Cellular pharmacology and potency of HIV-1 nucleoside analogs in primary human macrophages

Christina Gavegnano, Mervi Detorio, Leda Bassit, Selwyn J. Hurwitz, Thomas North and Raymond F. Schinazi

Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA, and Veterans Affairs Medical Center, Decatur, GA 30033, USA

ABSTRACT

Understanding the cellular pharmacology of antiretroviral agents in macrophages and subsequent correlation to antiviral potency provides a sentinel foundation for definition of the dynamics between antiretroviral agents and viral reservoirs across multiple cell types, with the goal of eradication of HIV-1 from these cells. Various clinically relevant nucleoside antiviral agents, and the integrase inhibitor Raltegravir, were selected for this study. The intracellular concentrations of the active metabolites of the nucleoside analogs were found to be 5- to 140-fold lower in macrophages versus lymphocytes, and their antiviral potency was significantly lower in macrophages constitutively activated with m-CSF during acute infection compared to resting macrophages (EC50 0.4-9.42 μM versus 0.03-0.4 μM, respectively). Although tenofovir-treated cells displayed significantly lower intracellular drug levels versus its prodrug tenofovir disoproxil fumarate treated cells, the levels of tenofovir-diphosphate for tenofovir-treated cells were similar in lymphocytes and macrophages. Raltegravir also displayed significantly lower intracellular concentrations in macrophages versus lymphocytes, independent of...
activation state, but had similar potency in resting and activated macrophages. These data underscore the importance of delivering adequate levels of drug to macrophages to reduce and eradicate HIV-1 infection.

INTRODUCTION

Macrophages are a major target of HIV-1 infection, and infected cells must be selectively destroyed to achieve HIV-1 eradication since they act as potential long-term HIV reservoirs (1). Macrophages are recruited, or already localized to the mucosal site of initial HIV-1 transmission (2-5), and due to high surface expression of both CD4 and CCR5 (6, 7), represent a target for early establishment and maintenance of latent and chronic HIV-1 infection. These cells are also key mediators of both innate and acquired immunity (5), thus HIV-1 orchestrated depletion of macrophages is catastrophic to the global immune response.

Macrophages and macrophage-like cells are found in every tissue and organ (5), and display various activation states, largely dictated by the presence or absence of infection coupled with corresponding autocrine and paracrine cytokine milieus. These factors confer many distinct and often concomitant microenvironments with heterogeneous exposures to extracellular drug concentrations relative to systemic CD4+ T lymphocytes. Macrophages are a target for early infection (1, 3, 8), and are present in multiple sites of primary transmission including the gut and genital tract (1, 2, 4). Infection in macrophages often results in subpopulations of latent infection, which can occur across any tissue or organ containing these cells, such as the brain or lymphoid tissues, rendering them a ubiquitous source for reactivation of virus later in disease progression. Although the antiviral profile of antiretroviral therapy (ART) is partially defined in macrophages (2, 9-11), a direct link between potency and cellular pharmacology had not been reported. The relationship between cellular pharmacology of...
ART and macrophages can impact viral loads, emergence of resistant HIV-1, and long-term survival of infected individuals (8, 12). For these reasons, understanding the dynamics of ART pharmacology in macrophages, and subsequent targeted elimination of both these cells and the virus that they harbor, is critical to eliminating HIV-1 infection.

Nucleoside-containing regimens are the backbone of standardized treatment for HIV-1, with tenofovir disoproxil fumarate (TDF) and emtricitabine [(-)-FTC] representing two of the three drugs in the most prescribed antiviral combination therapy (Atripla). One of the most widely used integrase inhibitors is Raltegravir (RAL; Merck) although most recently Elvitegravir, as part of the quad pill Stribild™ (Gilead) was approved and could become an alternative choice for persons infected with HIV-1. As nucleoside-containing regimens will likely remain a sentinel tool in HIV-1 management and treatment for the foreseeable future, and integrase inhibitors provide a novel addition to combination ART (cART), various nucleoside analogs as well as RAL were selected for this study. The antiviral potency of the protease inhibitor Atazanavir was also evaluated as a control, since it represents a class of antiretroviral agents which does not require metabolic activation, and is also known to demonstrate potency in both acutely and chronically infected lymphocytes and macrophages.

Additionally, consideration was taken for the fact that lymphocytes and macrophages are found across various activation states in vivo, and activation state can alter a variety of cellular functions including levels and concentrations of dNTP/rNTP and cellular kinases in these cells. Therefore it follows that activation-state driven modulation of dNTP/rNTP or kinase levels can alter either antiviral potency of NRTI, or NRTI-TP formation because 1) NRTI-TP compete with endogenous dNTP/rNTP for incorporation into the growing viral DNA strand, and 2) different levels of kinases responsible for phosphorylation of NRTI to NRTI-TP in activated versus resting cells can impact the rate of formation of NRTI-TP. Therefore, it follows within this manuscript that
“states of activation” in lymphocytes refers to presence or absence of mitogen/cytokine (PHA/IL-2), where presence of these activators confers a hyper-activated state wherein cellular division, dNTP/rNTP levels, immunologic activation, and a variety of other factors occur. The term “states of activation” in macrophages refers to the presence or absence of macrophage-colony stimulating factor (M-CSF), where M-CSF activates multiple pathways that drive production of pro-inflammatory, pro-HIV cytokines.

Although previous reports have established differential antiviral potency in acutely infected macrophages versus lymphocytes, we report for the first time a link between antiviral activity and cellular pharmacology in these cells. A previous report by Szebeni et al (13) measured concentrations of AZT-MP, AZT-DP, or AZT-TP in lymphocytes and macrophages, however data were reported as a sum of AZT-MP + AZT-DP + AZT-TP, mitigating the ability to delineate concentrations of the active metabolite, AZT-TP. Additionally, antiviral potency was not reported. Although other reports have defined the antiviral potency of NRTI against acute and chronic infection in macrophages and lymphocytes, the data reported are the first to correlate concentrations of the active metabolite of NRTI directly to antiviral potency. Together, these data provide a robust foundation for design of novel therapeutics with greater intracellular concentration and potency across macrophages of all activation states.

MATERIALS AND METHODS

Cellular pharmacology:

For macrophage cultures, 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). Wells were seeded at a density of 1.0 x 10^6 cells/well for 1 hr at 37°C and 5% CO₂ to confer plastic adherence prior to repeated...
washes with 1 x PBS. For cellular pharmacology studies, activated macrophages were maintained in medium containing 100 U/mL macrophage colony-stimulating factor (m-CSF, R&D Systems, Minneapolis, MN), supplemented with 20% fetal calf serum (heat inactivated for 30 min, 56°C) (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) for 7 days (37°C, 5% CO₂) prior to testing. For resting macrophages, cells were maintained in medium containing m-CSF for 18 hr prior to two washes with 1 x PBS (to remove m-CSF) and subsequent culture in m-CSF free medium supplemented with 20% heat inactivated fetal calf serum and 1% penicillin/streptomycin for six 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%.

Primary human peripheral blood mononuclear (PBM) cells were isolated from buffy coats derived from healthy donors (Lifesouth, Dunwoody, GA). Resting PBM cells were maintained in PHA-free RPMI media supplemented with 20% heat inactivated fetal calf serum, 1% penicillin/streptomycin and 2% L-glutamine (Sigma Aldrich, San Jose, CA) for 72 hr prior to cellular pharmacology studies. Activated PBM cells were maintained analogously with the exception that medium was supplemented with 6 µg/ml phytohemagglutinin (Cape Cod associates, East Falmouth, MA).

After preparation of cells as described above, activated or resting cells were exposed to 10 µM AZT, abacavir (ABC), carbovir (CBV), (−)-FTC, TDF, tenofovir (TFV), (−)-beta-D-dioxolane-guanine (DXG), DXG plus AZT (1:1 ratio) or RAL for 4 hr, at 37°C in a 5% CO₂ atmosphere. Extracellular application of drug at concentrations equal to the EC₅₀ prohibits accumulation of NRTI-TP above the limit of detection of the LC-MS/MS. Therefore, 10 µM NRTI was applied, as it is within the concentration that can be reached in vivo, and the assumption of linearity relative to extracellular drug
concentration: intracellular accumulation of NRTI-TP for can be made, providing a mechanistic and physiologically relevant rationale for using that concentration.

Then, extracellular media was removed and cells were washed twice with ice-cold 1 x phosphate buffered saline (PBS) to remove any residual medium. Cells were resuspended in 60% CH₃OH overnight, and extracts were centrifuged at 18,000 x g for 10 min. Supernatants were dried under a flow of air, re-dissolved in mobile phase for LC/MS-MS analysis (14). For each condition experiments were conducted in duplicate 12-well plates (BD Biosciences, San Jose, CA) at a density of 1.0 x 10⁶ cells/well. Duplicate control wells to determine total cell number of viable cells (trypan blue exclusion stain) were also prepared. Cell volumes of 0.32 µm³ and 2.66 µm³ for lymphocytes and macrophages, respectfully, (15) were used to calculate the intracellular concentrations for the active metabolites of each drug.

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 hr prior to infection and cultured for 2 hr in medium with various concentrations of AZT, ABC, CBV, (-)-FTC, TDF, TFV, DXG, RAL, or Atazanavir prior to removal of drug-containing medium and 4 hr infection with HIV-1_BaL at 0.1 MOI in the absence of drug. Then, virus was removed and drug-containing medium was returned to each culture. For chronic infections, resting or activated cells were serum starved for 8 hr, and then infected for 4 hr with HIV-1_BaL at 0.1 MOI prior to removal of virus and culture in drug containing medium for 7 days. For both acute and chronic infections, supernatants were collected on day 7 post-infection and HIV-1 p24 was quantified by ELISA (Zeptometrix Corporation, Buffalo, NY). The median effective concentration (EC₅₀) was determined 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\textsubscript{BaL}. Supernatants were collected on day 7 after infection and HIV-1 p24 was quantified by ELISA as described above.

For lymphocytes, testing was performed using at least three independent assays performed in duplicate. Cells were incubated in RPMI medium (HyClone, Logan, Utah) containing HR-IL2 (26.5 units/mL) (Chiron Inc., Emeryville, CA) and 20% heat inactivated fetal calf serum. Infections were performed by adding HIV-1\textsubscript{LAI} followed by a further incubation at 37°C, 5% CO\textsubscript{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°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 (16).

\textbf{Statistical Methods}

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

\textbf{RESULTS}

\textit{Cellular Pharmacology:}

The concentration of nucleoside analog triphosphate, the active form of the nucleoside (NRTI-TP) in activated and resting macrophages were significantly lower in macrophages \textit{versus} activated lymphocytes and were independent of activation state \((p < 0.05)\), with the exception of TFV, (Figure 1). The intracellular concentration of AZT-TP was 18- and 35-fold lower in resting macrophages than in resting and activated lymphocytes, respectively (Figure 1A, Table 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) (Figure 1A, Table 1).

For resting cells treated with ABC, the intracellular concentration of carbovir triphosphate (CBV-TP), the active metabolite of ABC, was 27.5- and 7.5-fold lower in resting macrophages versus activated and resting lymphocytes, respectively (Figure 1B, Table 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 (Figure 1B, Table 1). Resting cells treated with CBV demonstrated an intracellular concentration of CBV-TP that was 12.2- 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 (Figure 1C, Table 1).

In resting cells treated with (-)-FTC, the intracellular concentration of (-)-FTC-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 activated lymphocytes (Figure 1F, Table 1).

For resting cells treated with TDF, the intracellular concentration of TFV-DP, the active metabolite of both TDF and 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 (Figure 1D, Table 1).

Contrarily, the exception to the overall trend for NRTI was found with TFV. In TFV-treated cells, the intracellular concentration of TFV-DP was not significantly different in
The intracellular concentrations of TFV-DP for TDF treated cells was not unexpected versus that for TFV treated cells, as TDF is a prodrug of TFV, and is more lipophilic, allowing for greater intracellular accumulation.

3TC-treated resting cells demonstrated an intracellular concentration of 3TC-TP that was 140-fold and 70-fold lower in resting macrophages versus resting lymphocytes, respectfully. For activated cells treated with 3TC, the intracellular concentration of 3TC-TP was 93-fold lower in activated macrophages versus lymphocytes (Figure 1G, Table 1). 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, respectively. For activated cells treated with DXG, the intracellular concentration of DXG-TP was 4-fold lower in activated macrophages versus lymphocytes (Figure 1H, Table 1).

Co-incubation of cells treated with 10 µM DXG together with AZT (ratio 1:1) was also assessed. 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 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 2).

RAL-treated resting cells conferred an intracellular concentration that was 8-fold lower in resting macrophages versus lymphocytes. For activated cells treated with RAL, the intracellular concentration was 93-fold lower in activated macrophages versus
lymphocytes (Figure 1I, Table 1).

Antiviral potency:

For chronically infected macrophages, the EC50 of all NRTI were > 50 µM. This was expected, as the primary mechanism of action of NRTI is to inhibit HIV-1 RT mediated DNA synthesis, a mechanism that is largely ineffective against chronic, already-established infection. Protease inhibitors primarily target virus that is extracellular and affect maturation of the virus, whereas NRTI only target virus inside of cells. Therefore, it follows that PI are able to inhibit chronic infection, which is hallmarked by the presence of integrated provirus.

During acute-infection of constitutively activated macrophages, EC50 of NRTI ranged from 0.4-9.42 µM, compared to 0.03-0.40 µM in resting macrophages (Table 1). As expected, Atazanavir, displayed great potency against both acute resting, acute activated, and chronic HIV-1 infection in macrophages (EC50 was 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 was inactive even at 50 µM against chronic HIV-1 infection in macrophages. For comparison, the antiviral potency of NRTI in activated lymphocytes during acute infection ranged from 0.001-0.3 µM. (Table 1).

DISCUSSION

The importance of delivering adequate concentrations 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. As the mechanism of action of NRTI is upstream of integrated provirus found in latently infected cells, therapeutic modalities with novel mechanisms of action designed to eliminate virus across all states of...
activation, latency, tissue distribution and localization are necessary to achieve systemic eradication of HIV-1. To that end, delivery of NRTI to adequate concentrations to inhibit de novo infection across multiple sites remains a significant obstacle, and, if achieved, could provide a mitigating agent to ongoing de novo replication in concert with novel therapeutic modalities.

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 (17-21). Therefore, this mechanistic study was performed to investigate the potency of antiretroviral agents in macrophages, since these cells become infected early during HIV transmission in the majority of individuals, and may be involved in the infection of more shielded organ sites including tissues protected by the blood-brain barrier. The antiretroviral potencies in macrophages were compared to potencies in activated lymphocytes, another primary HIV-1 target cell. 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.

Multiple reports define ongoing chronic inflammation and immune activation in HIV-infected patients, even in those with well-controlled viremia (22-24). Elevated levels of IL-6, TNF-α, and IL-1α/β largely hallmark these events. Relative to the activation states conferred in these data, “resting” populations are largely absent of IL-6, TNF-α, and IL-1α/β. “Activated” populations as conferred by addition of PHA/IL-2 (lymphocytes) or M-CSF (macrophages) (7) triggers intrinsic activation of each population of cells, which, upon infection, as occurs in vivo, cross activates infected and bystander cells, resulting in the pro-inflammatory milieu hallmarked by IL-6, TNF-α, and IL-1α/β. Therefore, the “activation” conferred herein mirrors a key event in immune activation.
Although it is a long and uncertain leap going from *in vitro* to *in vivo* studies, these *in vitro* experiments were designed to recapitulate *in vivo* dynamics as closely as possible by using primary cells and physiologically relevant concentrations of drug.

Intracellular concentrations of all NRTI, except TFV, were 5-140-fold lower in macrophages than in lymphocytes independent of activation state. We further demonstrate that compared to macrophages maintained in the absence of m-CSF, acutely-infected cells that are constitutively maintained in the presence of m-CSF displayed significantly diminished antiviral potency (0.03-0.40 μM versus EC₅₀ 0.4-9.42 μM, respectively). These data are affirmed by previous studies (2, 8, 10) on antiviral potency of NRTI 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 (10). The cellular pharmacology studies also demonstrated for the first time that the intracellular concentration of RAL was significantly lower in macrophages versus lymphocytes, independent of activation state.

Although RAL is not confined intracellularly as are NRTI-TP, RAL-mediated export from cells can be conferred by efflux transporters p-gp/MDR-1 (p-glycoprotein/multi-drug resistance transporter 1) (25). P-gp/MDR-1 activity is active at 37°C but is inactive at 4°C (25-27). All washes were performed with ice-cold PBS at 4°C, taking care to ensure that RAL could not be effluxed by this mechanism. Additionally, as washing procedures for cells were performed analogously for macrophages and lymphocytes, this allows for direct comparison between data from each cell type without concern for differential handling or efflux that could skew results. Additionally, we demonstrated that RAL was not potent against chronic HIV-1 infection in macrophages, similar to the profile displayed by NRTI. In contrast to the NRTI tested, RAL did not display any difference in potency against acute HIV-1 infection in resting versus activated macrophages. These
data are in general agreement to that recently reported by Scopelliti, et al (28) for acutely infected macrophages, although potency of RAL against chronic infection in macrophages was not reported.

A primary mechanism of action for NRTI-TP is through competition with the natural dNTP and rNTP (in PBM cells and macrophages, respectively) for HIV-1 RT (29, 30). Therefore, it is possible that the reduced accumulation of NRTI-TP may be partially compensated by reduced macrophage concentrations of natural dNTP, namely, dCTP, dTTP, dATP or dGTP, or dUTP, and natural ribonucleoside triphosphates (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 from our group demonstrated that macrophages harbor 22-320 fold lower overall dNTP concentrations than dividing target cells, as well as a significantly lower disparity between dNTP and rNTP concentrations versus that observed in resting and activated lymphocytes (14, 29, 30).

Herein, we demonstrated that activation state of macrophages does not significantly alter intracellular concentrations of nucleoside analogues and that constitutive exposure to m-CSF (to ensure a constitutively activated phenotype), significantly decreases the potency of NRTI versus macrophages maintained without constitutive m-CSF exposure. 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 NRTI. 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 (31, 32). 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.

As expected, the accumulation of AZT-TP, a cell cycle dependent NRTI was significantly (3.5-fold) higher in activated lymphocytes versus resting lymphocytes (p < 0.05) (33-36). In contrast, ABC- or CBV-treated lymphocytes demonstrated a 2-fold increase in intracellular CBV-TP in resting versus activated lymphocytes (p < 0.01). 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 by various cytosolic enzymes, and then to CBV-TP by cellular kinases (37). 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 (37, 38). 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 NRTI tested which did not display significantly lower concentrations of active nucleotide concentrations in macrophages than in lymphocytes. TFV is a weak inhibitor of HIV-1 because it may not penetrate cells efficiently due to the negatively charged phosphate moiety. 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 negative charges of TFV. Therefore, it is not surprising that TFV-DP levels were significantly higher following TDF than TFV incubation (Fig. 1). TFV is converted to TFV-MP by AMPK (AMP-activated protein kinase or AMPK or 5'
adenosine monophosphate-activated protein kinase), and to TFV-DP via NDPK (nucleoside diphosphate kinase) or creatine kinase) (39, 40). 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 they 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(39).

Other reports have assessed concentrations of TFV-DP or (-)-FTC-TP in lymphocytes or CD14+ cells (monocytes) isolated from healthy volunteers orally administered either TDF or (-)-FTC (41). Results indicated that concentrations of both (-)-FTC-TP and TFV-DP were higher in monocytes versus lymphocytes for some time-points tested, however ranges reported overlapped in some cases. Although it appears that (-)-FTC-TP and TFV-DP concentrations were higher in the monocyte subset, it was difficult to directly correlate these data to ours, as CD14+ monocytes are a markedly different cell type than CD11b+ terminally differentiated macrophages.

Since AZT and DXG are not cross-resistant, AZT can prevent the emergence of the K65R mutation, and the combination of the two drugs are synergistic in vitro and in humans (42). DXG was evaluated in the presence of AZT in macrophages (42, 43). 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 (44).

In summary, our findings demonstrated that significantly lower levels of NRTI-TP are observed in macrophages versus lymphocytes and demonstrate the relative impact of activation state upon antiviral potency. We also demonstrated that TFV displays
similar intracellular concentrations of drug across both lymphocytes and macrophages, and that CBV-treated resting macrophages, TDF- or (-)-FTC-treated resting or activated macrophages, and 3TC-treated resting macrophages displayed EC_{50} values (potencies) that are significantly below the corresponding intracellular concentration of drug in these cells, necessitating consideration for these drugs in targeting virus in macrophage-derived viral sanctuaries (30). These data underscore the importance of delivering adequate levels of drug to primary HIV-1 target cells and viral reservoirs with antiviral potency of drugs across macrophages of various activation states. Definition of these key facets provides a powerful foundation for better understanding how activation states \textit{in vivo} can impact ability to achieve systemic HIV-1 eradication.

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Figure 1. Intracellular concentrations of ART are significantly lower in macrophages (Mϕ) versus PBM cells independent of activation state (A-D, F-I), with the exception of TFV, which was similar in both PBM cells and Mϕ. For all NRTI tested, EC₅₀ for chronically infected Mϕ was > 50 µM (data not shown), and constitutive activation significantly diminished potency versus resting Mϕ (A-I). Potency of Raltegravir and Atazanavir (data not shown) and Raltegravir (I) was similar for resting and activated Mϕ. Data represent mean and standard deviation calculated from at least 5 independent experiments conducted with duplicates within each experiment. * Indicates statistically significant difference relative to PBM cells (p < 0.01). † Indicates significant difference relative to resting PBM cells (p < 0.05).
## Table 1. Intracellular concentrations and antiviral potency of ART in resting or activated PBM cells and macrophages.

<table>
<thead>
<tr>
<th>Drug Tested</th>
<th>State of Cells</th>
<th>Active metabolite measured</th>
<th>Intracellular NTP or drug in PBM cells (µM)</th>
<th>Intracellular NTP or drug in PBM cells (pmol/10⁶ cells)</th>
<th>EC₅₀ in acutely infected PBM cells (µM)</th>
<th>Intracellular NTP or drug in MΦ (µM)</th>
<th>EC₅₀ in acutely infected MΦ (µM)</th>
<th>EC₅₀ in chronically infected MΦ (µM)</th>
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<td>AZT</td>
<td>Activated</td>
<td>AZT-TP</td>
<td>3.5 ± 0.9**</td>
<td>1.1 ± 0.3</td>
<td>0.004 ± 0.002</td>
<td>0.3 ± 0.3*</td>
<td>0.8 ± 0.8</td>
<td>0.4 ± 0.04**</td>
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<td>AZT-TP</td>
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<td>Activated</td>
<td>TFV-DP</td>
<td>1.25 ± 0.89</td>
<td>400.0 ± 286</td>
<td>0.01 ± 0.01</td>
<td>160 ± 123</td>
<td>425.6 ± 132</td>
<td>1.7 ± 1.3**</td>
</tr>
<tr>
<td>TDF</td>
<td>Resting</td>
<td>TFV-DP</td>
<td>4.58 ± 1.764</td>
<td>1,460.0 ± 564</td>
<td>N/A</td>
<td>107 ± 144</td>
<td>284.6 ± 383*</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>TFV</td>
<td>Activated</td>
<td>TFV-DP</td>
<td>0.7 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>1.6 ± 1.2</td>
<td>0.7 ± 0.6*</td>
<td>1.9 ± 1.1</td>
<td>2.3 ± 0.9**</td>
</tr>
<tr>
<td>TFV</td>
<td>Resting</td>
<td>TFV-DP</td>
<td>0.7 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>N/A</td>
<td>0.8 ± 0.4*</td>
<td>2.1 ± 1.5</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>(-)-FTC</td>
<td>Activated</td>
<td>(-)-FTC-TP</td>
<td>49.2 ± 41.8</td>
<td>15.7 ± 3.4</td>
<td>0.008 ± 0.007</td>
<td>2.2 ± 2.0*</td>
<td>5.9 ± 5.0*</td>
<td>0.4 ± 0.2**</td>
</tr>
<tr>
<td>(-)-FTC</td>
<td>Resting</td>
<td>(-)-FTC-TP</td>
<td>113 ± 18.8</td>
<td>36.2 ± 6.0</td>
<td>N/A</td>
<td>1.0 ± 0.6*</td>
<td>2.7 ± 1.6*</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>3TC</td>
<td>Activated</td>
<td>3TC-TP</td>
<td>56 ± 21</td>
<td>17.9 ± 6.7</td>
<td>0.06 ± 0.04</td>
<td>0.6 ± 0.4*</td>
<td>3.6 ± 1.0*</td>
<td>0.6 ± 0.3**</td>
</tr>
<tr>
<td>3TC</td>
<td>Resting</td>
<td>3TC-TP</td>
<td>84.5 ± 36</td>
<td>27.0 ± 11.5</td>
<td>N/A</td>
<td>0.8 ± 0.3*</td>
<td>2.1 ± 0.8*</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>DKG</td>
<td>Activated</td>
<td>DKG-TP</td>
<td>0.2 ± 0.1</td>
<td>0.06 ± 0.03</td>
<td>0.3 ± 0.2</td>
<td>0.05 ± 0.04*</td>
<td>0.1 ± 0.1</td>
<td>0.6 ± 0.2**</td>
</tr>
<tr>
<td>DKG</td>
<td>Resting</td>
<td>DKG-TP</td>
<td>0.5 ± 0.2</td>
<td>0.2 ± 0.06</td>
<td>N/A</td>
<td>0.04 ± 0.03*</td>
<td>0.1 ± 0.06*</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>RAL</td>
<td>Activated</td>
<td>Raltegravir</td>
<td>80.9 ± 14.7</td>
<td>25.9 ± 4.7</td>
<td>0.001 ± 0.002</td>
<td>10.5 ± 4.1*</td>
<td>27.9 ± 11.0</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>RAL</td>
<td>Resting</td>
<td>Raltegravir</td>
<td>55.3 ± 24.1</td>
<td>17.7 ± 7.9</td>
<td>N/A</td>
<td>7.0 ± 0.2*</td>
<td>38.6 ± 0.5*</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td>ATV</td>
<td>Activated</td>
<td>Atazanavir</td>
<td>ND</td>
<td>ND</td>
<td>0.007 ± 0.002</td>
<td>ND</td>
<td>ND</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>ATV</td>
<td>Resting</td>
<td>Atazanavir</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
<td>ND</td>
<td>ND</td>
<td>0.03 ± 0.04</td>
</tr>
</tbody>
</table>

Error bars indicate standard deviation. * indicates significant difference relative to PBM cells (p < 0.01). ** indicates significant difference relative to resting PBM cells (p < 0.05). *** indicates significant difference relative to resting macrophages (p < 0.05). All data represent at least 5 independent experiments containing pooled cells from 8 donors, and duplicates in each experiments. EC₅₀ refers to effective concentration 50, which is the concentration of drug required to inhibit 50 % of viral replication.
Table 2. Intracellular concentrations of AZT-TP or DXG-TP for activated or resting macrophages or PBM cells treated with 10 µM in a 1:1 ratio of AZT:DXG. The presence of AZT did not antagonize DXG-TP levels in macrophages or PBM cells, independent of activation state, and the presence of DXG did not antagonize AZT-TP levels in macrophages or PBM cells independent of activation state.