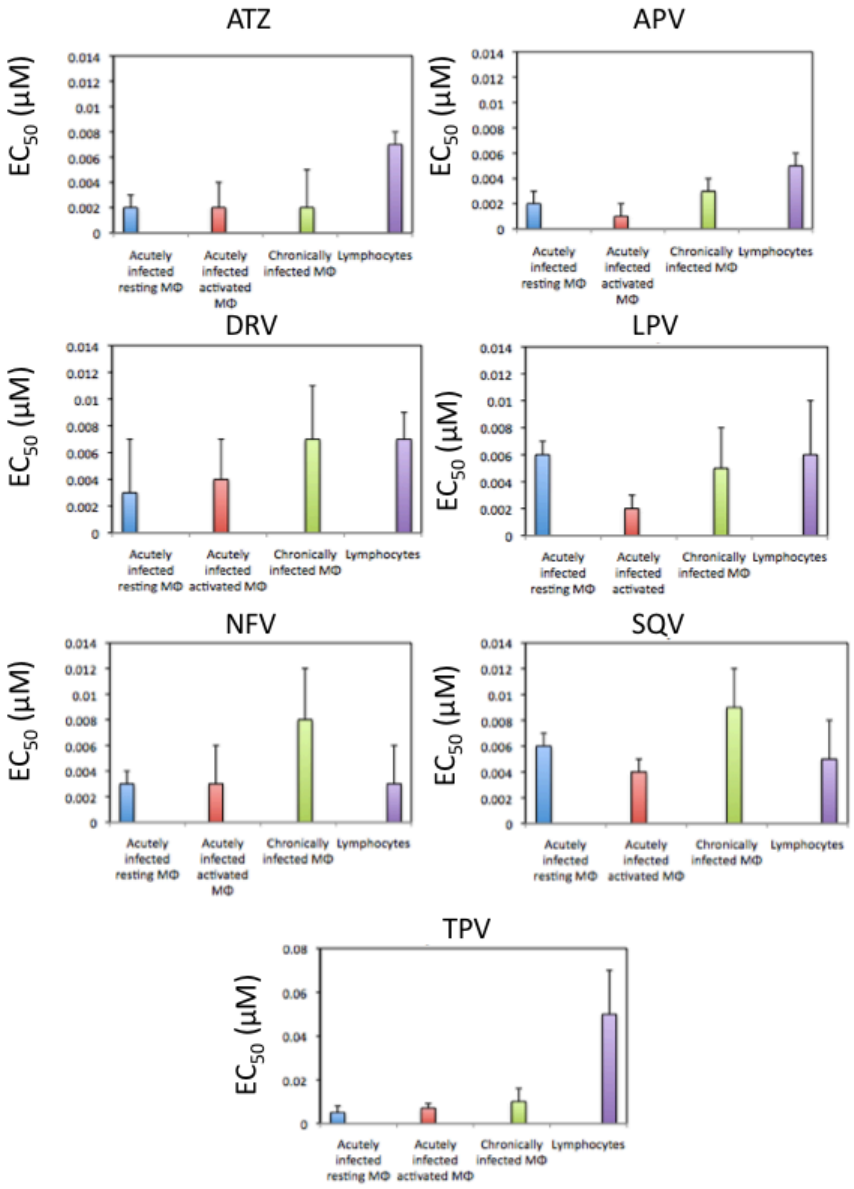


Figure 3.3: Antiviral potency and of various HIV-1 protease inhibitors in acutely or chronically infected primary human macrophages. The median effective concentration (EC_{50}) for ATZ, APV, DRV, LPV, NFV, and SQV was not significantly different for activated *versus* resting macrophages, or acutely infected or chronically infected macrophages (0.001- 0.006 µM), and was not significantly diminished in macrophages *versus* PBM cells (A-F). TPV was significantly more potent in macrophages (EC_{50} 0.005 ± 0.003 µM and 0.007 ± 0.002 µM) in resting and activated cells, respectively, *versus* 0.05 ± 0.04 µM in lymphocytes ($p < 0.05$) (F). * indicates significant difference compared to lymphocytes.

PI tested	2 % serum EC_{50} (μM)	5 % serum EC_{50} (μM)	10 % serum EC_{50} (μM)
APV	0.006 ± 0.001	0.04 ± 0.02	0.05 ± 0.03
LPV	0.006 ± 0.001	0.04± 0.02	0.05 ± 0.04
SQV	0.006 ± 0.001	0.02 ± 0.03	0.04 ± 0.03
TPV	0.005 ± 0.003	0.03 ± 0.03	0.06 ± 0.05

Table 3.4: Antiviral potency of HIV-1 PI in acutely infected resting macrophages at various concentrations of extracellular serum. Antiviral potency for APV, LPV, SQV, and TPV was approximately one log less potent for macrophages maintained in 5.0 % or 10.0 % serum (0.005-0.006 *versus* 0.02-0.06 μM, respectively). There was no significant difference in antiviral potency for macrophages maintained in 5.0 % *versus* 10.0 % serum containing media.

Antiviral potency and toxicity of cellular factor inhibitors:

The NFAT inhibitor was potent against acute infection in macrophages (EC_{50} 1.4 ± 1.7 μM), but was not potent against chronic infection in macrophages (EC_{50} > 100 μM). The EC_{50} for the NFAT inhibitor was 1.9 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was > 100 μM for lymphocytes, CEM, and Vero cells (Table 3.5).

The lck kinase inhibitor was potent against acute infection in macrophages (EC_{50} 2.5 ± 0.7 μM), and was weakly potent against chronic infection in macrophages (EC_{50} 14.5 ± 2.8 μM). The EC_{50} for the lck kinase inhibitor was 0.03 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was 9.5, 10.3, and 38.2 μM for lymphocytes, CEM, and Vero cells, respectively (Table 3.5).

The Akt inhibitor miltefosine was potent against acute infection in macrophages (EC_{50} 4.8 ± 1.8 μM), but was not potent against chronic infection in macrophages (EC_{50} > 100 μM). The EC_{50} for miltefosine was 18.8 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was 22.4, 3.4, and 27.6 μM for lymphocytes, CEM, and Vero cells, respectively (Table 3.5).

The Akt/NFκB inhibitor hyaluronin was not potent against acute or chronic infection in macrophages (EC_{50} > 100 μM), The EC_{50} for hyaluronin was > 100 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was > 100 μM for lymphocytes, CEM, and Vero cells (Table 3.5).

The p38 MAPK inhibitor dorapimod was potent against acute infection in macrophages (EC_{50} 15.5 ± 3.4 μM), but was not potent against chronic infection in macrophages (EC_{50} > 100 μM). The EC_{50} for dorapimod was 72.6 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was 38.4, 33.2, and > 100 μM for lymphocytes, CEM, and Vero cells, respectively (Table 3.5).

The Merck Akt inhibitor, which is currently under clinical investigation for treatment of cancer, was weakly potent against acute infection in macrophages (EC_{50}

31.4 ± 6.4 μM), and was not potent against chronic infection in macrophages (EC_{50} > 100 μM). The EC_{50} for the Merck Akt inhibitor was 43.9 ± 1.9 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was 18.5 ± 6.6, 5.4 ± 5.7, and 21.7 ± 13.9 μM for lymphocytes, CEM, and Vero cells, respectively (Table 3.5).

The Pfizer Jak3 inhibitor, Tofacitinib, internally coded as RS-1294, which is currently under phase 3 clinical investigation for treatment of rheumatoid arthritis, was potent against acute infection in macrophages (EC_{50} 0.2 ± 0.08 μM), and was equally potent against chronic infection in macrophages (EC_{50} 0.3 μM). The EC_{50} for Tofacitinib was 0.08 ± 0.06 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was 1.9 ± 0.8, > 100, and > 100 μM for lymphocytes, CEM, and Vero cells, respectively (Table 3.5).

The Incyte Jak2 inhibitor, Jakafi, internally coded as RS-1374, which recently received FDA approval for treatment of myelofibrosis, and is under clinical investigation for treatment of rheumatoid arthritis, was potent against acute infection in macrophages (EC_{50} 0.3 ± 0.1 μM), and was equally potent against chronic infection in macrophages (EC_{50} 0.3 μM). The EC_{50} for Tofacitinib was 0.02 ± 0.05 μM in lymphocytes. The IC_{50} (toxicity measured by MTT assay) was 2.1 ± 1.1, 11.8 ± 9.8, and 29.3 ± 6.8 μM for lymphocytes, CEM, and Vero cells, respectively (Table 3.5).

Antiviral potency and toxicity of various Jak inhibitors:

Antiviral potency and toxicity profile for Tofacitinib and Jakafi are described above. RS-1531 (Astra Zeneca's AZD2480), RS-1532 (Eli Lily's LY2784544), and RS-1533 (YM Bioscience's CYT387), all of which are under clinical investigation, were evaluated for potency and toxicity in the system described above. Antiviral potencies (EC_{50}) ranged from 0.1-2.8 μM, and toxicity (IC_{50}) ranged from < 0.1-26.9 μM, depending on the inhibitor tested (Table 3.6 A). Each drug presented with a narrow therapeutic

window of < 1 (Table 3.6 B), which may preclude further consideration for RS-1531, RS-1532, and RS-1533 for treatment of HIV-1 infection.

Viability of macrophages exposed to various concentrations of Jak inhibitors:

Primary human macrophages were exposed to 0.1-100 μM of RS-1294, RS-1374, RS-1531, RS-1532, and RS-1533 for 5 days. There was no significant difference between cells maintained in drug containing medium up to 10 μM versus cells maintained in drug free medium (Figure 3.4).

Viability and proliferation assays in lymphocytes:

Lymphocytes were maintained in PHA or PHA+IL-2 containing medium for 5 days in the presence of 0.1-100 μM of RS-1294, RS-1374, RS-1531, RS-1532, and RS-1533. For PHA stimulated lymphocytes, there was no significant difference in viability or proliferation *versus* cells maintained in drug-free medium for all concentrations tested (Figure 3.5A, B). For PHA+IL-2 stimulated lymphocytes, there was no significant difference in viability *versus* cells maintained in drug-free medium for all concentrations tested (Figure 4C), however there was a significant reduction in proliferation *versus* cells maintained in drug-free medium between 0.1 and 1.0 μM for all inhibitors tested (Figure 4D).

Antiviral potency of combination of Tofacitinib+Jakafi:

Antiviral potency of Tofacitinib+Jakafi was assessed in lymphocytes at a ratio of EC_{50}:EC_{50}, which is 1:4. Antiviral assays were performed as described above, and assessment of synergy or antagonisim was performed using Calcusyn (BioSoft Corporation, Cambridge, UK). Combination administration of Tofacitinib+Jakafi decreased the EC_{50} by 5-fold and the EC_{90} by 117-fold (Figure 3.6).

Antiviral potency in rhesus macaque lymphocytes and macrophages:

The potency of Tofacitinib and Jakafi was not significantly different against RT-SHIV in rhesus macaque lymphocytes or macrophages. EC_{50} ranged from 0.02-0.35 μM and EC_{90} ranged from 0.32-3.1 μM.

Target of inhibitor	Target of inhibitor	EC_{50} Acutely infected Resting Mϕ (μM)	EC_{50} in Chronically infected Mϕ (μM)	EC_{50} in lymphocytes (μM)	IC_{50} in lymphocytes (μM)	IC_{50} in CEM cells (μM)	IC_{50} in Vero cells (μM)
NFAT	NFAT	1.4 ± 1.7	> 100	1.9	> 100 (0)	> 100 (8.5)	> 100 (13.1)
Commercial lck	lck kinase	2.5 ± 0.7	14.5 ± 2.8	0.03	9.5	10.3	38.2
Miltefosine	Akt	4.8 ± 1.8	> 100	18.8	22.4	3.4	27.6
Hyaluronin	Akt/NFkB	> 50	> 100	> 50	> 100 (3.9)	> 100 (0)	> 100 (5.9)
Dorapimod	p38 MAP kinase	15.5 ± 3.4	> 100	72.6	38.4	33.2	≥ 100 (45.4)
Merck Akt	Akt	31.4 ± 6.4		43.9 ± 1.9	18.5 ± 6.6	5.4 ± 5.7	21.7 ± 13.9
Tofacitinib	Jak	0.2 ± 0.08	0.3	0.08 ± 0.06	1.9 ± 0.8	> 100	> 100
Jakafi	Jak	0.3 ± 0.1	0.3	0.02 ± 0.05	2.1 ± 1.1	11.8 ± 9.8	29.3 ± 6.8

Table 3.5: Antiviral potency and toxicity of cellular factor inhibitors. Antiviral potency of inhibitors of NFAT, lck, PI3K/Akt, NFκB, p38MAPK, or Jak in macrophages or lymphocytes, and toxicity in lymphocytes, CEM, and Vero cells. EC_{50} (potency) ranged from 0.02- > 100 μM and IC_{50} (toxicity) ranged from 1.9 to > 100 μM, depending upon inhibitor tested.

A.

Compound	Lymphocytes $EC_{50/90}$ (μM)	MΦ $EC_{50/90}$ (μM)	MΦ IC_{50} (μM)	PHA stimulated lymphocytes IC_{50} (μM)	PHA + IL-2 stimulated lymphocytes IC_{50} (μM)	CEM cells IC_{50} (μM)	Vero cells IC_{50} (μM)
Cycloheximide	ND	ND	ND	0.65	0.57	< 0.1	< 0.1
RS-1294	0.08/1.9	0.2/1.9	> 100	2.1	1.2	> 100	> 100
RS-1374	0.02/0.32	0.3/4.8	20.8	9.5	2.4	11.8	29.3
RS-1531	1.1/10.1	0.1/1.8	19.7	3.8	2.5	16.3	36.6
RS-1532	1.3/2.5	0.3/4.2	13.4	0.56	0.4	1.2	10.1
RS-1533	2.8/11.1	0.09/1.3	26.9	2.1	1.04	4.2	22.9

ND = not determined

B.

Compound	Therapeutic Index
RS-1294	15
RS-1374	120
RS-1531	< 1
RS-1532	< 1
RS-1533	< 1

Table 3.6: Evaluation of potency and toxicity of various investigational Jak inhibitors (A) and corresponding therapeutic index (B). Jakafi (RS-1374) demonstrated the most favorable antiviral and toxicity profile of all compounds tested (A), and demonstrated the widest therapeutic window (120). Tofacitinib (RS-1294) also demonstrated a favorable antiviral and toxicity profile (A), and a therapeutic window of 15 (B). All other compounds tested demonstrated toxicity across either CEM, Vero, or lymphocytes (A), and had a therapeutic window of <1 (B).

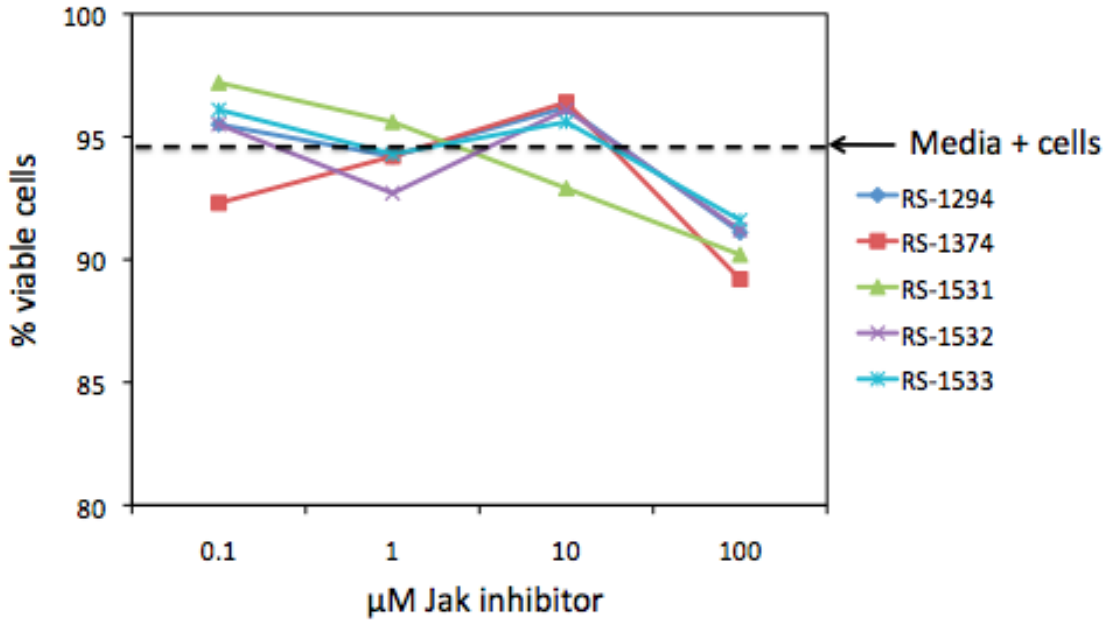


Figure 3.4: Viability of macrophages exposed to various concentrations of Tofacitinib (RS-1294), Jakafi (RS-1374), Astra Zeneca's AZD 1480 (RS-1531), Eli Lily's LY2784544 (RS-1532), or YM Bioscience's CYT387 (RS-1533). Viability was not significantly reduced *versus* macrophages maintained in drug-free medium after 5 days incubation of resting primary human macrophages with 0.1-100 µM drug.

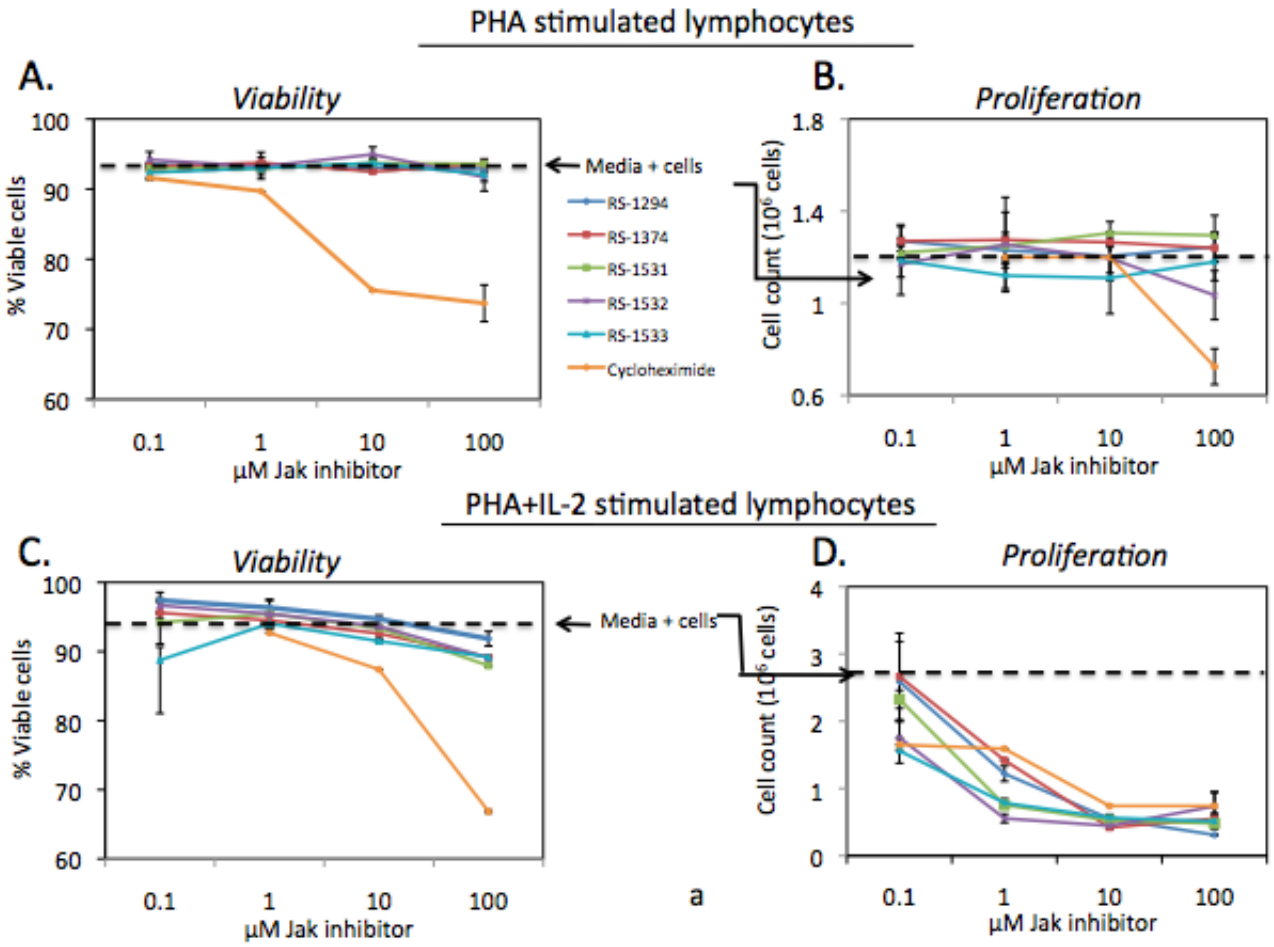


Figure 3.5: Viability and proliferation of PHA or PHA+IL-2 stimulated primary human lymphocytes. For PHA stimulated lymphocytes, viability and proliferation were not significantly different than that of cells exposed to media alone for all concentrations and for all drugs tested (A, B). For PHA+IL-2 stimulated lymphocytes, viability was not significantly different than that of cells exposed to media alone for all concentrations and all drugs tested (C), however proliferation was significantly inhibited by 1.0 µM for all drugs tested (D). RS-1294 is Pfizer's Tofacitinib, RS-1374 is Incyte's Jakafi, RS-1531 is Astra Zeneca's AZD1480, RS-1532 is Eli Lilly's LY2784544, and RS-1533 is YM bioscience's CYT387.

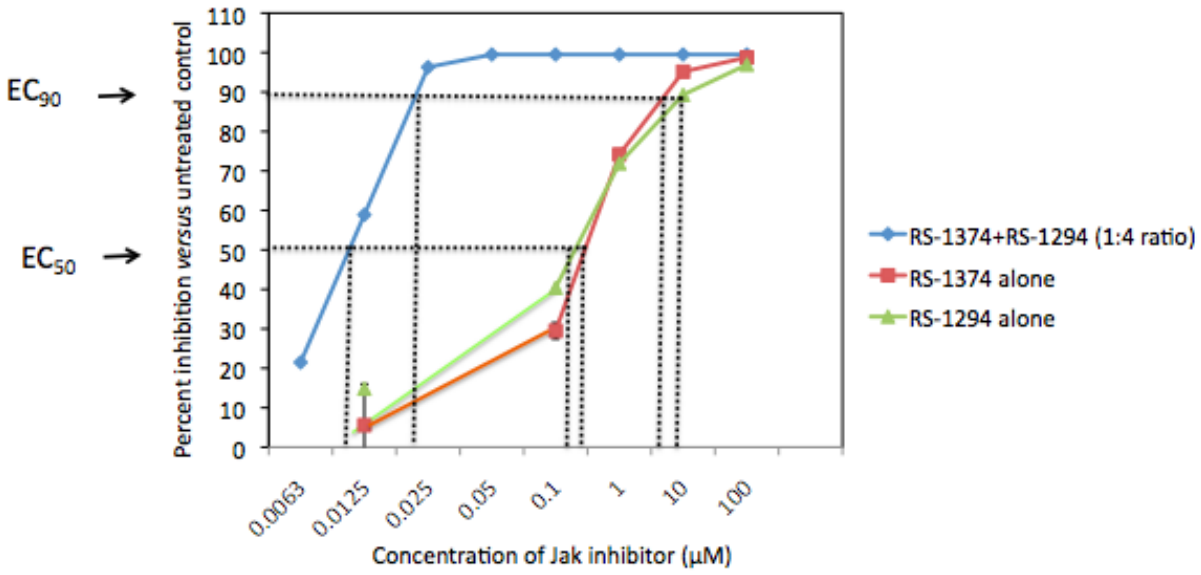


Figure 3.6: Assessment of antiviral potency of the combination of Jakafi+Tofacitinib. Co-incubation of Jakafi+ Tofacitinib (RS-1374+RS-1294) at a ratio of 1:4 decreases the EC_{50} and EC_{90} by 5-fold and 117-fold, respectively.

Code	Virus type	EC_{50} (μM)	EC_{90} (μM)	Fold Increase 50 (FI_{50})	Fold Increase 90 (FI_{90})
AZT Control	xxLAI	0.0001	0.005		
	K65R	0.0004	0.013	4	3
	L74V	0.02	0.061	200	12
RS-1294	xxLAI	.05	0.6		
	K65R	0.05	0.5	1	1
	L74V	0.43	5.4	9	9
RS-1374	xxLAI	0.14	3.9		
	K65R	0.13	2.4	1	2
	L74V	0.29	15.4	2	4

Table 3.7: Antiviral potency of Tofacibinib (RS-1294) and Jakafi (RS-1374) against drug resistant HIV-1 in primary human lymphocytes. Tofacibinib and Jakafi display similar potency against nucleoside analog-resistant mutations K65R and L74V mutations. L74V may confer a decrease in potency, however this experiment is an n = 1, and must be repeated to determine deviation of inter-assay variability.

Id	Identity of compound	Jak 1 IC_{50} (nM)	Jak 2 IC_{50} (nM)	Jak 3 IC_{50} (nM)	Tyk2 IC_{50} (nM)
RS-1294	Pfizer's Tofacitinib	24	57	69	609
RS-1374	Incyte's Jakafi	2.9	2.0	323	21
RS-1531	Astra Zeneca's AZD1480	36	44	2383	220
RS-1532	Eli Lilly's LY2784544	7.4	0.4	161	19
RS-1533	YM Bioscience's CYT387	81	48	1936	86
INCB018424	Jakafi provided by Incyte3.7	3.7	2.6	384	19
INCB028050	Unknown Incyte compound	4.3	3.6	434	51

Table 3.8: Jak1, Jak2, Jak3, or Tyk2 inhibition by various investigational Jak inhibitors. All compounds tested inhibited Jak1, Jak2, Jak2, and Tyk2, with IC_{50} values ranging from 0.4-2,383 nM. RS-1294 inhibits Jak/Tyk2 in the following rank order: Jak1 > Jak2 > Jak3 > Tyk2. RS-374 inhibits Jak/Tyk2 in the following rank order: Jak2 > Jak1 > Tyk2 > Jak3. RS-1531 inhibits Jak/Tyk2 in the following rank order: Jak1 > Jak2 > Tyk2 > Jak3. RS-1532 inhibits Jak/Tyk2 in the following rank order: Jak2 > Jak1 > Tyk2 > Jak3. RS-1533 inhibits Jak/Tyk2 in the following rank order: Jak2 > Jak1 > Tyk2 > Jak3. These data were obtained in collaboration with Incyte.

Drug	Acute infection in rhesus macaque macrophages $EC_{50/90}$ (μM)	Acute infection in rhesus macaque lymphocytes $EC_{50/90}$ (μM)	Acute infection in human lymphocytes $EC_{50/90}$ (μM)	Acute infection in human macrophages $EC_{50/90}$ (μM)
AZT	0.083/0.921	0.002/0.03	0.002/0.05	0.03/0.5
(-)FTC	0.042/0.35	ND	0.001/0.02	0.08/1.0
RS-1294	0.27/3.1	0.3/2.9	0.08/1.9	0.2/1.9
RS-1374	0.35/4.2	0.09/1.27	0.02/0.32	0.3/4.8

Table 3.9: Antiviral potency of Tofacibinib (RS-1294) and Jakafi (RS-1374) against RT-SHIV in primary rhesus macaque lymphocytes and macrophages. Tofacitinib and Jakafi demonstrate similar potency in human *versus* rhesus macaque macrophages and lymphocytes, with EC_{50} ranging from 0.02-0.35 μM and EC_{90} ranging from 0.32-3.1 μM.

Inhibition of reactivation of latent HIV-1 in primary human lymphocytes:

Jak inhibitors Tofacitinib (RS-1294), Jakafi (RS-1374), Astra Zeneca's AZD 1480 (RS-1531), Eli Lily's LY2784544 (RS-1532), or YM Bioscience's CYT387 (RS-1533) were applied at various concentrations to latently infected primary human lymphocytes that were stimulated to reactivate HIV-1 infection by CD3/CD28 mAb treatment [43]. The effect of each compound to inhibit reactivation was measured by intracellular staining for p24 via flow cytometry. Tofacitinib inhibited reaction at concentrations between 10-100 μM, Jakafi inhibited reactivation at concentrations between 1.0-10 μM, AZD1480 inhibited reactivation at concentrations between 10-100 μM, and LY2784544 and CYT387 inhibited reactivation at concentrations between 1.0-10 μM (Figure 3.7).

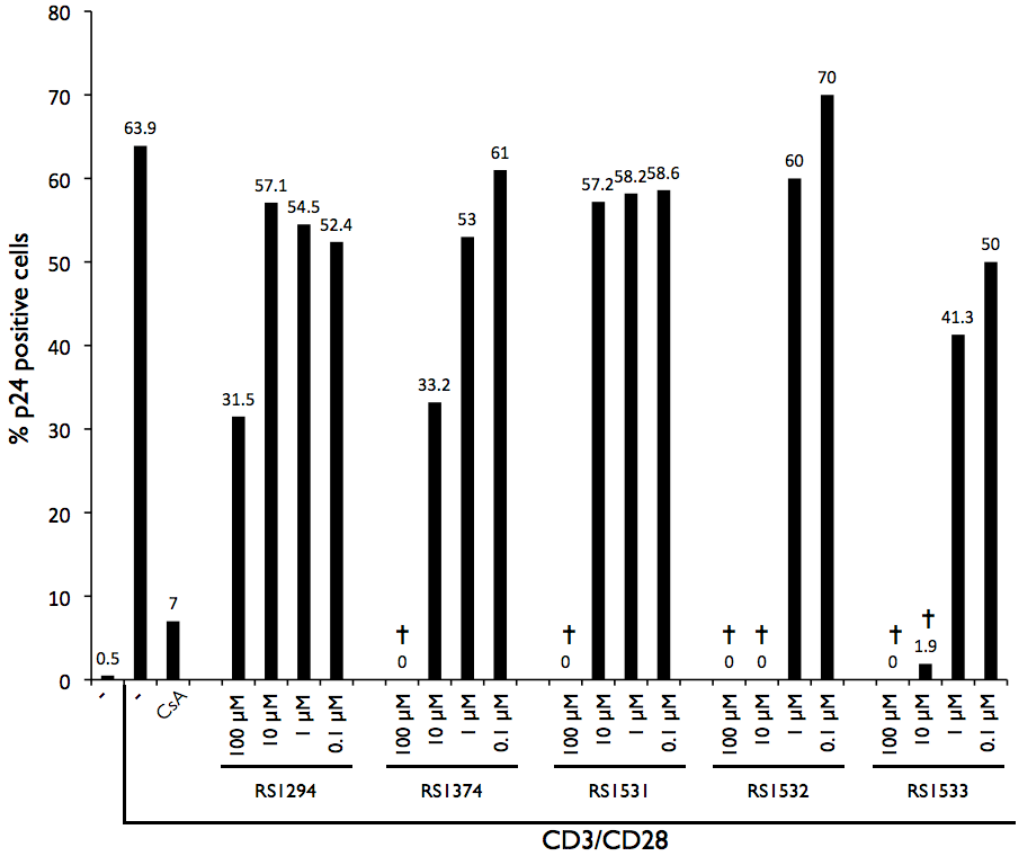


Figure 3.7: Inhibition of reactivation of latent HIV-1 in primary human lymphocytes. Jak inhibitors Tofacitinib (RS-1294), Jakafi (RS-1374), Astra Zeneca's AZD 1480 (RS-1531), Eli Lily's LY2784544 (RS-1532), or YM Bioscience's CYT387 (RS-1533) were applied at various concentrations to latently infected primary human lymphocytes that were stimulated to reactivate HIV-1 infection by CD3/CD28 mAb treatment [43]. Reactivation was measured by intracellular p24 production (via flow cytometry). Tofacitinib inhibited reaction at concentrations between 10-100 µM, Jakafi inhibited reactivation at concentrations between 1.0-10 µM, AZD1480 inhibited reactivation at concentrations between 10-100 µM, and LY2784544 and CYT387 inhibited reactivation at concentrations between 1.0-10 µM. Control of CsA (cyclosporine A), a calcinurin inhibitor, demonstrated potent inhibition of reactivation. Data obtained in collaboration with Dr. V. Planelles group at the University of Utah.

Levels and ratios of dNTP/rNTP in activated and resting lymphocytes and macrophages:

Known amounts of $^{13}C/^{15}N$-labeled individual standards were used to determine dNTP and rNTP quantities in the samples with LC-MS/MS, and the cellular nucleotide concentrations were calculated using their cell volumes, which were previously reported [13]. Macrophages were found to harbor a much lower dNTP concentration *versus* lymphocytes, independent of activation state. The dNTP concentration in macrophages is 22–133-fold lower than in lymphocytes (Figure 3.8 A, B, Table 3.10). In contrast, the rNTP concentration in macrophages was only 4–7-fold lower than lymphocytes, independent of activation state (Figure 3.8 C, D, Table 3.10), supporting the idea that non-dividing cells still maintain high rNTP concentrations, presumably due to multiple roles of rNTPs in various cellular events, such as transcription, cell signaling, and cellular metabolism, which also occur in non-dividing cells.

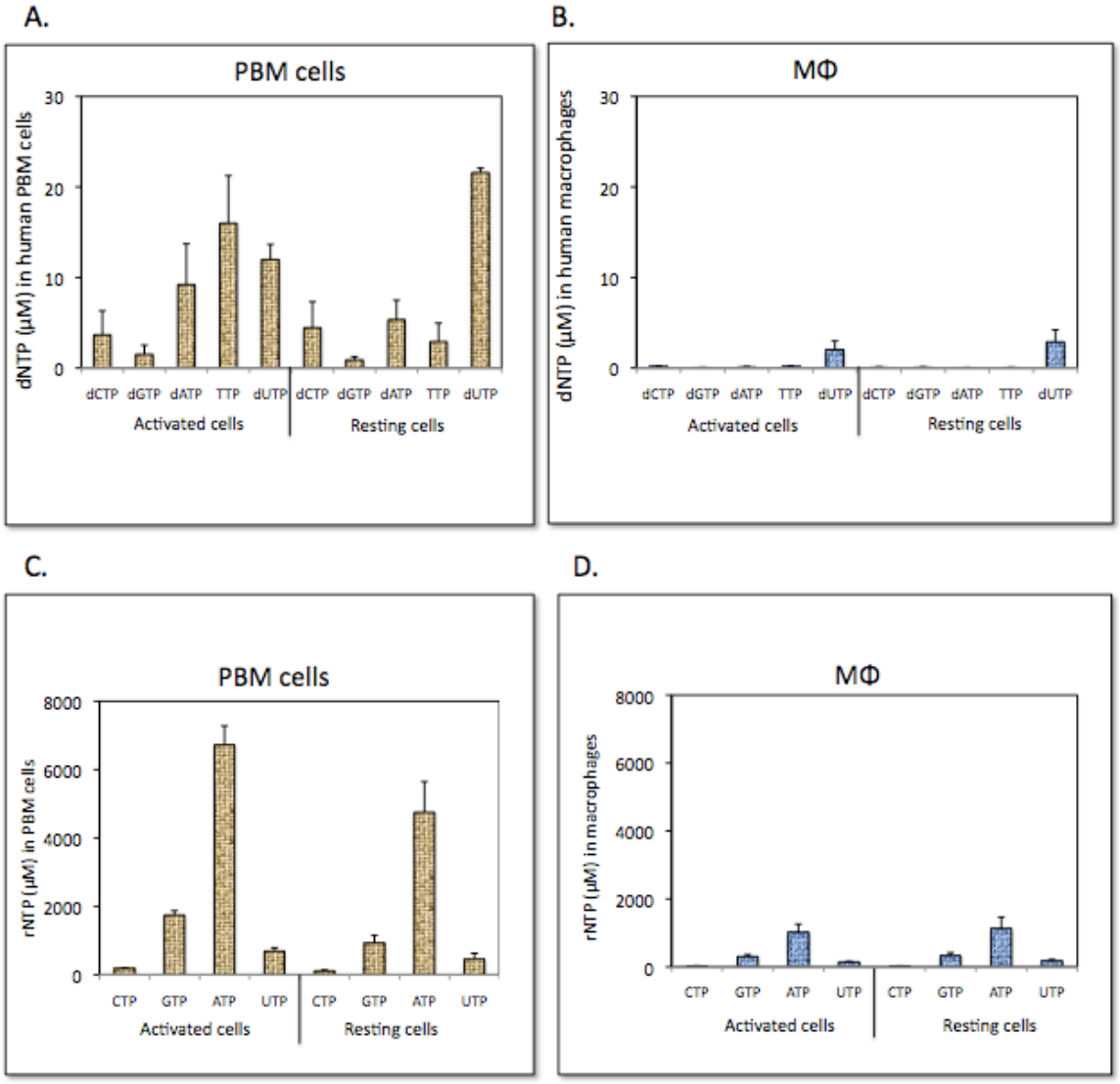


Figure 3.8: Concentrations of dNTP or rNTP in activated or resting lymphocytes or macrophages. dNTP are 22-133 fold lower in macrophages *versus* lymphocytes (A, B), but rNTP are only 4-7 fold lower in macrophages *versus* lymphocytes (C, D) independent of activation state.

	dCTP	dGTP	dATP	TTP	dUTP
			μM		
Activated lymphocytes	3.67 ± 2.65	1.52 ± 1.01	9.22 ± 4.5	16.0 ± 5.25	11.99 ± 1.67
Activated macrophages	0.15 ± 0.10	0.05 ± 0.03	0.10 ± 0.07	0.15 ± 0.10	2.04 ± 9.54
Fold difference between activated lymphocytes *versus* activated macrophages	25	30	92	107	6
Resting lymphocytes	4.46 ± 2.86	0.91 ± 0.35	5.32 ± 2.18	2.92 ± 2.04	21.60 ± 0.48
Resting macrophages	0.07 ± 0.05	0.07 ± 0.05	0.04 ± 0.03	0.05 ± 0.04	2.88 ± 1.34
Fold difference between resting lymphocytes *versus* resting macrophages	64	13	133	58	8

	CTP	GTP	ATP	UTP
		μM		
Activated lymphocytes	182 ± 24	1,745 ± 128	6,719 ± 560	690 ± 100
Activated macrophages	27 ± 8	303 ± 60	1,011 ± 247	141 ± 17
Fold difference between activated lymphocytes *versus* activated macrophages	7	6	7	5
Resting lymphocytes	111 ± 30	923 ± 234	4,753 ± 896	453 ± 174
Resting macrophages	25 ± 8	323 ± 95	1,124 ± 339	173 ± 47
Fold difference between resting lymphocytes *versus* resting macrophages	4	3	4	3

Table 3.10: Concentrations of dNTP or rNTP in activated or resting lymphocytes or macrophages. Concentrations (μM) of dNTP or rNTP are 22-133 fold lower in macrophages *versus* lymphocytes (top panel), but rNTP are only 4-7 fold lower in macrophages *versus* lymphocytes (bottom panel) independent of activation state.

DNA synthesis profiles of HIV-1 RT in cellular dNTP pools of lymphocytes or macrophages:

The dNTP concentration-dependent DNA synthesis profile of HIV-1 RT was investigated in the presence of dNTP and rNTP pools intended to simulate the cellular microenvironments of macrophages *versus* lymphocytes. As shown in Figure 3.9, the polymerase reaction of HIV-1 RT with dNTPs alone did not generate any visible bands in the absence of rNTP, which was expected. However, fully extended, radiolabeled products (F) were detected in reactions performed using simulated macrophage dNTP/rNTP pools (Figure 3.9). In contrast, no radiolabeled extension products were detected in reactions performed using simulated lymphocyte dNTP/rNTP pools (Figure 3.9). Control experiments with a 5'-end ^{32}P-labeled primer confirmed the efficient extension of the primer and the production of full-length products in both the simulated lymphocyte and simulated macrophage environments (Figure 3.10). Thus, the absence of a radiolabeled extension product under the lymphocyte condition in Figure 3.9 reflects a lack of rNTP incorporation and not a failure to complete DNA synthesis. Quantification of the radioactively labeled extended products in Figure 3.9 revealed that rNTP incorporation in the simulated macrophage environment was 22 times more efficient than in the simulated lymphocyte environment.

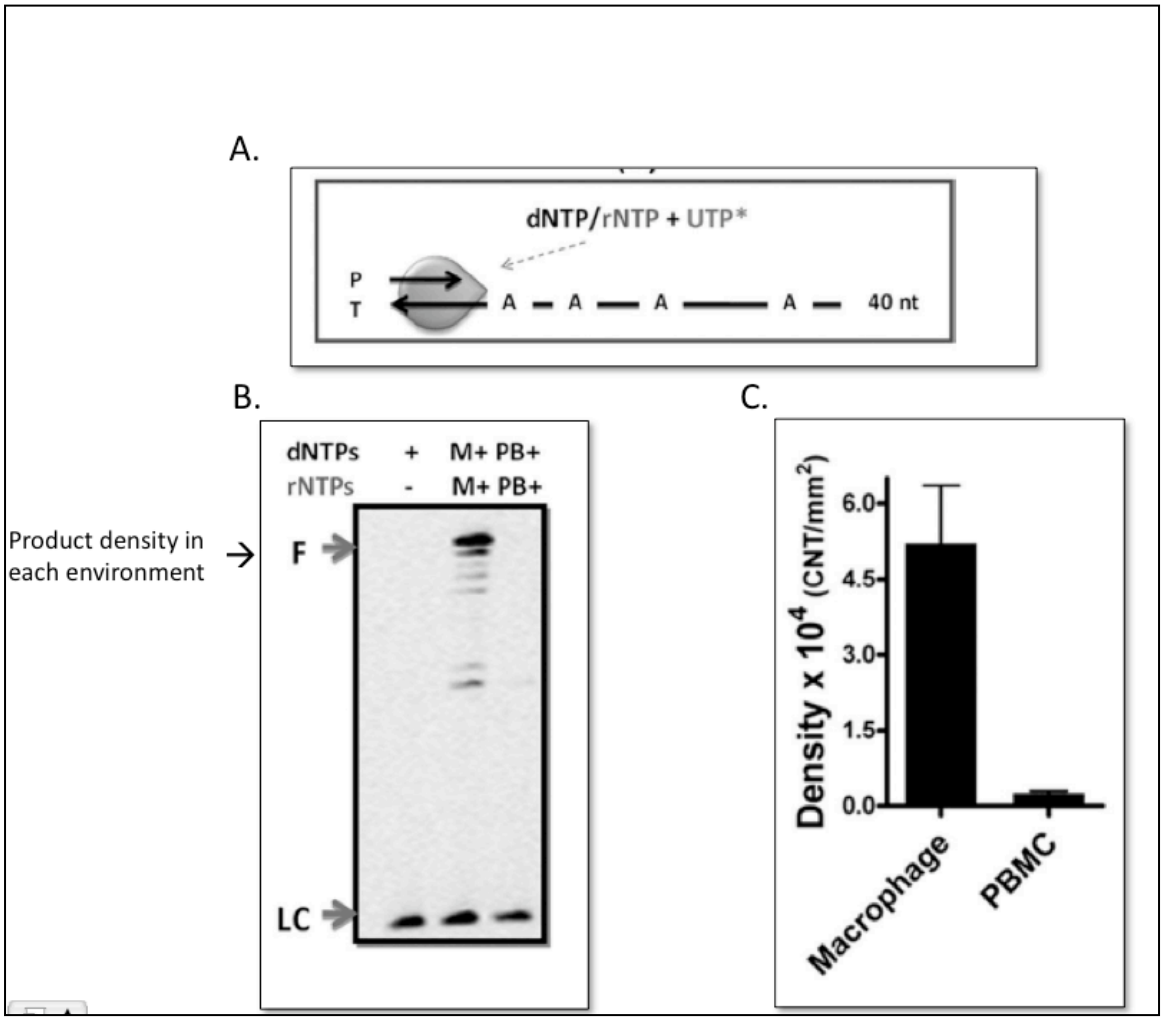


Figure 3.9: rNTP are more efficiently incorporated into the growing viral DNA strand in the macrophage but not the lymphocyte simulated environment. Polymerase reaction of HIV-1 RT with dNTPs alone (reaction in A) did not generate any visible bands in the absence of rNTP, as expected. However, fully extended, radiolabeled products (F) were detected in reactions performed using simulated macrophage dNTP/rNTP pools (B, C). In contrast, no radiolabeled extension products were detected in reactions performed using simulated lymphocyte dNTP/rNTP pools (B, C).

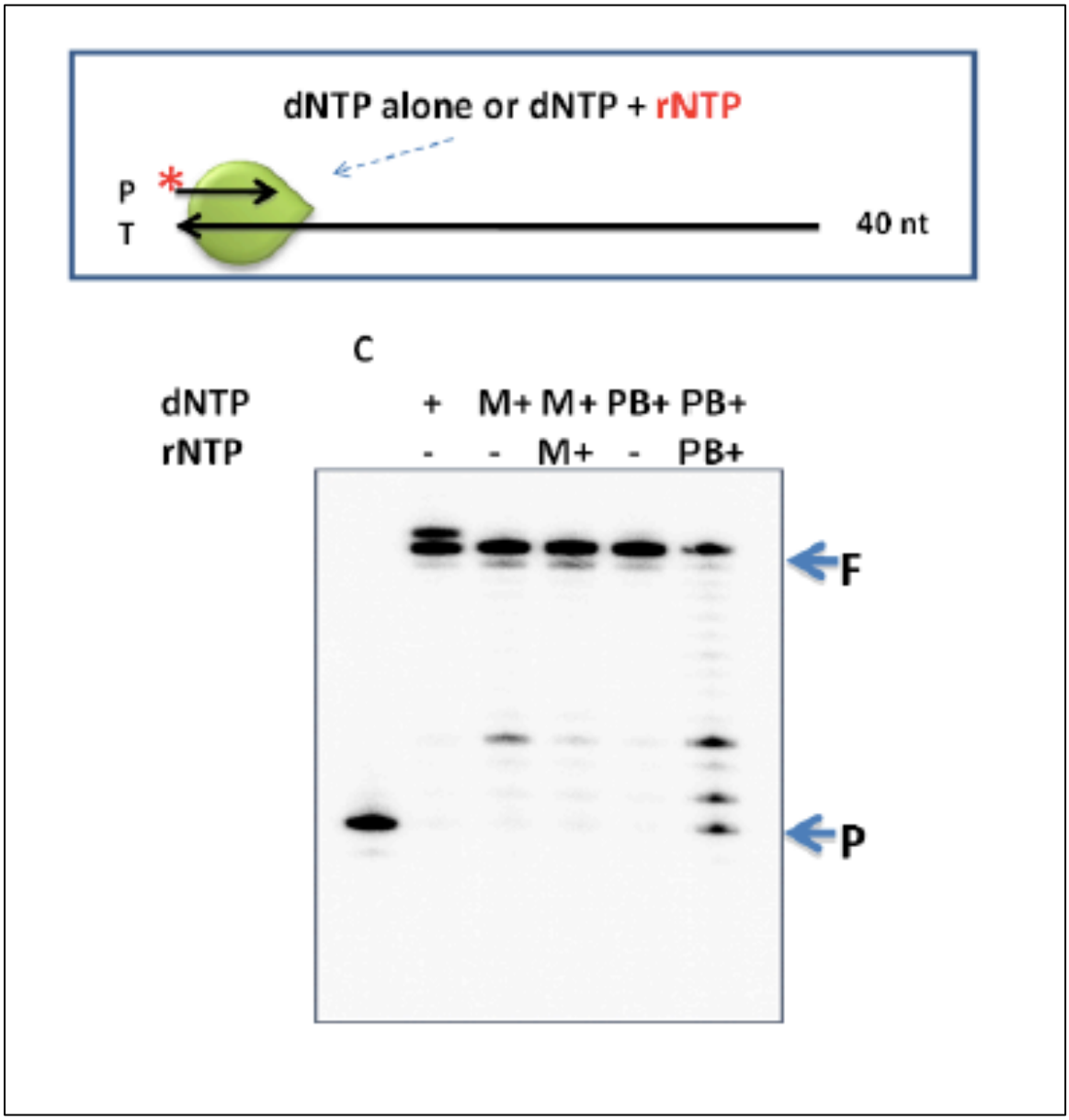


Figure 3.10: Efficient extension of primer to confirm DNA synthesis under conditions used in Figure 3.9. Control experiments with a 5'-end ^{32}P-labeled primer confirmed the efficient extension of the primer and the production of full-length products in both the simulated lymphocyte and simulated macrophage environments.

Antiviral potency of ribonucleoside inhibitors in lymphocytes and macrophages:

An existing library of ribonucleoside inhibitors from the Laboratory of Biochemical Pharmacology were subjected to toxicity (CEM, Vero, lymphocytes, and macrophages) and potency (lymphocytes and macrophages) screening (Table 3.11).

Two ribonucleoside inhibitors, RS-1285 and RS-1292, demonstrated potent inhibition of viral replication in a dose dependent manner in the in vitro assay for antiviral potency in macrophages (EC_{50} of 19.8 ± 7.2 μM and 5.5 ± 4.4 μM), but were not potent in lymphocytes (EC_{50} > 100 μM). Drugs were not toxic (IC_{50} > 100 μM in Vero, CEM, primary human lymphocytes, or macrophages) (Table 3.11).

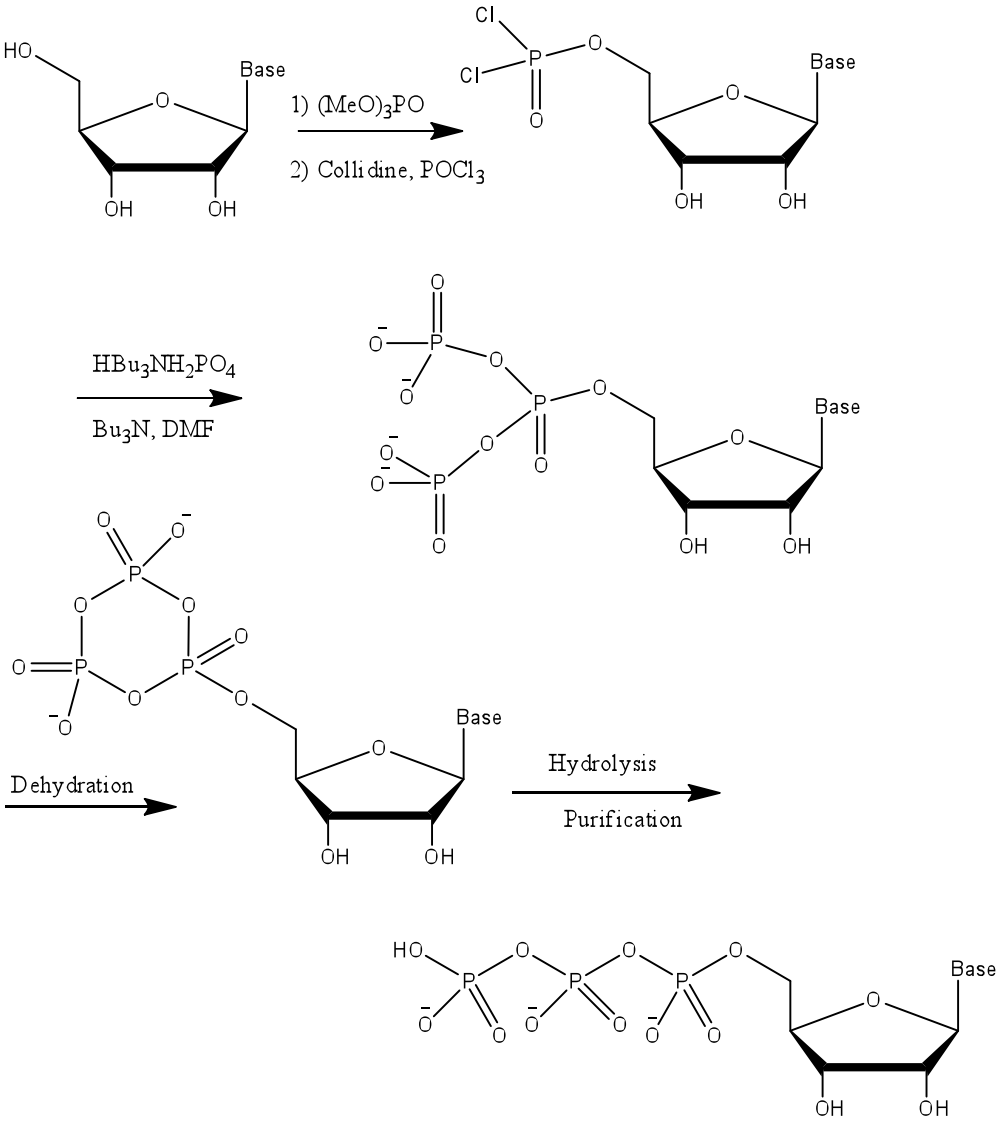
Target of inhibitor	EC_{50} Acutely infected resting macrophages (μM)	EC_{50} in chronically infected macrophages (μM)	EC_{50} in lymphocytes (μM)	IC_{50} in VERO cells (μM)	IC_{50} in CEM cells (μM)	IC_{50} in lymphocytes (μM)
Drug A	> 100	> 100	> 100	> 100	> 100	> 100
Drug B	> 100	> 100	> 100	> 100	> 100	> 100
Drug C	> 100	> 100	> 100	> 100	> 100	> 100
Drug D	> 100	> 100	> 100	> 100	> 100	> 100
Drug E	> 100	> 100	> 100	> 100	> 100	> 100
Drug F	> 100	> 100	> 100	> 100	> 100	> 100
Drug G	> 100	> 100	> 100	> 100	> 100	> 100
Drug H	> 100	> 100	> 100	> 100	> 100	> 100
Drug I	> 100	> 100	> 100	> 100	> 100	> 100
Drug J	> 100	> 100	> 100	> 100	> 100	> 100
Drug K	> 100	> 100	> 100	> 100	> 100	> 100
Drug L	> 100	> 100	> 100	> 100	> 100	> 100
Drug M	> 100	> 100	> 100	> 100	> 100	> 100
Drug N	> 100	> 100	> 100	> 100	> 100	> 100
Drug O	> 100	> 100	> 100	> 100	> 100	> 100
Drug P	**19.8 ± 7.2**	**> 100**	**> 100**	**> 100**	**> 100**	**> 100**
Drug Q	**5.5 ± 4.4**	**> 100**	**> 100**	**> 100**	**> 100**	**> 100**
Drug R	> 100	> 100	> 100	> 100	> 100	> 100

Table 3.11: Antiviral potency and toxicity screen of ribonucleoside inhibitors from the Laboratory of Biochemical Pharmacology. Existing ribonucleoside inhibitors from the Laboratory of Biochemical Pharmacology were subjected to antiviral potency (macrophages, lymphocytes), and toxicity (lymphocytes, CEM, Vero cells). Two ribonucleoside inhibitors, RS-1285 (drug P), and RS-1292 (drug Q) demonstrated selective potency against HIV-1 infection in macrophages but not lymphocytes (19.8 ± 7.2 and 5.5 ± 4.4 μM, respectively in macrophages).

Synthesis of triphosphorylated RS-1285 and RS-1292 for use in cell free biochemical assays:

Synthesis of triphosphorylated RS-1292 and RS-1285 were performed as previously described [45]. Synthesis of RS-1292-TP was successful, however synthesis of RS-1285-TP could not be performed yet. Further analysis revealed that RS-1285 was degraded both before and during the process of triphosphate formation, preventing triphosphate synthesis of RS-1285-TP (Figure 3.11).

Nucleoside Triphosphates



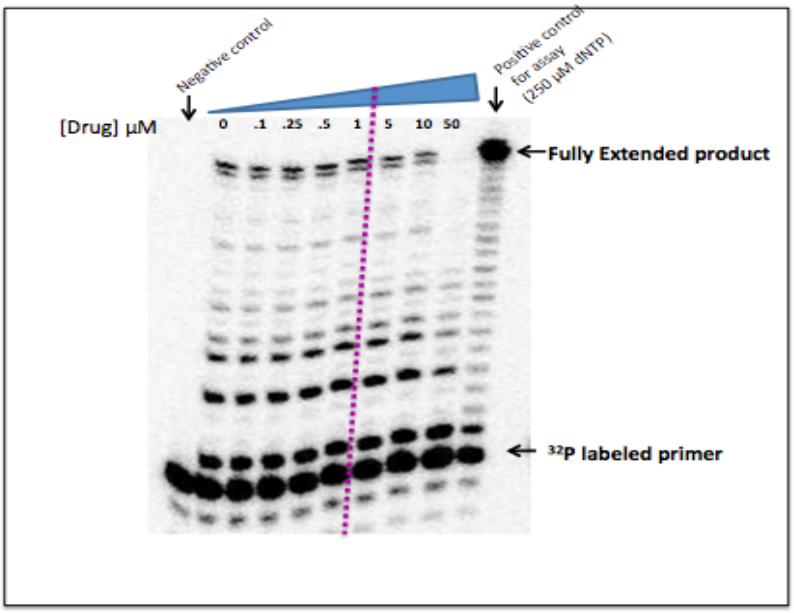
Ref : *Chem. Rev.* **2000**, 100, 2047-2059

Figure 3.11: Synthesis of triphosphorylated ribonucleosides. The above synthesis methodology was used to synthesize the triphosphate of RS-1292 (RS-1292-TP).

DNA synthesis profiles of HIV-1 RT in cellular dNTP pools of macrophages in the presence of RS-1292-TP:

The dNTP concentration-dependent DNA synthesis profile of HIV-1 RT was investigated in the presence of dNTP and rNTP pools intended to simulate the cellular microenvironments of macrophages *versus* lymphocytes. RS-1292-TP inhibits HIV-1RT mediated DNA synthesis in the macrophage dNTP/rNTP simulated microenvironment in a dose dependent manner, beginning at 5 μM, with complete inhibition observed at 50 μM (Figure 3.12).

A.



B.

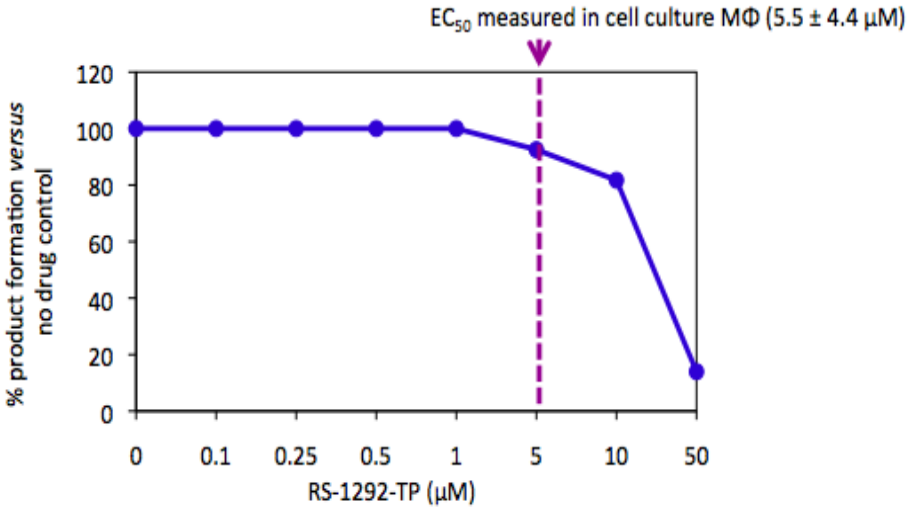
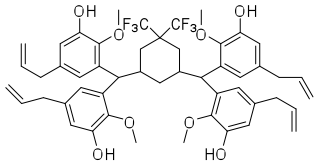
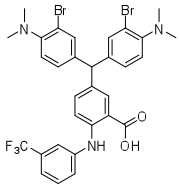
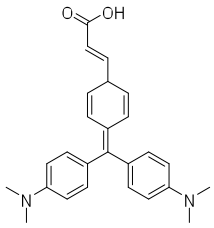
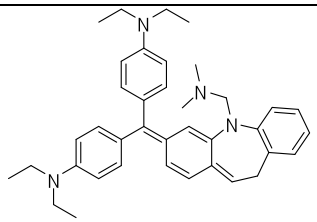
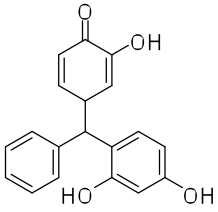
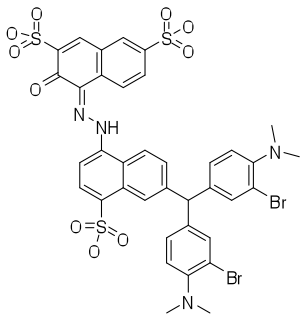
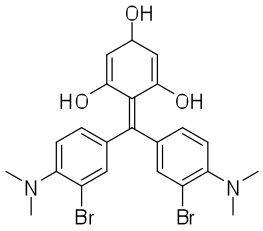


Figure 3.12: RS-1292-TP inhibits HIV-1 RT mediated DNA synthesis in the macrophage dNTP/rNTP simulated environment. RS-1292-TP completely inhibits HIV-1 RT mediated DNA synthesis at 50 µM, with inhibition observed beginning at 5 µM (A, B). Percentage inhibition of HIV-1 RT mediated DNA synthesis.

Antiviral potency and toxicity of Fosamax, Boniva, or investigational MDA provided by collaborator:

Both Fosamax and Boniva were potent inhibitors of HIV-1 infection in macrophages (EC_{50} < 0.1 μM), but were not potent against HIV-1 infection in lymphocytes (EC_{50} > 50 μM), and were not toxic across lymphocytes, CEM, or Vero cells (IC_{50} 9.8 - > 100 μM) (Table 3.12). Most investigational inhibitors demonstrated weak potency against HIV-1 infection in macrophages (EC_{50} 0.03- > 100 μM) but demonstrated narrow therapeutic windows and were toxic in most cell types tested (IC_{50} < 1.0 μM in at least 1 cell type for most drugs) (Table 3.12, Table 3.13). Interestingly, three investigational inhibitors, JA-075, 076, and 081 demonstrated low micromolar potency against both acute and chronic HIV-1 infection in macrophages only, and were not toxic in lymphocytes, CEM, or Vero cells (Table 3.13, red highlight).

Compound	EC_{50} Acutely infected Resting Mϕ (μM)	$EC_{50/90}$ Acutely infected lymphocytes (μM	IC_{50} in lymphocytes (μM)	IC_{50} in Vero cells (μM)	IC_{50} in CEM cells (μM)
Boniva	< 0.2	> 50	43.9	9.8	> 100 (47.5)
Fosamax	< 0.1	> 50	> 100 (4.5)	57.5	> 100 (0)
JA-057 	45.4	> 100 (24.5)	> 100 (26.4)	45.8	> 100 (11.4)
JA-058 	13.8	> 100 (40.7)	> 100 (29.6)	23.1	27.3
JA-059 	2.9	4.0/19.1	10.9	< 1 (96.7)	< 1 (53.4)
JA-060	1.8	0.36/1.7	< 1 (80.8)	< 1 (97.3)	1.4

					
JA-061 	2.7	7.2/56.2	> 100 (29.6)	2.9	4.7
JA-062 	18.9	32.5/> 100 (58.8)	> 100 (0)	48	> 100 (0.7)
JA-063 	0.07	0.14/1.8	> 100 (29.6)	< 1 (96.3)	< 1.0 (90.9)
JA-064	1.2	7.2/51.1	> 100 (29.6)	< 1 (77.7)	1.2

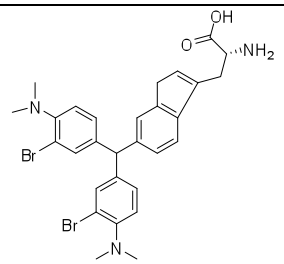
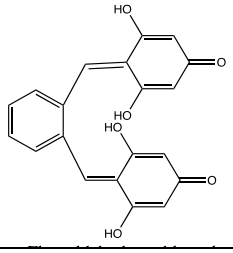
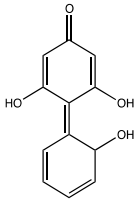
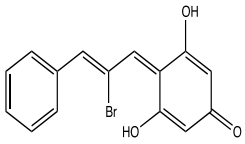
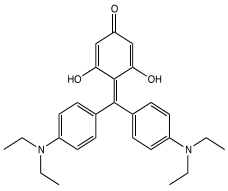
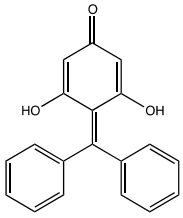
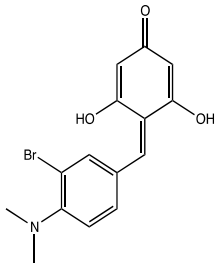
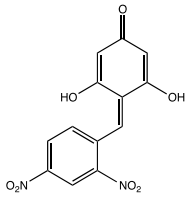
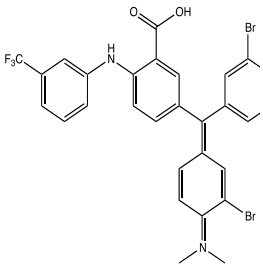
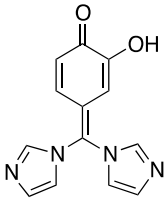
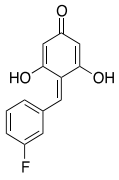
					

Table 3.12: Antiviral potency and toxicity of various macrophage depleting agents. Fosamax and Boniva, two FDA approved therapies were potent and selective inhibitors of HIV-1 replication in macrophages but not lymphocytes, and were not toxic in lymphocytes, CEM, or Vero cells. Investigational MDA agents demonstrated a modest antiviral activity against HIV-1 infection in macrophages, with a narrow therapeutic window and were toxic across in either CEM, Vero, or lymphocytes. Number in parenthesis indicates percentage inhibition observed at the concentration indicated; for example 100 (24.2) indicates that at 100 μM, 24.2 % inhibition was observed.

Name of Inhibitor	Structure	EC_{50} Acutely infected MΦ (μM)	EC_{50} in Chronically infected MΦ (μM)	EC_{50} in PBM cells (μM)	IC_{50} in PBM cells (μM)	IC_{50} in CEM cells (μM)	IC_{50} in Vero cells (μM)
JA-069 fluroaldehyde + phloroglucinol		16.4	17.8	45.4	> 100	> 100	> 100
JA-070 catechol + phloroglucinol		> 50	> 50	> 100	> 100	> 100	> 100
JA-071 alpha bromocinnamaldehyde +phloroglucinol		26.7	38.5	> 100	> 100	> 100	> 100
JA-072 ethyl michler ketone + phloroglucinol		0.9	1.7	2.4	3.7	2.9	100
JA-073 2,4-dihyfroxybenzophenone		> 50	> 50	> 100	> 100	> 100	> 100

JA-074 BrDimethylaminobenzaldehyde		12.5	16.4	29.2	80.2	26.6	> 100
JA-075 2,4 dinitrobenzaldehyde + phloroglucinol		0.8	0.3	1.2	> 100	> 100	> 100
JA-076 flufenamic acid + Brmmk		0.7	1.1	3.6	36.4	25.9	> 100
JA-077 carbonyldiimidazole + catechol		> 50	> 50	> 100	> 100	> 100	> 100
JA-078 3-fluorobenzaldehyde		3.9	14.5	29.0	31.5	52.4	> 100
JA-079 carbonydiimidazole + imipramine		2.7	6.5	24.4	15.4	16.2	24.2

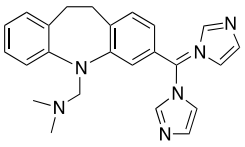
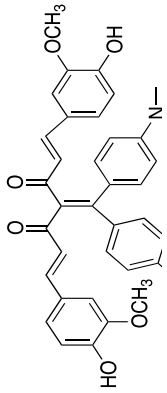
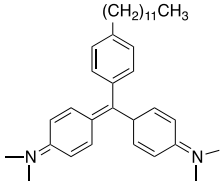
JA-079 carbonydiimidazole + imipramine		2.7	6.5	24.4	15.4	16.2	24.2
JA-080 curcmin + MMK		0.2	0.3	1.4	< 1 (68.0)	< 1 (92.8)	7.0
JA-081 dodecylbenzene		**1.1**	**0.9**	**4.5**	6.5	3.8	50.4

Table 3.13: Antiviral potency and toxicity of investigational MDA agents. Most compounds demonstrated a narrow therapeutic window, however three compounds, JA-075, JA-076, and JA-081 (red), demonstrated low micromolar inhibition of HIV-1 replication against both acute and chronic infection in macrophages and were not toxic in lymphocytes, CEM cells, or Vero cells.

Discussion

Understanding the complex relationship between macrophages or lymphocytes, HIV-1, and approved or investigational anti-HIV therapies necessitates a multi-faceted approach to assess each variable. Obstacles such as delivery of adequate levels of drug to inhibit viral replication, cell-specific factors that can alter potency or provide grounds for design of cell-specific inhibitors, or activation of pro-HIV pathways demonstrate that therapies aimed at eradication must consider all of these caveats.

Discussion about intracellular drug concentrations and antiviral potency of nucleoside analogs and RAL:

The importance of delivering adequate levels of antiretroviral drug to permissive HIV-1 target cells in all viral reservoirs is a primary goal for the development of regimens for eventual viral eradication. Certain organ sites including the brain and genitourinary tissues are more shielded from drug delivery. However, antiretroviral agents may also demonstrate non-uniform potency in the various permissive cell types. Therefore, this mechanistic study was performed to investigate the potency of antiretroviral agents in macrophages, since these cells are believed to be the initial cells to become infected during HIV transmission in the majority of individuals, and may be involved in the infection of more shielded organ sites including tissues behind the blood-brain barrier. The antiretroviral potencies in macrophages were compared to potencies in activated lymphocytes since they are the most studied cells. These data suggest that certain cells including macrophages may require higher concentrations of certain antiretroviral agents than those needed to inhibit infection in activated lymphocytes.

Intracellular concentrations of all nucleoside analogs except TFV were 5-198-fold lower in macrophages than in activated lymphocytes independent of activation state, and that constitutively activated acutely-infected cells displayed significantly diminished

antiviral potency (EC_{50} 0.4 - 9.42 μM *versus* 0.03 - 0.40 μM, respectively). These data are supported by previous studies (Perno *et al* and Aquaro *et al*), on antiviral potency of nucleoside analogs in chronically-infected macrophages, and are also in agreement with reports demonstrating the potency of protease inhibitors against chronically and acutely infected lymphocytes and macrophages. The cellular pharmacology studies also demonstrated for the first time that the intracellular concentration of RAL, an integrase inhibitor, is significantly lower in macrophages *versus* lymphocytes, independent of activation state, and that RAL is not potent against chronic HIV-1 infection in macrophages, similar to the profile displayed by nucleoside analogs. In contrast to the nucleoside analogs tested, RAL did not display any difference in potency against acute HIV-1 infection in resting *versus* activated macrophages.

A primary mechanism of action for nucleoside analog-TP is through competition with the natural dNTP and rNTP (in lymphocytes and macrophages, respectively), for HIV-1 RT [14, 44]. Therefore, it is likely that the reduced potency of these agents in macrophages could result from differing concentrations of natural 2'-deoxynucleoside-5'-triphosphates dNTP (e.g., (dNTP), namely, dCTP, dTTP, dATP or dGTP, or dUTP), and rNTP near the active site of HIV-1 RT, in activated lymphocytes relative to macrophages. Activated lymphocytes are considered replicating cells, while macrophages are primarily in a resting G1 state. Therefore, the relative concentrations of dNTP tend to be higher in activated lymphocytes since they are needed for DNA replication. Recent reports demonstrate macrophages harbor 22-133 fold lower dNTP concentrations and a greater disparity between rNTP and dNTP concentrations than dividing target cells [40, 44].

The data herein demonstrate that activation state of macrophages does not significantly alter intracellular concentrations of nucleoside analogues and that activation state prior to acute infection does not significantly alter antiviral potency. We further demonstrate that constitutive exposure to m-CSF (to ensure a constitutively activated

phenotype), significantly decreases the potency of nucleoside analogs. These results could be explained by m-CSF differentially modulating the cellular milieu resulting in different cytokine-based events that could alter antiviral potency independent of influencing the cellular pharmacology, of nucleoside analogs. M-CSF is secreted by macrophages upon HIV-1 infection, and has been implicated as a factor that promotes infection and triggers a Th2 pro-inflammatory cytokine milieu, namely TNF-α and IFN-γ, that is associated with increased viral loads, decreased CD4 T cell counts, and progression to AIDS. A cytokine-based mechanism that impacts viral replication independent of cellular pharmacology could explain the differential potencies of antiretroviral agents in activated *versus* resting macrophages [27].

As expected, the accumulation of AZT-TP, a cell cycle dependent nucleoside analogs was significantly (3.5-fold) higher in activated lymphocytes *versus* resting lymphocytes ($p < 0.05$). In contrast, ABC- or CBV-treated lymphocytes demonstrated a 2.0-fold increase in intracellular CBV-TP in resting *versus* activated lymphocytes ($p < 0.01$), which is presently unexplained. However, the metabolism of these agents is complex. For instance, ABC is converted to ABC-MP via adenosine phosphotransferase, which is then converted to CBV-MP *via* various cytosolic enzymes, and then to CBV-TP via cellular kinases. An alternate pathway of metabolism is conversion of ABC to 6-amino-CBV, then phosphorylation to 6-amino-CBV-MP by AMP deaminase, and subsequent phosphorylation to CBV-TP by cellular kinases. Alternatively, 6-amino-CBV can be converted to CBV by adenosine deaminase, and then to CBV-MP by 5'-nucleotidase, an inosine phosphotransferase [46]. Therefore, modulation of the activation state of the cell may alter the levels of various enzymes responsible for the complex intracellular metabolism of ABC or CBV to their active metabolite, CBV-TP, in lymphocytes and macrophages.

TFV was the only nucleoside analog tested which did not display significantly lower concentrations of active nucleotide concentrations in macrophages than in lymphocytes. TFV is positively charged. Therefore, it is possible that the effect of its polarity may be reduced in macrophages, since they demonstrate a high degree of phagocytic activity that could bypass traditional mechanisms of entry. The prodrug TDF is lipid soluble and masks the positive charges of TFV. Therefore, it is not surprising that TFV-DP were significantly higher following TDF than TFV incubation (Panel D and E, Figure 3.3, Table 3.3). TFV is converted to TFV-MP by AMPK, and to TFV-DP via NDPK or creatine kinase [12, 47]. TDF follows this same metabolic pathway, but is rapidly converted to TFV by carboxylesterase or phosphodiesterase. TFV circumvents the need for carboxylesterase or phosphodiesterase to convert TDF to TFV. :However, since these enzymes are not rate limiting, it is unlikely that these enzymes are responsible for the lack of significant difference in TFV-DP across lymphocytes in macrophages. It is more likely that lower levels of TFV, conferred by differences in polarity and charge of TFV *versus* TDF, confer lower enzymatic activity and rate of conversion of TFV to its active metabolite, TFV-DP.

Since AZT and DXG demonstrate complementary resistance profiles and are synergistic *in vitro*, DXG is undergoing clinical testing co-administered with AZT [48]. Therefore, the cellular pharmacology of AZT and DXG was assessed in macrophages in this study. The cellular accumulation of DXG-TP and AZT-TP were unaffected by co-incubation (Table 2), in support of similar findings in activated lymphocytes, lending further support for the development of this drug combination [49].

The relationship between extracellular [nucleoside analog], intracellular nucleoside analog-TP, EC_{50} and K_i during steady-state incubation *in vitro*, using at [nucleoside analogs] similar to the 2-4 hr C_{max} observed for most nucleoside analogs following oral administration (10 μM) [12]. Linear regression of $\log_{10}(EC_{50}/K_i)$ *versus* $\log_{10}$

[nucleoside analog] /(nucleoside analog-TP per 10^6 cells) (Eq.4, Fig. 2.2), for the various nucleoside analogs in activated macrophages produced slopes similar to unity, suggesting that [nucleoside analog]: nucleoside analog-TP per 10^6 cells was proportional to EC_{50}:K_i. The apparent pharmacodynamic volume containing viral RT (V_{cell}, based on the published values of K_i) calculated as the anitlogorithm of the y-intercept, were 0.056 and 1.68 pl per activated lymphocyte and macrophage respectively (volume ratio between cell types = 36). Typical actual cell volumes for activated lymphocytes and alveolar macrophages are 0.31 and 5.0 pl, respectively (ratio between cell types = 16), suggesting that the nucleoside analog-TP may be 3 to 6 fold higher in the cell compartment in which HIV-1 RT is located [13, 50]. The scatter observed in Figure 3.2 is not surprising since the K_i used were measured in different laboratories and K_i measurements for nucleoside analog-TP versus HIV-1 RT are sensitive to a variety of factors, including the use of homopolymer/heteropolymer templates, pH variations, competing levels of natural dNTP's including ATP, and possibly other experimental conditions. Levels of HIV-1 RT heterodimer p66/p51 and homodimer form p66/p66 can also affect the K_i values. Therefore, estimates of V_{cell} should be considered approximate. However, it is possible that the use of linear regression may "smoothe" out some errors inherent in observations. Proportionality between nucleoside analog: nucleoside analog-TP and EC_{50}:K_i was not demonstrated for resting macrophages, as the slope differed significantly from unity. Reasons for this deviation are unknown, since the duration of incubation (4 hr) is expected to produce equilibration between extracellular nucleoside analog and intracellular nucleoside analog-TP accumulation. However, it may result in part from differing dNTP/rNTP contents in resting and activated macrophages, as dNTP and rNTP levels are: activated lymphocytes > activated macrophages > resting macrophages [40, 44].

These findings demonstrate that significantly lower levels of nucleoside analogues are observed in macrophages than in activated lymphocytes, and demonstrate the relevant impact of activation state of macrophages on drug potency. Definition of these key facets could provides a foundation for better understanding how activation states *in vivo* can impact ability to achieve systemic HIV-1 eradication.

Discussion about antiviral potency of HIV-1 PI in acute or chronic infection of resting or activated macrophages

Prior to this work, significant gaps in knowledge existed relative to the potency of various ART, including PI, across activation states of macrophages, and during acute and chronic infection. The mechanism of action of PI focuses on inhibition of HIV-1 PR. PR cleaves HIV-1 Gag and Gag-Pol, resulting in a mature, infectious virion. PI compete for binding in the active site with the natural substrate. Once bound, PI cannot usually be cleaved, resulting in inactivation of the enzyme. Due to this mechanism, it follows that PI are the only class of approved therapy that is potent against HIV-1 infection in chronically infected lymphocytes. Whether potency of PI would also withstand against chronic infection in macrophages, and whether activation state would alter potency of PI in these cells, was not known.

In vivo, macrophages comprise multiple microenvironments, conferred as a function of many factors such as physical location, tissue localization, systemic viral loads, paracrine/autocrine inflammatory responses within the microenvironment, and presence of latent infection or ongoing low-level viral replication. Definition of the potency of PI across macrophages in all of these microenvironments allows for better understanding which existing therapies can target viral replication in macrophages *in vivo*, and concomitantly provides a foundation for design of optimized therapy regimens targeting macrophage derived viral sanctuaries. As PI are highly plasma protein bound, and high plasma protein levels is an *in vivo* factor that is encountered, it is also important

to understand how various concentrations of plasma proteins that might be encountered impact antiviral potency of PI. Prior to this work, definition of this factor was unknown. Maintenance of PI in 2.0 % serum results in antiviral potency that is approximately one log more potent than that observed for PI maintained in either 5.0 or 10.0 % serum. Antiviral potency for the 5.0 and 10.0 % serum study was not significantly different, suggesting that a maximum level of protein binding occurs at approximately 5.0 % serum concentration, and that further serum saturates the system and does not confer any additional decrease in antiviral potency (Table 3.3).

These data are the first to show that 1) activation state of macrophages does not alter antiviral potency of PI in macrophages, 2) potency of PI in activated macrophages was similar to lymphocytes, except TPV, which was more potent in macrophages, and 3) concentration of plasma proteins significantly alters potency. Together, this information demonstrates that HIV-1 PI are a class of ART that is equally potent across both lymphocytes and macrophages at various activation states, all of which can be found *in vivo*. Additionally, it is important to define that varying levels of plasma proteins can significantly alter the potency of PI, which should be considered when evaluating potency of PI across various tissues and organs, including the CNS and brain, which may have altered levels of plasma proteins. Thus, these data support the use of currently approved PIs to control viremia in resting or activated macrophages, which is a significant viral reservoir.

Discussion about targeted inhibition of pro-HIV cellular factors

Current therapy cannot eliminate virus from all tissues and reservoirs, and is not capable of achieving either systemic eradication or a functional cure to HIV-1 infection. The optimal addition to current antiretroviral therapy would be able to meet all currently unmet goals, including 1) render uninfected HIV-1 target cells “non-susceptible” to HIV-1

infection, 2) mechanism to prevent “re-establishment” of infection in long-lived, latently infected “memory” lymphocytes, 3) mechanism to eliminate virus from long lived cells that harbor virus for years (macrophages, memory lymphocytes). To this end, inhibitors of cellular factors that favor a pro-HIV milieu, both within infected cells, and extracellularly via promotion of a global hyperactive state systemically, were studied because they represent a potential class of anti-HIV therapy that could meet the “unmet goals” necessary for eradication.

The NFAT inhibitor was weakly potent in lymphocytes and acutely infected macrophages, although its low micromolar potency is likely not enough to render therapeutically relevant application, coupled with the lack of activity against chronic infection in macrophages, and potential to lack potency against any chronically infected cells, present with significant limitations when considering this target for use *in vivo*.

The lck kinase inhibitor demonstrated submicromolar potency in lymphocytes (0.03 μM), and weak potency in acutely infected macrophages (2.5 ± 0.7 μM), however its significantly diminished potency against chronic infection in macrophages (14.5 ± 2.8 μM), is disappointing and limits the clinical relevance of this compound, especially relative to elimination of virus from macrophage derived reservoirs. Additionally, the target of lck kinase, which directly interacts with the CD4 receptor upon engagement of CD4 by any CD4 agonist without discrimination, could result in systemic immunosuppression by ablation of CD4 mediated adaptive immunity. Although this drug demonstrates submicromolar potency against HIV-1 infection against acute infection in lymphocytes, these limitations, most notably relative to interference with adaptive immunity, would likely preclude use of this drug therapeutically *in vivo*.

Hyaluronin, a dual Akt/NFkB inhibitor, although not toxic in any cell type tested, was not potent against either acute or chronic infection in lymphocytes or macrophages (EC_{50} > 50 μM). As no other NFκB inhibitors were tested, it is difficult to determine from

this study alone whether NFκB is a favorable or unfavorable target relative to inhibition of HIV-1 replication, or whether the results are a function of the specific drug chosen. Independent of this fact, the promiscuity of NFκB in a variety of key signaling events, independent of HIV infection, and its activation by a multitude of other upstream pathways, could present with significant obstacles relative to specifically targeting HIV-infected cells only.

Dorapimod, a p38 MAPK inhibitor, was not potent in lymphocytes, or chronically infected macrophages, and was only weakly potent against acute infection in macrophages (15.5 ± 3.4 μM), demonstrating that this molecule is not a target for therapeutic application for treatment of HIV infection.

Miltefosine, an Akt inhibitor, and a second Akt inhibitor under clinical investigation by Merck, were not potent in lymphocytes, or chronically infected macrophages, and only weakly potent against acute infection in macrophages. Although previous reports identified Akt as a potential target for inhibition that could inhibit HIV replication, data obtained from these two molecules suggest that targeted inhibition of Akt may not represent a clinically relevant option.

Tofacitinib and Jakafi, which inhibit Jak1, Jak2, Jak2, and Tyk2, with varying activity (Table 3.8), demonstrated submicromolar potency across both lymphocytes and macrophages (0.2-0.3 μM in macrophages, for both acute and chronic infection, and 0.02-0.08 μM in lymphocytes). The therapeutic window for these compounds ranges from 69 to > 100, depending on the cell type and compound, and assessment of viability demonstrated no significant difference in viability *versus* lymphocytes or macrophages maintained in drug-free medium at concentrations 2-3 logs above the EC_{50} (Figure 3.4, 4.4). For PHA stimulated lymphocytes, there was no significant reduction in proliferation *versus* lymphocytes maintained in drug free medium for all concentrations tested (Figure 3.5 B). For PHA+IL-2 stimulated lymphocytes, a significant reduction in proliferation was

observed between 0.1-1 μM for all Jak inhibitors tested (Figure 3.5 D). All other Jak inhibitors demonstrated a narrow therapeutic window and will likely not be considered for future evaluation for treatment of HIV-1 infection.

Although the mechanism of action of Jak inhibitors' antiviral potency against HIV-1 is not fully understood, it is hypothesized that inhibition of this pathway, which is characterized as a positive modulator of activation of the cell and promotion of a pro-inflammatory cytokine milieu, which in turn reduces proliferation of hyper-activated cells. Therefore, it follows that a reduction in proliferation would be observed in an in vitro system containing IL-2, which is a potent activator of proliferation both in vivo and in vitro (Figure 3.5 D). As PHA activates cells to a lesser extent than PHA+IL-2, it also follows that a cell population activated by PHA alone, which is not proliferating as much as that of a population exposed to IL-2, would not display a significant reduction in proliferation when exposed to Jak inhibitors (Figure 3.5 B).

Tofacitinib and Jakafi also demonstrated potent inhibition of HIV-1 replication for two HIV-1 containing nucleoside-analog resistant mutations (K65R and L74V), which were similar to that observed for wild type drug sensitive HIV-1, providing a proof of principle wherein these compounds could potently inhibit replication of drug-resistant HIV-1. The potency against L74V was diminished relative to that observed for wt HIV-1 (FI50/90 of 2-9 versus wt HIV-1), however these data represent one experiment, and inter-assay variability could be responsible for observed results. More specifically, assays are performed in primary cells, with pooled donors, and each experiment as a function of the primary cells and repetition, confers a standard deviation in results. This experiment must be repeated at least 3 independent times, in order to determine the statistical significance of observed results, all of which are currently underway. This work must also be expanded to include HIV-1 containing a variety of mutations, including those conferring resistance against nucleoside analogues, NNRTI, PI, and IN inhibitors,

however these preliminary data provide a foundation for further work elucidating the potency of Jak inhibitors against drug-resistant HIV-1.

Although Jakafi is FDA approved, and Tofacitinib is in late phase 3 clinical trials, neither drug has been evaluated for treatment of HIV-1 infection in humans, necessitating that further work must be done in a non-human primate model to fully understand the impact of Jak inhibition upon viral loads, inflammation, reduction of latent viral reservoirs, emergence of resistance, and systemic safety/tolerability. Therefore, it is important to define *in vitro* whether an inhibitor designed to target a human cellular factor will confer potency in a system containing cells from a non human primate. Jakafi and Tofacitinib demonstrated similar potency against RT-SHIV in rhesus macaque lymphocytes and macrophages, providing a proof of concept wherein Jakafi and Tofacitinib can be evaluated in our non human primate model, with the expectation that they will confer equal potency to that observed in humans.

Jakafi recently received FDA approval, and Tofacitinib is in late phase 3 clinical trials, underscoring the potential safety and therapeutic application of these compounds in humans. The mechanism of action of these drugs relative to inhibition of HIV-1 replication is incompletely understood, although it is believed that inhibition of the Jak-STAT signaling pathway, which is implicated as a modulator of hyperactivation, proliferation, and a pro-inflammatory cytokine milieu, could render uninfected bystander cells “pseudo resting”, and therefore non supportive of productive viral replication. Additionally, as the Jak-STAT pathway is activated during HIV infection [51-53], conferring transcription and translation of a variety of gene products that confer activation of the infected cell, and therefore are “pro-HIV” gene products, targeted inhibition of this pathway could serve to render infected cells less supportive of productive replication. Together, blockade of bystander cells from infection, while down regulating the pro-HIV Jak-STAT pathway, could also reduce production of pro-

inflammatory cytokines, which are a hallmark of increased viral loads, decline in CD4 T cell counts, rapid progression to AIDS, and death, *in vivo*.

Although the Jak-STAT pathway has been implicated as a modulator of many factors relating to HIV-1 infection and overall hyperactivation, it is unknown whether blockade of Jak-STAT signaling by Jak inhibitors would confer inhibition of reactivation of latent HIV-1. Reactivation of virus from latently infected cells allows for high viremia to be reintroduced, establishing new infection in both lymphocytes and macrophages, while re-establishing and maintaining new latent viral reservoirs. Together, reactivation, without a mechanism to eliminate the newly produced virus, is a significant obstacle in ability to clear virus from all cells and tissues systemically. If a Jak inhibitor prevents reactivation in addition to the observed antiviral potency, then in theory a Jak inhibitor administered in combination with traditional HAART could 1) confer additional antiviral potency to existing HAART, 2) render bystander cells unsupportive of productive viral replication via establishment of a “pseudo-resting” state, and 3) prevent reactivation of latent HIV-1. In performing these three actions concomitantly, the hypothesis is that latently infected cells would not reactivate to produce virus, and would ultimately be removed by normal cell turnover.

To test whether Jak inhibitors are also inhibitors of reactivation, latently infected primary human lymphocytes were reactivated in the presence of various concentrations of Jak inhibitors. Although Jakafi and Tofacitinib are submicromolar inhibitors of HIV-1 replication, inhibition of reactivation occurred at concentrations significantly higher, ranging from 1.0 to > 10 μM. For AZD1480, LY2784544, and CYT387 demonstrated inhibition of reactivation between 1-10 μM (Figure 3.7). The concentrations that inhibit reactivation for all Jak inhibitors is significantly higher than that which confers antiviral potency, demonstrating that the mechanism of action for antiviral potency is likely distinct from inhibition of reactivation, and may include a complex set of events that

ultimately confers antiviral activity. Whether addition of a Jak inhibitor *in vivo* could confer inhibition of reactivation in some cell subsets is unknown, and whether addition of a Jak inhibitor in combination with traditional HAART could confer synergism is currently not known, however synergy studies with various ART are underway. As a pilot study, a combination study with combination of Jakafi+Tofacitinib applied at a 1:4 ratio (ratio of EC_{50}:EC_{50}) resulted in a significant increase in potency as defined by a 5-fold decrease in EC_{50} and a 117-fold decrease in EC_{90} in primary human lymphocytes. Together this information provides a proof-of-principle wherein synergism occurs as a function of combination therapy. Assessment of toxicity across multiple cell types upon combination administration of this regimen and other combinations identified as synergistic must occur, with the ultimate goal of employing the optimized therapy regimen in a rhesus macaque SHIV model.

Together, these data merit further work to elucidate the exact mechanism by which these drugs confer potency against HIV infection in both lymphocytes and macrophages. Additionally, as the mechanism of action is independent of direct interference with the viral replication cycle, further work must be done to define the full scope of antiviral potency of Jak inhibitors against drug resistant HIV-1. These data demonstrate for the first time that inhibitors targeting a cellular factor, as is the case with Jak inhibitors Tofacitinib and Jakafi, and not a step within the viral replication cycle, can confer potent inhibition of HIV-1 replication across HIV-1 target cells. The novel mechanism of action (target of cellular factor and not a step in the viral replication cycle), along with preliminary data described above, suggests that these compounds could be potent against drug resistant HIV-1, which could significantly increase treatment options for patients with drug resistant HIV-1 who have severely limited treatment options. These data provide an encouraging, potentially clinically applicable therapy that could address the “unmet goals” needed to achieve systemic eradication or a functional cure

of HIV-1, and provide key information necessary to achieve the ultimate goal of systemic eradication of HIV-1.

Discussion about levels/ratios of dNTP:rNTP and discovery of a novel class of ART that specifically targets HIV-1 replication in macrophages

Whereas dNTPs exclusively serve as building blocks of DNA during chromosomal DNA replication and DNA repair, rNTPs are more versatile and are used for not only RNA synthesis but also cellular energy transfer (e.g. ATP) and as substrates of various signal transduction cascades (e.g. ATP and GTP). All nucleotides are clearly not compartmentalized in either the nucleus or the cytoplasm [54], so both cellular and viral DNA and RNA polymerases have evolved to efficiently differentiate dNTPs and rNTPs. Specifically, this chemical difference between these two nucleotides is the 2'-OH of their sugar moiety. This can be attributed in part to the fact that the dNTP binding sites of cellular DNA polymerases possess a highly conserved gate control mechanism using a bulky amino acid (e.g. tyrosine) that confers steric hindrance, which prevents entrance of the 2'-OH of rNTPs and effectively eliminates their binding to the active site [55, 56].

In dividing cells, the rNTP concentration is much higher than that of dNTPs. These data show that this disparity is even greater in terminally differentiated macrophages, simply because the dNTP concentration difference (22–133-fold) between these two cell types is much larger than the rNTP concentration difference (4–7-fold). Viruses, such as HIV-1, which require DNA synthesis upon infection of macrophages, a cell type wherein DNA synthesis is largely absent, encounter not only very low dNTP concentrations that can restrict viral genomic DNA synthesis but also a very large dNTP/rNTP disparity. These findings prompted the hypothesis that rNTP may be more efficiently incorporated into the growing viral DNA strand in the macrophage but not lymphocyte simulated dNTP/rNTP microenvironment.

These data confirm that although dNTPs are 6-133 fold lower in macrophages *versus* lymphocytes, independent of activation state, levels of rNTP are only 4-7 fold lower in macrophages (Table 3.10, Figure 3.8). These reports were complemented by the finding that rNTP are preferentially incorporated into proviral DNA in the macrophage but not the lymphocyte dNTP:rNTP simulated microenvironment in a biochemical simulation assay [6], a finding that is distinct from the previously accepted canon that dNTP are incorporated into the growing viral DNA strand exclusively. This report demonstrates that rNTPs are incorporated into the proviral DNA strand in macrophages, and also predicts that ribonucleoside chain terminators could be specific inhibitors of HIV-1 replication in macrophages, wherein their mechanism of action would be dictated by the unique landscape of dNTP:rNTP found in macrophages. These data do not exclude the fact that dNTP are incorporated into HIV-1 proviral DNA, as dNTP-based inhibitors demonstrate anti-HIV activity in macrophages, although potency is diminished for most nucleoside analogs *versus* lymphocytes [21, 26, 57]. The fact that rNTP are preferentially incorporated into the growing viral DNA strand in the biochemical simulation of the macrophage cellular environment could in part be responsible for the fact that deoxy-based nucleoside analogs are not as potent in macrophages compared to lymphocytes, where dNTP are preferentially incorporated, especially with respect to chronic infection [21, 26, 57].

Last, as these data imply that ribonucleoside inhibitors could demonstrate potency against HIV-1 infection in macrophages specifically, by way of preferential rNTP incorporation into the growing viral DNA strand in the macrophage but not lymphocyte environment, screen to identify ribonucleoside based inhibitors targeting viral replication in macrophages was performed. Two ribonucleoside inhibitors, RS-1285, and RS-1292, were identified as potent, non-toxic specific inhibitors of viral replication in macrophages (Table 3.11, Figure 3.12). To better understand the mechanism

responsible for inhibition of viral replication, the triphosphorylated form of RS-1285 was synthesized for use in the cell free biochemical assay to determine if RS-1292-TP is a chain terminator. Synthesis of RS-1285-TP could not be completed due to instability of the parent compound. RS-1292-TP inhibits HIV-1RT mediated DNA synthesis in the macrophage dNTP/rNTP simulated microenvironment in a dose dependent manner, confirming that RS-1292-TP is a chain terminator (Figure 3.12). Together, these data demonstrate that the biochemical landscape of HIV-1 replication in macrophages is distinct from that observed in lymphocytes, and that ribonucleoside chain terminators represent a new class of anti-HIV-1 agents that specifically inhibit viral replication in macrophages.

Although dNTPs are frequently incorporated into DNA, two phosphate groups are cleaved, with the resulting energy used to create the phosphodiester bond that functions to attach the single remaining phosphate to the growing DNA strand. Therefore, upon discovery that rNTP are preferentially incorporated into the growing viral DNA strand in macrophages, it follows that the incorporated rNTP may undergo the same bi-phosphate cleavage, potentially resulting in incorporation of rNMP into the growing viral DNA strand in macrophages.

Recent reports demonstrate that rNMP is incorporated into HIV-1 proviral DNA, as determined by the presence of 2 LTR circles with quantitative real-time PCR, at a rate of 1/146 nucleotides in macrophages. Additionally, macrophages possess significantly diminished capacity for repairing monoribonucleotides *versus* that observed in activated lymphocytes, and rNTP incorporation in the template strand preceding the 3' terminus causes pausing during DNA synthesis, which is a known correlate of mutagenesis. Taken together, the presence of rNMP in the HIV-1 proviral genome, suboptimal levels of repair machinery to remove rNMP in macrophages *versus* activated lymphocytes, and the established correlation between site-specific incorporation and pausing in DNA

synthesis, provide an environment in macrophages that could be a source for increased production of mutagenic HIV-1 [58, 59]. Additionally, data presented in Chapter 2 demonstrates that intracellular levels of nucleoside analog-TP, are significantly lower in macrophages *versus* lymphocytes, and is often not delivered at adequate levels to inhibit viral replication [27]. Suboptimal levels of drug delivered to macrophages, could provide selective pressure for emergence of drug resistant HIV-1, and together with the established environment in macrophages that correlates with increased production of mutagenic HIV-1, point to macrophages as a cell-specific microenvironment that could in theory result in emergence of drug resistant HIV-1 (Figure 3.13).

rNMP incorporation occurs at a rate of 1/146 nucleotides in the HIV-1 proviral genome in macrophages, and dNTP are clearly still incorporated, raising questions about the relative impact of rNMP incorporation in macrophages and its systemic implications *in vivo*. Macrophages are found in every tissue and organ, and due to high CCR5 expression, presence in mucosal sites that often confer primary infection, and rapid localization to the site of infection, all represent significant rationale for *in vivo* relevance of rNMP incorporation into the growing viral DNA strand in macrophages.

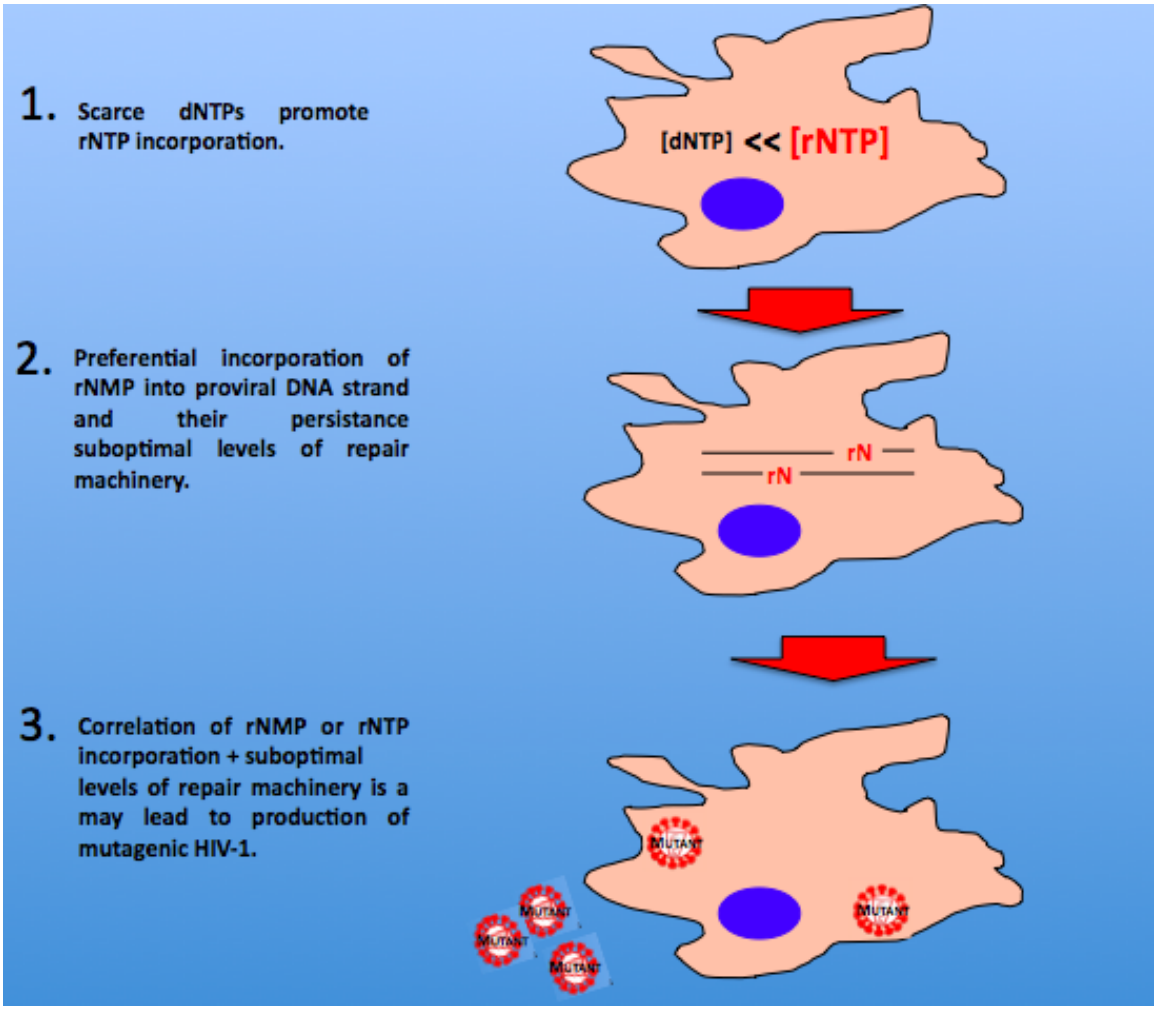


Figure 3.13: **Potential impact of levels/ratios of dNTP:rNTP in macrophages upon emergence of mutagenic HIV-1.** Similar ratios of dNTP:rNTP (point 1) confer preferential incorporation of rNTP and rNMP into the growing viral DNA strand (point 2). Together, with suboptimal levels of repair machinery found in macrophages, these incorporations are a known correlate for production of mutagenic HIV-1 (point 3). In the presence of increased production of mutagenic HIV-1, it is possible that this microenvironment could result in production of drug resistant HIV-1 in the presence of HAART (point 4).

Discussion about identification of macrophage depleting agents for use in a rhesus macaque model

As recent reports have demonstrated the importance of macrophages in HIV-1 infection *in vivo*, and the necessity to eliminate virus in macrophages as sentinel in achieving systemic eradication of HIV-1 [25], development of drugs aimed at elimination of virus in these cells must be explored. One method to eliminate both existing and future infection in macrophages is to deplete macrophages systemically, without discrimination for infected *versus* uninfected macrophages, eventually allowing for repopulation of newly differentiated, uninfected macrophages by the bone marrow as a function of time. This therapy could be used in combination with traditional HAART as a tool to eliminate virus harbored in macrophages.

Previous work demonstrates that clodronate confers specific suicide of macrophages first via endocytosis or phagocytosis of clodronate by the macrophage or macrophage precursor, and subsequent intracellular metabolism of the clodronate to the toxic ATP [5'-(β,γ-dichloromethylene) triphosphate], conferring selective apoptosis of phagocytic cells only (Figure 6.1 A, B) [35-38], and recent reports demonstrate that addition of nitrogen-containing side chains to clodronate [38] confer up to a 500-fold increase in potency, as measured against inhibition of osteoclast viability.

To this end, we hypothesized that clodronate-based drugs with nitrogen containing side chains would also significantly increase potency relative to specific depletion of macrophages. A literature search was performed to determine if any FDA approved drugs with this profile existed, and two drugs were identified: Fosamax and Boniva (Table 6.1). The data demonstrated that both Fosamax and Boniva are potent inhibitors of HIV-1 replication in macrophages, but not lymphocytes, and are not toxic in lymphocytes, CEM, or Vero cells (non phagocytic cells), as expected.

This work defines both Fosamax and Boniva, which are clodronate-based drugs with nitrogen containing side chains, as potent and selective inhibitors of HIV-1 replication in macrophages, likely as a function of depletion of macrophages via apoptosis (mechanism described in Figure 6.1). Additionally, discovery of investigational inhibitors that demonstrate low micromolar inhibition of both acute and chronic infection in macrophages (Table 3.13) provides a foundation for further drug discovery to invent safe, potent MDA agents.

To further define the relative impact of systemic macrophage depletion on HIV-1 infection, disease progression, viral loads, CD4 T cell counts, progression to AIDS, or ability to functionally cure a patient, further work must be done within an *in vivo* system. Experiments designed to answer these questions are currently under development for use in our rhesus macaque monkey model. Definition of the role of macrophages during primary infection, which has not been explicitly defined in an *in vivo* system, could be clearly elucidated by systemic macrophage depletion before infection with SHIV. Additionally, the impact of systemic macrophage depletion upon established chronic SHIV infection could be defined by addition of Fosamax or Boniva after SHIV infection. Understanding this information, which is only minimally defined *in vivo*, would provide critical information necessary to design therapy regimens targeting viral reservoirs that persist in the absence of macrophages.

Together, this information, along with addition of an MDA in the presence of HAART, could represent a novel mechanism that could reduce viral loads, delay disease progression, prevent establishment or maintenance of macrophage derived viral reservoirs, eliminate latent reservoirs that can reactivate and subsequently repopulate the periphery, or potentially allow for systemic eradication of HIV-1.

Summary Conclusions

Current HAART cannot achieve systemic eradication of HIV-1, and latent viral reservoirs such as macrophages represent a significant obstacle in achieving eradication. The goal of this thesis is to identify mechanisms to eliminate HIV-1 in macrophages. This goal was explored by a multifaceted approach exploiting specific factors that are specific to the macrophage environment, targeting pro-HIV pathways, identification of which currently approved antiretroviral therapies display the greatest potency and intracellular accumulation in macrophages, and identification of potent and selective macrophage depleting agents.

More specifically, a variety of factors exist that represent a barrier to achievement of eradication, including: 1) inability to render uninfected HIV-1 target cells "non-susceptible" to HIV-1 infection, 2) inability to prevent re-establishment of infection in long-lived, latently infected "memory" lymphocytes and macrophages, and 3) inability to eliminate virus from long lived cells that harbor virus for years (macrophages, memory lymphocytes). To that end, the portion of this thesis focusing on novel antiretroviral agents targeting viruses in macrophages was designed to satiate as many of these facets as possible, with the idea that combination therapy along with traditional HAART could overcome these obstacles.

Relative to assessment of intracellular drug concentrations and antiviral potency, of all nucleosides tested, TDF, 3TC, and AZT accumulate to intracellular levels necessary to eliminate HIV-1 replication in macrophages and lymphocytes. With respect to potency of existing therapy in macrophages, HIV-1 PI demonstrated equal potency for both acute and chronic infection in macrophages, and potency was not altered as a function of activation state of macrophages.

Assessment of antiviral potency of HIV-1 PI demonstrated no significant difference in potency for 1) macrophages *versus* lymphocytes, 2) acutely infected resting

versus activated macrophages, and 3) acute *versus* chronic infection in macrophages. This information provided key information about PI, clearly defining them as a class of HAART that can target HIV-1 infection across multiple cell types at various stages of activation. Assessment of concentrations of extracellular serum upon potency of PI, a highly plasma protein bound class of drugs, demonstrated that potency is decreased approximately one log for cells maintained in 5.0 or 10.0 % serum *versus* 2.0 %. No significant change was observed for 5.0 *versus* 10.0 % serum, suggesting that a saturation of plasma protein binding occurs at or between 5.0 and 10.0 % serum (Table 3.3).

This information provides insight into understanding the potency of PI across various microenvironments and sub-compartments, such as the CNS and brain, which may present with different levels of plasma proteins. Additionally, assessment of antiviral potency of PI in macrophages can be employed to better understand dynamics of infection and antiviral potency in the brain and CNS, using pharmacokinetic modeling. These data were used to model which PI are most potent against HIV-1 infection in the brain, and which PI should be used to prevent or reverse HIV-associated neurocognitive impairment [60]. As CNS infection is largely conferred by macrophage-like cells/microglia, these data provide an *in vitro* model that can be applied to confer *in vivo* relevance.

Discovery of significantly different levels and ratios of dNTP:rNTP in macrophages *versus* lymphocytes led to the discovery that rNTP are preferentially incorporated into the growing viral DNA strand in macrophages but not lymphocytes, a finding that was used to discover that ribonucleoside inhibitors are specific inhibitors of HIV-1 replication in macrophages but not lymphocytes. These data provided a foundation for reconsideration of the previously accepted canon that deoxy based inhibitors are the only class of nucleosides that are capable of inhibiting HIV-1

replication, and provide a rationale for further discovery of ribonucleoside inhibitors, which can allow specific targeting of HIV-1 within macrophages.

With respect to cellular factor inhibitors, two inhibitors of the Janus Activating Kinase (Jak) (Incyte's Jakafi and Pfizer's Tofacitinib were identified as safe, potent sub micromolar inhibitors of HIV-1 replication in both lymphocytes and macrophages, and combination of both compounds confers a significant increase in potency. Although the exact mechanism by which Jak inhibitors confer inhibition of viral replication is not defined, and inhibition of reactivation occurred at concentrations significantly above the EC_{50}, the fact that activation of the Jak-STAT pathway is directly associated with cellular activation and production of pro-inflammatory cytokines, all of which represent events that favor HIV-1 replication, inhibition of this pathway could render cells unsupportive of productive viral replication.

An alternative approach that was explored is systemic macrophage depletion conferred by a drug-based mechanism. Two FDA approved drugs, Fosamax and Boniva, were identified as potent inhibitors of HIV-1 replication in macrophages, achieved by depletion of macrophages via apoptosis of macrophages by generation of a toxic ATP metabolite. As each of these compounds are FDA approved, it follows that these compounds could be used in vivo to deplete macrophages at various stages of disease progression, while in combination with traditional HAART, to eliminate latent HIV-1 within macrophages. Additionally, assessment of investigational MDA identified two inhibitors with submicromolar potency against both acute and chronic infection in macrophages, providing a foundation for further work to define novel, safe, and potent MDAs.

The goal of this thesis was to define which currently approved drugs are able to efficiently eliminate virus in macrophages, while concomitantly identifying novel inhibitors to target HIV-1 replication in macrophages. The data generated herein suggest that

therapy regimens aimed at elimination of HIV-1 in macrophages, with a goal of systemic eradication, should consider a therapy regimen of TDF, 3TC, or AZT + PI + ribonucleoside inhibitor + Jak inhibitor, potentially in combination with a macrophage depleting agent such as Fosamax or Boniva. Combinations of these therapies will be evaluated in our rhesus macaque monkey model, to determine appropriate dosing required to achieve reduction or elimination of virus from macrophages and/or the periphery, while maintaining a wide therapeutic window of safety, with a long-term goal of applying optimized regimens to humans.

Appendices

List of all published, submitted, and prepared papers to date

1. **Christina Gavegnano** and Raymond Schinazi. Antiviral therapy in macrophages: implication for eradication. *Antiviral Chemistry and Chemotherapy*. 2009 Oct 19;20(2):63-78.

2. Raymond Schinazi, Leda Bassit, and **Christina Gavegnano**. HCV drug discovery aimed at eradication. *J Viral Hepat.* 2010 Feb 1;17(2):77-90.

3. Emilie Fromentin, **Christina Gavegnano**, Aleksandr Obikhod, and Raymond Schinazi. Simultaneous quantification of intracellular natural and antiretroviral nucleosides and nucleotides by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 2010 Mar 1;82(5):1982-9.

4. Edward M. Kennedy*, **Christina Gavegnano***, Laura Nguyen, Rebecca Slater, Amanda Lucas, Emilie Fromentin, Raymond Schinazi, and Baek Kim. Biochemical simulation of human immunodeficiency virus type 1 reverse transcription with physiological nucleotide pools found in macrophages and $CD4^+$ T cells. *J Biol Chem.* 2010 Dec 10;285(50):39380-91.**** denotes equal contribution by each author.***

5. Edward Kennedy, Waaqo Daddacha, Rebecca Slater, **Christina Gavegnano**, Emilie Fromentin, Raymond F. Schinazi, and Baek Kim. Abundant non-canonical dUTP found in primary human macrophages drives its frequent incorporation by human immunodeficiency type-1 reverse transcriptase. *J Biol Chem Jul 15;286(28):25047-55.*

6. Cecelia Shikuma, Beau Nakamoto, Bruce Shiramizu, Chin-Yuan Liang, Victor DeGruttola, Kara Bennett, Robert Paul, Kalpana Kallianpu, Dominic Chow, **Christina Gavegnano,** Selwyn Hurwitz, Raymond Schinazi, and Victor Valcour. Antiretroviral monocyte efficacy score linked to cognitive impairment in HIV. *Antiviral Therapy, in press.*

7. **Christina Gavegnano,** Edward M. Kennedy, Baek Kim, and Raymond F. Schinazi. The impact of macrophage nucleotide pools on HIV-1 reverse transcription, viral replication, and the development of novel antiviral agents. *Submitted, Molecular Biology International.*

8. **Christina Gavegnano,** Mervi Detorio, Leda Bassit, Emile Fromentin, and Raymond Schinazi. Cellular pharmacology and potency of HIV-1 nucleoside analogs in primary human macrophages: implication for eradication. *Submission April, 2012.*

9. **Christina Gavegnano,** Catherine Montero, Mervi Detorio, and Raymond F. Schinazi. HIV-1 protease inhibitors are equally potent in resting and activated primary human macrophages. *Submission August, 2012.*

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