THE EFFECTS OF HIV INFECTION ON THE EXPRESSION OF THE DRUG EFFLUX PROTEINS P-GLYCOPROTEIN AND BREAST CANCER RESISTANCE PROTEIN IN A HUMAN INTESTINE MODEL

BY

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Submitted to the graduate degree program in Preventive Medicine and Public Health and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science in Clinical Research.

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Abstract

Background: Emerging evidence suggests poor antiretroviral penetration within human gastrointestinal (GI) tissues may contribute to HIV persistence within reservoirs despite effective therapy. We hypothesize that HIV infection induces an upregulation of drug efflux pumps P-glycoprotein and Breast Cancer Resistance Protein (BCRP) within GI lymphocytes which may limit antiretroviral accumulation within these cells. The mechanism of upregulation may be related to the HIV-1 protein Tat, which generates oxidative stress.

Methods: Primary lymphocytes were isolated from human blood and co-cultured with Caco2 cells in a Transwell® configuration to model the *in vivo* environment of the human intestine. Cells were infected with HIV and triplicate samples were lysed over a 7 day time course. Primary lymphocytes were also treated with 0.1nM Tat for 24 hours. Lymphocyte and Caco2 samples were immunoblotted for P-glycoprotein and BCRP expression using β-actin to normalize. Tat treated samples were additionally probed for peroxiredoxin sulfate, a marker for oxidative stress marker, and standardized to 2-cys- peroxiredoxin.

Results: P-glycoprotein expression was increased in both HIV-infected, activated and resting lymphocytes compared to uninfected controls. BCRP expression was also increased in HIV-infected resting lymphocytes compared to controls. Additionally, P-glycoprotein expression was increased by 43% in lymphocytes treated with Tat ($p < 0.05$). No difference in BCRP expression was observed in Tat exposed lymphocytes (8% increase, $p<0.05$). Peroxiredoxin sulfate trended toward being increased (27%) in Tat treated lymphocytes ($p>0.05$). Little change was observed in the expression of P-glycoprotein and BCRP in Caco2 cells exposed to HIV.

Conclusions: These data demonstrate an upregulation of two drug efflux proteins important in HIV pharmacology, P-glycoprotein and BCRP, in HIV-infected primary lymphocytes grown in

an *in vitro* intestinal model. Furthermore, Tat may contribute to the increased expression of P-glycoprotein through oxidative stress as indicated by the elevation of peroxiredoxin sulfate in Tat treated cells. These results suggest a mechanism to explain decreased antiretroviral concentrations within the lymphoid rich regions of the GI tract, an important viral reservoir. Understanding the mechanisms of decreased antiretroviral accumulation within reservoir tissues will be critical in developing therapies to modulate and optimize HIV treatment.

Acknowledgements

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Additionally I would like to thank Dr. Tracey Taylor for your assistance in my research endeavors and in my life in general. Jerry Marlin, you have taught me that extreme organization and meticulous protocols matter and you keep our lab functioning at a level of excellence. Greg Rice, thank you for your continual hard work and willingness to help. Jenni Gillespie, you demonstrated great dedication to this project and I could not have isolated any lymphocytes without you. Dr. Patrick Clay, you changed my life with a single meeting. Thank you for presenting me with the opportunity to participate in the T32 program.

Thank you to Anita Macan, Dr. Theresa Shireman, Dr. Matt Mayo and all of the faculty and staff at KUMC with whom I have had the pleasure of working and studying this year.

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Background

Introduction

Although there have been many advances in treatment, there is currently no cure for HIV-1. Since the beginning of this epidemic 31 years ago, over 60 million people have been infected with HIV and 25 million have died from HIV-related causes.[1] HIV-infected individuals suffer from a high disease burden and are required to remain on combinations of drugs throughout their lifetime. Therapies can result in a decrease in viral load to below the limit of detection but if antiretroviral therapy is discontinued, viral replication resumes. This is due in part to the presence of viral reservoirs, tissues where the virus is able to persist despite "effective" therapy.[2] Viral reservoirs include the central nervous system (CNS), male and female genital tract, seminal fluids, and the gastrointestinal (GI) tract. The GI tract is known to be an important reservoir because the Gut Associated Lymphoid Tissue (GALT) is disproportionately infected with HIV compared to peripheral blood, even in patients receiving therapy.[3]

One new and emerging theory for the mechanism of reservoir maintenance is that antiretroviral drug penetration is decreased in reservoir tissues. Indeed, variable drug concentrations have been seen in the lymphoid tissues of the GI tract. Schacker *et al.* recently reported data (from 5 antiretroviral naïve subjects) indicating that despite the fact that initiation of highly active antiretroviral therapy (HAART) resulted in a drop in HIV viral concentrations (to undetectable limits in blood), virus could still be detected in gut tissues.[4] Furthermore, Fletcher et al. have reported that concentrations of the antiretroviral drugs within these same gut lymph tissues was variable and often did not reach therapeutic concentrations.[4]

Given these significant discoveries, it is important to consider potential mechanisms of decreased antiretroviral accumulation with HIV reservoir tissues including the gastrointestinal tract. Decreased drug concentrations within selected reservoirs could be related to increased efflux of antiretroviral drugs. Increased expression of drug efflux pumps such as P-glycoprotein and Breast Cancer Resistance Protein (BCRP), which are responsible for the efflux of many antiretroviral drugs, by HIV infection could result decreased accumulation of antiretroviral drugs within reservoir tissues. (see Table 1 for a complete list of antiretroviral substrates [5, 6]) Both P-glycoprotein and BCRP are normally expressed in healthy tissues including lymphocytes[7, 8]. Alterations in drug efflux proteins within reservoir cells (ie, lymphocytes of the GI tract) could be responsible for restricting antiretroviral accumulation within reservoir tissues because of increased drug transport out of cells.

Table 1: Antiretroviral drugs known to be substrates of P-glycoprotein and BCRP

Substrates of P-glycoprotein	Substrates of BCRP
Protease Inhibitors (PIs)	
Saquinavir	Atazanavir
Ritonavir	Lopinavir
Indinavir	Nelfinavir
Nelfinavir	
Amprenavir	
Lopinavir	
Atazanavir	
Tipranavir	
Darunavir	
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	
None	Delavirdine
Nucleoside Reverse Transcripatase Inhibitors (NRTIs)	
Zidovudine*	Zidovudine
Abacavir	Stavudine*
Tenofovir DF	Lamivudine
	Abacavir
Entry inhibitors	
Raltegravir	None
Elvitegravir	
Maraviroc	

* indicates conflicting evidence in the literature
Summarized from Weiss (2010)[5] and Szakacs (2008) [6]

There are several possible mechanisms by which HIV infection could enhance activity and/or expression of drug efflux pumps. The HIV-1 protein transactivator of transcription (Tat) enhances the efficiency of HIV transcription and replication and is known to increase the expression of cytokines and promote infection.[9] Within the brain, Tat has been shown to increase the expression of P-glycoprotein and another important efflux pump (MRP1) in the microvascular endothelium of the blood brain barrier.[10] However, to date, Tat effects on drug efflux proteins within the GI reservoir have not been examined.Tat effects, Because Tat is

known to disrupt pathways that maintain intracellular oxidative balance, any Tat related effects on drug efflux protein expression could be related to elevated levels of oxidative stress, [11, 12].

Study Objective

Despite being capable of suppressing viral concentrations to below detectable limits in blood plasma, current antiretroviral therapies cannot completely eradicate HIV from infected individuals. This is due, in part, to the presence of viral reservoirs throughout the body. Given the important roles of P-glycoprotein and BCRP in the transport of therapies involved in HIV infection, it is likely that an increase in expression of either of these drug efflux proteins in lymphoid tissue of the GI tract would result in decreased accumulation of many antiretrovirals within this important reservoir site. Therefore the effect of HIV infection on the expression of these proteins is of interest to understanding the maintenance of the viral reservoir in the gastrointestinal tract.

This study was designed to investigate the mechanisms which dictate diminished distribution of antiretroviral drugs into GI lymphoid tissue. Our **hypothesis** is that HIV infection of the lymphoid rich regions of the gastrointestinal tract (ie, the reservoir) causes increases in the expression of the drug efflux proteins P-glycoprotein and BCRP, which are responsible for the transport of HIV drugs out of target cells. Changes in the expression of these proteins may result in decreased antiretroviral accumulation within the reservoir. We anticipated increases in drug efflux protein expression would only occur in the lymphocytes and not in enterocytes, as increased efflux out of enterocytes could impair systemic absorption. The **objective** of this study was to measure the effects of HIV infection on drug transport protein expression in an *in vitro* co-culture model of the human intestinal tract using isolated primary human lymphocytes and

Caco2 cells (enterocyte cell line) and to examine the role of the HIV protein Tat as a potential mechanism for observed changes in drug efflux protein expression.

Materials and Methods

Chemicals

Interleukin-2, phytohaemagglutinin (PHA), Tumor Necrosis Factor-Alpha (TNF-α), and gluteraldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Ficoll-Paque Plus was purchased from GE Healthcare (Pittsburgh, PA). Potassium ferrocyanide and potassium ferricyanide were purchased from RICCA (Arlington, TX). Magnesium chloride and Fetal Bovine Serum (FBS) were purchased from Thermo Fisher (Waltham, MA). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was purchased from Life Technologies (Carlsbad, CA). Formaldehyde was purchased from VWR (Radnor, PA).

Cell Lines and Virus

Cell culture reagents were obtained from Life Technologies (Carlsbad, CA) unless otherwise indicated. The following agent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: J1 from Dr. Thomas Folks. J1.1 cells are a Jurkat derived cell line that produces HIV (CXCR4 tropic LAI strain) upon induction with TNF-α (10ng/mL). Jurkat E6-1 cells, a human leukemic T cell lymphoblast line, were purchased from American Type Culture Collection (ATCC) #TIB-152 (Manassas, VA), infected with HIV and used to propogate the CXCR4-tropic LAI strain of HIV. MDCK-MDR1 cells were a generous gift from Dr. Jeff Krise (University of Kansas School of Pharmacy, Lawrence, KS). This cell line originates from Madin Darby canine kidney cells, that overexpress P-glycoprotein due to a stable transfection with *MDR1,* and was used as a positive control for P-

glycoprotein. Caco-2 cells, derived from human colonic adenocarcinoma cells, were purchased from ATCC #HTB-37 and were used as an enterocyte model. Caco2 cells, a human colon cancer derived cell line, when cultured in a Transwell format for 21-24 days become polarized and develop appropriate localization of drug transport proteins and therefore serve as a model for intestinal enterocytes.[13] Caco-2 cells passaged at least 50 times were used as positive control for BCRP, as high passage Caco-2 cells have been shown to express a 3-fold increase in BCRP compared to lower passage Caco-2 cells.[14] The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. TZM-bl cells were used for virus quantification as they are indicator cells for a β-galactosidase assay. Primary lymphocytes were isolated from whole blood obtained from healthy volunteers using a Ficoll-Paque Plus method.[15] IRB Protocol #233110-1 approved by the Kansas City University of Medicine and Biosciences IRB 04/13/2011.

Caco-2, MDCK-MDR1 and TZM-bl cells were grown to at least 70% confluence in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin. J1.1, Jurkat E6-1, and primary lymphocytes were grown in RPMI medium (Mediatech, Manassas, VA). All cells were maintained at 37^{o}C in humidified 5% CO_2 with fresh medium replaced every 2 to 3 days.

Virus Production and Quantification

J1.1 cells were induced to produce HIV-1 with 10ng/ml TNF-α in RPMI at 37^{0}C for 48 hours. The cells were centrifuged at 277g for 10 minutes and the viral supernatant was collected. 1 x 10^6 Jurkat E6-1 cells were pelleted by centrifugation at 277g for 10 minutes. The pellet was

resuspended in 500 microliters of J1.1 viral supernatant and plated in a single well of a tissue culture grade 24 well plate. After incubating for 1 hour at 37°C and 5% CO_2, the Jurkat E6-1 cells were transferred to 4.5mL of RPMI. The infected Jurkat E6-1 cells were grown in RPMI at 37°C and 5% CO_2 for seven days. After seven days, the cells were centrifuged at 277g for 10 minutes and the viral supernatant removed and stored at -80°C.

TZM-bl cells were grown in DMEM at 37°C until 70% confluent. Then, TZM-bl cells were diluted to 2 x 10^5 cells/ml using DMEM with 1,000x DEAE Dextran. The virus solutions were diluted to 1:5, 1:25, 1:125, 1:625 and aliquoted to 96 well tissue culture grade plates. 50 microliters TZM-bl cells (2 x 10^5 cells/ml) were added to each well and the plate was incubated at 37°C for 48 hours. After 48 hours, media was removed and fixing solution [1% Formaldehyde, 0.2% gluteraldehyde, 1X PBS] was added to each well for 5 minutes. Cells were washed with 1X PBS and stained for one hour at room temperature using staining solution [1X PBS, 0.004M Potassium ferrocyanide, 0.004M Potassium ferricyanide, 0.002M $MgCl_2$, 0.53 µg/µl X-gal in DMSO (Sigma-Aldrich)]. Cells were washed with 1X PBS and virus was quantified by counting the number of β-galactosidase positive cells cells per 40x magnified field and using the following equation:

$$\beta - galactosidase\ positive\ cells\ \times \frac{Virus\ Dilution}{50ul} = Virion\ Concentration\ (\frac{Virions}{ml}).$$

Cell Co-Culture Configuration

The intestinal model used for this study consisted of both Caco-2 cells (an intestinal enterocyte model) and primary lymphocytes separated by a permeable membrane (Figure 1).

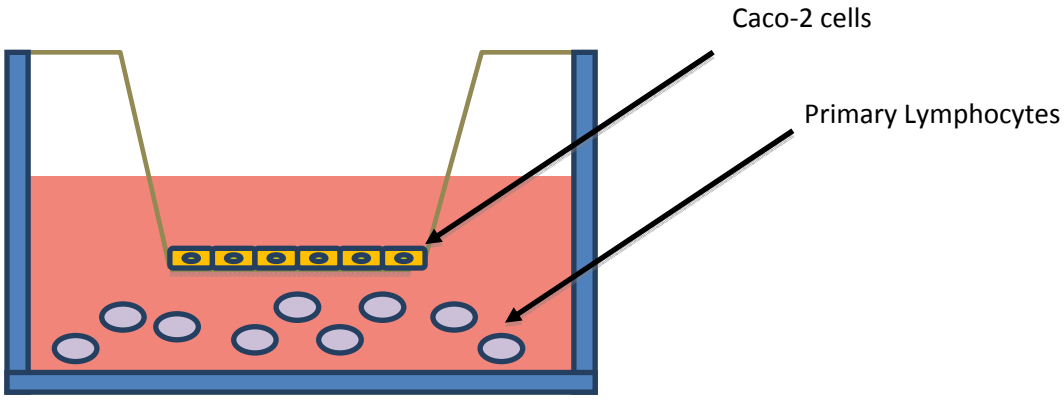

Figure 1. <u>Co-culture model used to mimic the relationship between the intestinal endothelial cells and the lymphoid cells of the GI reservoir.</u> Caco2 cells representing intestinal endothelial cells were grown to confluence on a permeable support in the top compartment of the Transwell plate. Primary lymphocytes were obtained from healthy volunteers and grown in RPMI media in the lower compartment.

The Caco-2 cells were plated at a density of $6x10^4$ cell/mL on a 12 Well Transwell (Corning Inc., Corning, NY) permeable support (0.4μm Polycarbonate). After 21 days of culturing the Caco2 cells on the membranes, media was changed from DMEM to RPMI. At this time, lymphocytes were isolated (see Lymphocyte Isolation/Activation section below) from whole blood and then cultured in suspension on the bottom compartment of the Transwell.

Lymphocyte Isolation/ Activation

Human lymphocytes were obtained via venous blood samples from healthy volunteers. Fresh heparinized human blood samples were separated at room temperature using Ficoll-Paque Plus (GE-Healthcare) and centrifuged at 400g for 30 minutes with the brake disengaged as described previously by Ting (1971).[15] The lymphocyte layer was drawn off and washed with 1X PBS and suspended in RPMI. Primary lymphocytes were counted using a hemacytometer and the cells were plated in the lower compartment of a 12-well transwell plate at a minimum

concentration of $3x10^6$ cells/well. Plates for experiments involving co-cultures with resting lymphocytes at this point were exposed to HIV (see protocol below), while lymphocytes for experiments involving co-cultures with activated lymphocytes were activated with phytohaemagglutinin (PHA) (0.002mg/mL) for 48 hours followed by the addition of 50 units/mL of IL-2 for the duration of the experiment. See Figure 2 for an overview of experiments performed.

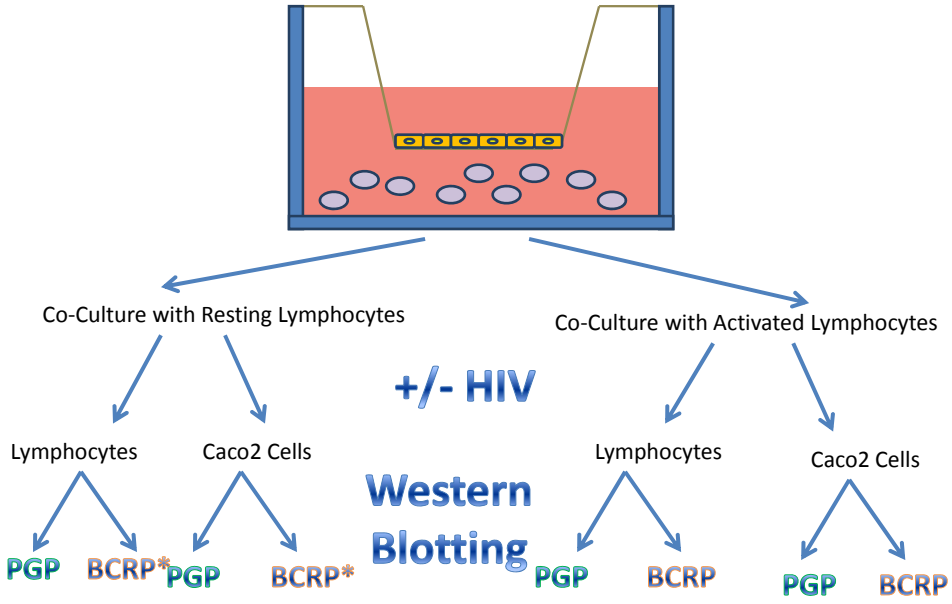

Figure 2: **Flow chart indicating experimental flow after lymphocyte isolation**. *Experiments involving resting lymphocytes and BCRP are ongoing.

Exposure to HIV-1 or HIV Tat

Caco-2 cells and primary lymphocytes were exposed to HIV by adding the viral supernatant at a concentration of 5,000 virions/ml to RPMI media with 1,000x DEAE Dextran. Cells were incubated over a time course (6, 18, 24, 48, 96 or 168 hours) at 37°C and 5% CO_2.

Additional plates of primary lymphocytes were exposed to HIV Tat by adding Tat to RPMI media at a final concentration of 0.1 nM (for 24 hours) at 37^{o}C and 5% CO_2. A time course with multiple time points and multiple concentrations of Tat was performed and treatment using 0.1nM Tat for 24 hours was selected as the time and concentration where Tat had the optimal effect (data not shown).

Cell Lysis of Lymphocytes and Caco2 Cells for Protein Content

To isolate total protein from the lymphocytes and Caco2 cells, cells were lysed at the designated time points (6, 18, 24, 48, 96, 168 hours) of a seven day time course. At each time point, primary lymphocytes were washed with 1X PBS and centrifuged at 600g for 10 min at room temperature to pellet cells. The supernatant was discarded and pelleted cells were resuspended in a NP40 lysis buffer [1% Igepal Ca-630 (Sigma-Aldrich), 150 mM Sodium Chloride (Thermo Fisher), 50mM Tris (Avantor, Center Valley, PA) HCl (EMD)pH 7.5, Sodium Hydroxide (EMD, Gibbstown, NJ) as needed to achieve pH of 8.0] and 1X Complete Protease Inhibitor (Roche, Mannheim, Germany). Caco2 cells were washed with 1X PBS and lysed on the tissue culture plate by scraping cells in the lysis buffer off the Transwell membrane with a cell spatula. Both lymphocytes and Caco2 cells were incubated for 5 minutes on ice, sonicated for 2-3 seconds, centrifuged at 18,188g (4^{o}C, 10 min) and stored at -80^{o}C. Total protein was quantified using BCA Protein Assay Kit (Pierce/Thermo).

Analysis of Protein Expression

To examine the effects of HIV infection and Tat exposure on the expression on drug efflux proteins in primary lymphocytes and enterocytes (Caco2 cells), we examined P-glycoprotein and BCRP expression via Western blotting using the methodology described below

(Immunoblotting) with primary antibodies for P-glycoprotein, BCRP, and β-actin. To measure oxidative stress in Tat treated samples we used primary antibodies for Peroxiredoxin-SO_3, 2-Cys-Peroxiredoxin. Peroxiredoxin is a collective term for a family of peroxidases (22-27kDa) that catalyze the reduction of hydrogen peroxide through the reactive Cys residues of the enzymes.[16] 2-cys-peroxiredoxin (2-cys-Prx) acts as a flood gate protecting cells against peroxide substances such as H_2O_2.[17] However, under high levels of oxidative stress, 2-cys-peroxiredoxin can be overoxidized to the sulfonic form ($PrxSO_3$).[17] With this conversion, the peroxidase activity of the peroxiredoxin is lost[18] and cells become susceptible to severe damage from peroxide molecules. Increased or altered distribution of peroxiredoxin proteins has been used as a marker for intracellular oxidative stress in studies of Parkinson's, Alzheimers, and other conditions.[16, 19, 20]

Immunoblotting

Protein expression was analyzed using Western blotting. For each sample, 30μg of total cellular protein in Laemmli Buffer [125mM Tris-HCL ph 6.8, 2mM EDTA (Sigma-Aldrich), 4% SDS (Avantor), 50% glycerol (Life Technologies), and 0.02% bromphenol blue(EMD) and 10% 2-Mercaptoethanol(Sigma-Aldrich)] was resolved with electrophoresis on a 12% Novex Tris-HCL gel (Life Technologies) with a 1X Tris-Glycine SDS running buffer and transferred onto Immoblion-P polyvinylidene difluoride (PVDF) 0.45um pore membranes (Millipore, Billerica, MA). Membranes were blocked for one hour at room temperature in 5% nonfat milk solution [2g of milk + 40ml 0.1% TTBS (0.025M Tris pH7.5, 0.15M NaCl, 0.1% Tween 20(Avantor)]. Then, membranes were incubated overnight at 4^{o}C with primary antibody diluted to the appropriate concentration in 3% Bovine Serum Albumin (BSA) (EMD) and 0.1% Na Azide in TTBS for the following proteins: P-glycoprotein, BCRP, β-Actin, Peroxiredoxin-SO_3 and 2-Cys-

Peroxiredoxin. Primary antibody information is summarized in Table 2. Blots were then incubated for one hour at room temperature with the horseradish peroxidase-conjugated anti-mouse (1:10,000 dilution in 5% nonfat milk/TTBS solution) secondary antibody. Signals were enhanced with chemiluminescence using SuperSignal West Dura System (Pierce/Thermo Fisher Scientific, Waltham, MA) and detected by exposure to Premium X-Ray Film (Phenix Research Products, Candler, NC). The chemiluminescence signal intensity was quantified using an Epson Expression 10,000 XL scanner and ImageJ software (NIH).

Table 2: Summary of primary and secondary antibodies used in western blot experiments

Primary Antibody	**Protein Size (kD)**	**Company**	**Dilution**	**Secondary Antibody**	**Company**	**Dilution**
C219 Monoclonal Antibody, Purified	170	Covance (Princeton, NJ)	1:200	Anti-mouse IgG, HRP-linked Antibody	Cell Signaling (Danvers, MA)	1:10,000
Breast Cancer Resistance Protein (human), mAb (BXP-21)	72	Enzo Life Sciences (Farmingdale, NY)	1:50	Anti-mouse IgG, HRP-linked Antibody	Cell Signaling (Danvers, MA)	1:10,000
Monoclonal Anti-β-Actin Clone AC-15	42	Sigma-Aldrich (St. Louis, MO)	1:100,000	Anti-mouse IgG, HRP-linked Antibody	Cell Signaling (Danvers, MA)	1:10,000
Anti-Peroxiredoxin-SO_3 antibody [10A1]	25	abcam (Cambridge, MA)	1:200	Anti-mouse IgG, HRP-linked Antibody	Cell Signaling (Danvers, MA)	1:10,000
Anti-2CysPeroxiredoxin antibody [6E5]	22	abcam (Cambridge, MA)	1:1,000	Anti-mouse IgG, HRP-linked Antibody	Cell Signaling (Danvers, MA)	1:10,000

Data Analysis

Scatter plots of all available data were generated with mean relative protein expression of repeated trials with standard error of the mean error bars. Predictive curves were generated and area under the curve (AUC) was calculated by the trapezoidal rule using SigmaPlot 12.0. Time courses were excluded from AUC calculations if data was missing from critical time points. Results are reported as the mean AUC with standard deviation and 95% confidence intervals. Wilcoxon Rank Sign Tests were performed to compare control AUCs to AUCs of HIV infected samples. To test for an increase in P-glycoprotein, BCRP and peroxiredoxin sulfate expression in response to Tat exposure one-tailed, paired Students *t*-tests were conducted between each treatment group and the appropriate control. Baseline criterion for statistical significance was $p < 0.05$.

Results

In this study we examined the effects of HIV infection on the expression of P-glycoprotein and BCRP in an *in vitro* co-culture model of the human intestinal tract. Additionally we investigated the role of the HIV protein Tat as a potential mechanism for observed changes in drug efflux protein expression.

HIV Effects on P-glycoprotein and BCRP Expression in Primary Lymphocytes

Time course experiments (6 time points over 7 days) were performed using isolated lymphocytes grown in co-culture with Caco2 cells exposed to HIV. Lymphocyte and Caco2 cell lysates from each time point were analyzed for P-glycoprotein and BCRP expression. Five time course experiments were completed with co-cultures of activated lymphocytes and three complete time course experiments were completed with co-cultures of resting lymphocytes. We

detected P-glycoprotein protein expression in isolated primary lymphocytes and Caco2 cells at approximately 170kDa using the mouse monoclonal antibody C219, raised against an internal epitope of human P-glycoprotein. MDCK-MDR1 cell lysate was used as a positive control for P-glycoprotein expression. BCRP expression was detected in isolated primary lymphocytes and Caco2 cells at approximately 72kDa using the mouse monoclonal antibody BXP-21. Lysate from high passage number (p50) Caco2 cells was used as a positive control. Samples were normalized for protein concentration using β-actin (42kDa) with a mouse monoclonal antibody (Figure 3).

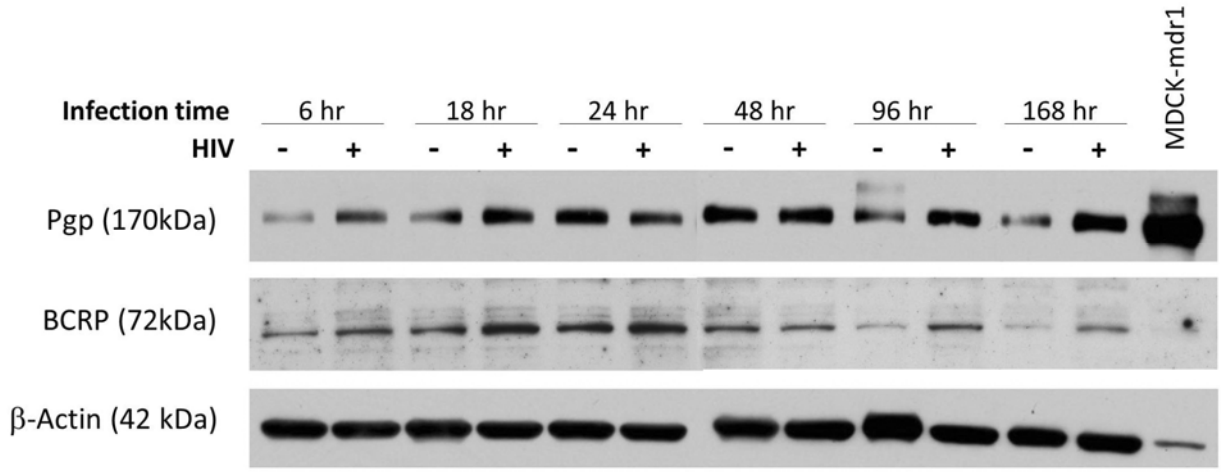

Figure 3: <u>Representative Blot of P-glycoprotein and BCRP Expression in Primary Lymphocytes Exposed to HIV</u> A representative blot of P-glycoprotein and BCRP expression from HIV infected, activated primary lymphocytes. MDCK-mdr1 cell lysate and high passage number Caco2 cells (P50) were used as positive controls for P-glycoprotein and BCRP, respectively. β-actin was used to normalize.

The blots represented in Figure 3 were quantified for relative P-glycoprotein expression, as were blots from experiments with resting lymphocytes and results are shown in Figure 4. Three to five time courses, with triplicate replicates at each time point, were analyzed for

experiments. Results are shown as the mean P-glycoprotein expression (normalized to β-actin) with standard error bars. Predictive curves were calculated to fit the data points. Overall, exposure to HIV resulted in an increased expression of P-glycoprotein in both the activated primary lymphocytes as well as resting lymphocyte experimental groups (Figure 4). Data for all experiments is summarized in Table 3, including the mean area under the curve (AUC) for P-glycoprotein expression in HIV infected activated lymphocytes which was 108.28 (95% CI 98.97-117.59) was higher than the control mean AUC of 54.52 (95% CI 24.49-84.54). A similar observation of a lesser magnitude was found in resting lymphocytes. P-glycoprotein expression AUC in HIV infected non-activated lymphocytes was 79.32 (95% CI -43.50 – 202.13) similar to that in the control group which had a mean AUC of 63.05 (95% CI 11.61-114.50).

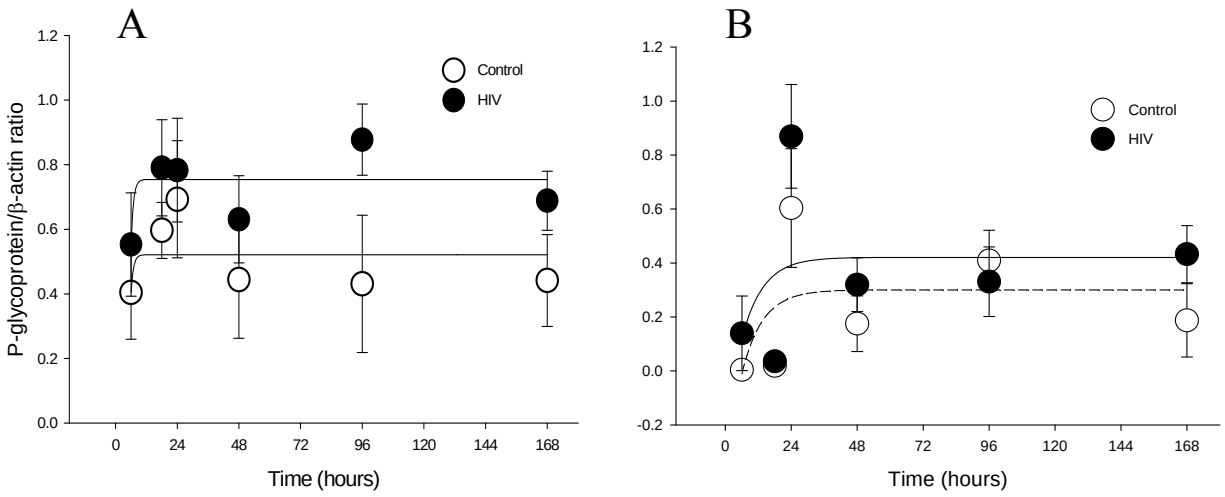

Figure 4: <u>Analysis of P-glycoprotein Expression in Primary Lymphocytes in Response to Exposure with HIV</u> Analysis from Western Blots revealing relative P-glycoprotein expression (normalized to β-actin) in activated primary lymphocytes cultured in a co-culture configuration with Caco2 cells. Panel A represents activated lymphocytes from the co-culture. Panel B represents resting lymphocytes from the co-culture. Graphs represent means from a minimum of 3 separate experiments (each performed in triplicate) with standard error bars.

Table 3 Area under the curve (AUC) summary data from co-culture experiments involving primary lymphocytes and Caco2 cells. Data represent mean AUC 2-3 complete time course experiments with standard deviation (St. Dev.) and 95% confidence intervals (CI).

	AUC Control			AUC HIV			p-value
Lymphocytes	Mean	St.Dev.	95% CI	Mean	St.Dev.	95% CI	
P-glycoprotein Expression in Activated Lymphocytes	54.52	15.32	(24.49 - 84.54)	108.28	4.75	(98.97 - 117.59)	0.1250/0.125
P-glycoprotein Expression in Resting Lymphocytes	63.05	26.25	(11.61 - 114.50)	79.32	62.66	(-43.50 - 202.13)	1.0000
BCRP Expression in Activated Lymphocytes	24.72	0.11	(24.52 - 24.93)	43.47	3.42	(36.76 - 50.18)	0.5000/0.2500
Caco2 Cells							
P-glycoprotein Expression in Caco2 cells co-cultured with Activated Lymphocytes	203.66	114.07	(-19.91 - 427.24)	265.05	109.67	(50.11 - 480.00)	0.2500/0.0625
P-glycoprotein Expression in Caco2 cells co-cultured with Resting Lymphocytes	156.43	51.18	(56.12 - 256.73)	164.86	28.52	(108.97 - 220.76)	1.0000
BCRP Expression in Caco2 cells co-cultured with Activated Lymphocytes	338.65	21.02	(297.44 - 379.85)	383.65	12.88	(358.41 - 408.89)	0.5000/0.2500

An increase in the expression of BCRP was observed in HIV infected activated lymphocytes compared to uninfected controls at all time points (Figure 5). The mean AUC for BCRP expression in HIV infected activated lymphocytes was 43.47 (95% CI 36.76 – 50.18) which is greater than the mean AUC of control samples [24.72 (95% CI 24.52-24.93)] (Table 3).

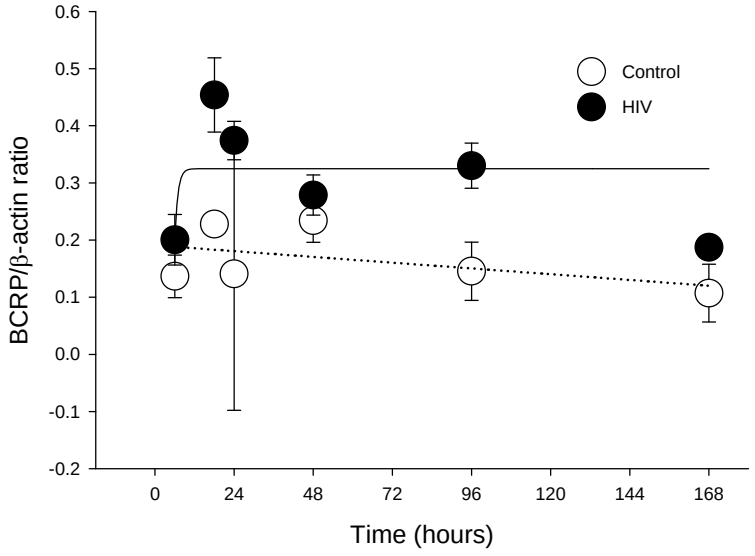

Figure 5: Analysis of BCRP Expression in Primary Lymphocytes in Response to Exposure with HIV Analysis from Western Blots revealing relative BCRP expression (normalized to β-actin) in activated primary lymphocytes culture in a co-culture configuration with Caco2 cells.

HIV Effects on P-glycoprotein and BCRP in Caco2 Cells

To determine if the HIV-induced increase in drug efflux protein expression was exclusive to the lymphocytes in our model system, we examined the expression of P-glycoprotein and BCRP in Caco2 cells grown in co-culture with isolated lymphocytes. We did not anticipate significant increases in the expression of these drug efflux pumps in the Caco2 cells, as this would impede normal drug absorption into circulation. We detected and analyzed protein

expression of P-glycoprotein and BCRP in HIV exposed Caco2 cells (Figures 6). The protein expression was quantified and normalized for loading using β-actin as described above.

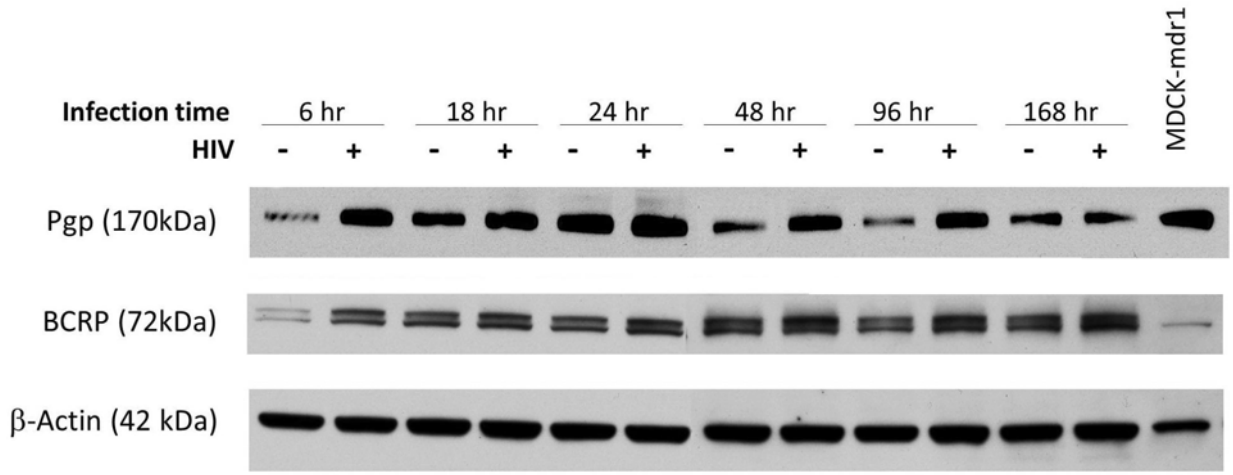

Figure 6: <u>Representative Blot of P-glycoprotein and BCRP Expression in Caco2 Cells Exposed to HIV</u> A representative blot of P-glycoprotein and BCRP expression from HIV exposed Caco2 cells. MDCK-mdr1 cell lysate and high passage number Caco2 cells (P50) were used as positive controls for P-glycoprotein and BCRP, respectively. β-actin was used to normalize.

Similar to lymphocytes, we analyzed the expression of P-glycoprotein and BCRP in Caco2 cells in response to HIV when grown in co-culture with activated and resting primary lymphocytes (Figure 7). The mean AUC for P-glycoprotein expression in Caco2 cells grown in co-culture with activated lymphocytes was 265.05 (50.11 – 480.00) compared to the control mean AUC of 203.66 (95% CI -19.91-427.24). In Caco2 cells grown in co-culture with resting lymphocytes, P-glycoprotein expression AUC in HIV infected samples was 164.86 (95% CI -108.97 – 220.76) compared to the control group which had a mean AUC of 156.43 (95% CI 56.12-256.73) (Table 3).

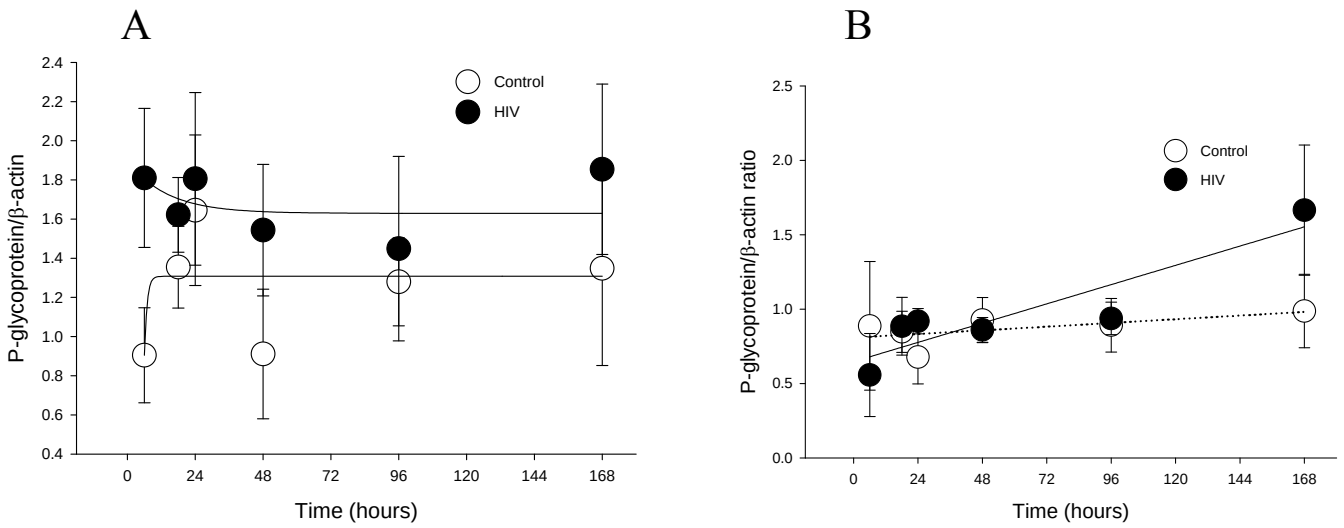

Figure 7: <u>Analysis of P-glycoprotein Expression in Caco2 cells in Response to Exposure with HIV</u>
Analysis from Western Blots revealing P-glycoprotein expression (normalized to β-actin) in Caco2 cells grown in co-culture with primary lymphocytes. Panel A represents Caco2 cells grown in co-culture with activated lymphocytes. Panel B represents Caco2 cells grown in co-culture with resting lymphocytes. Graphs represent means from a minimum of 3 separate experiments (each performed in triplicate) with standard error bars.

BCRP expression within Caco-2 cells grown in co-culture with activated lymphocytes does not appear to be altered upon exposure to HIV (Figure 8). The mean AUC for BCRP expression in HIV infected samples was 383.65 (95% CI 358.41-408.89). The mean AUC for BCRP expression in the control group was 338.65 (95% CI 297.44-379.85) (Table 3).

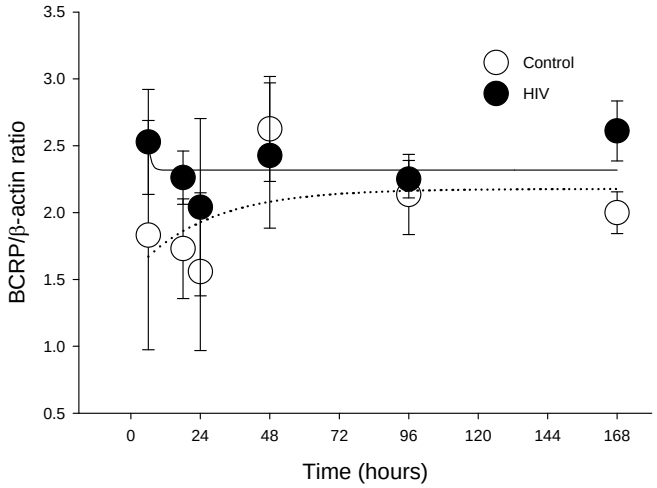

Figure 8: Analysis of BCRP Expression in Caco2 cells in Response to Exposure with HIV
Analysis from Western Blots revealing relative BCRP expression (normalized to β-actin) in Caco2 cells grown in co-culture with activated primary lymphocytes. Graphs represent data from 3 separate experiments (each performed in triplicate).

Relative Drug Efflux Pump Expression in Response to HIV-1 Tat Exposure

In order to investigate a potential mechanism of the upregulation of drug efflux proteins observed in response to HIV, we examined the effects of exposure to HIV-1 Tat on P-glycoprotein and BCRP expression in lymphocytes. In response to 0.1nM Tat exposure for 24 hours, P-glycoprotein expression in resting lymphocytes increased by 43% ($p < 0.05$) at 24 hours (0.1nM Tat) (Figure 9).

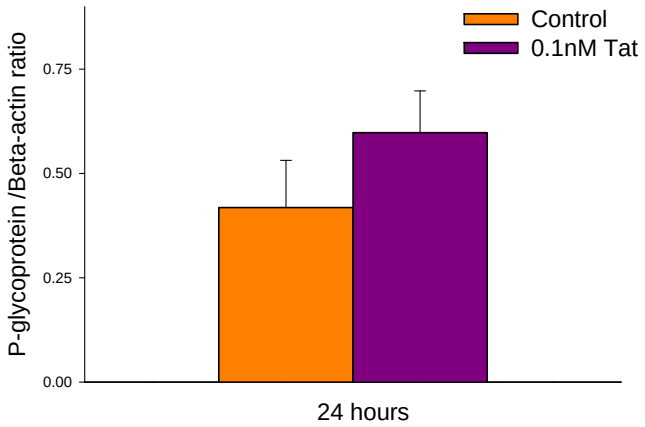

Figure 9: Exposure to Tat increases P-glycoprotein Expression in Primary Lymphocytes
Graph representing resting lymphocytes exposed to 0.1nM Tat for 24 hours, resulting in a 43% increase in P-glycoprotein expression ($p<0.05$) compared to untreated controls. Graphs represent data from 3 separate experiments (each performed in triplicate).

BCRP expression in resting lymphocytes exposed to 0.1nM Tat for 24 hours was not different from unexposed samples (8% increase in Tat-treated lymphocytes, $p=0.19$) (Figure 10).

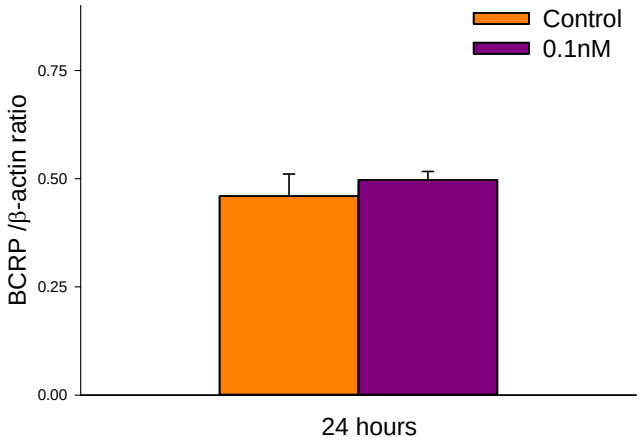

Figure 10: Exposure to Tat does not affect BCRP Expression in Primary Lymphocytes
Graph representing resting lymphocytes exposed to 0.1nM Tat for 24 hours, resulting in no significant change in BCRP expression ($p>0.05$) compared to untreated controls. Graphs represent data from 3 separate experiments (each performed in triplicate).

Expression of Oxidative Stress Marker in Tat-treated Lymphocytes

Oxidative stress is involved the pathogenesis of HIV[21, 22] and HIV-1 Tat is believed to be involved in this process. To examine if the observed increase in P-glycoprotein expression in Tat treated cells was associated with increased levels of oxidative stress, we analyzed differences in the expression of the protein peroxiredoxin sulfate, a known marker of oxidative stress, relative to 2-cys-peroxiredoxin. Increased ratios of peroxiredoxin sulfate to 2-cys-peroxiredoxin are the result of excess over-oxidation of the peroxiredoxin protein, indicating high levels of intracellular oxidative stress. We observed the over-oxidized form of the protein peroxiredoxin (peroxiredoxin sulfate) at approximately 25kDa using a mouse monoclonal antibody. We expressed this as a ratio of peroxiredoxin sulfate to the less oxidized form of peroxiredoxin (2-cys-peroxiredoxin) [Prx-SO_3 /2-cys-peroxiredoxin]. There was a 27% increase ($p=0.26$) in this marker of oxidative stress in lymphocytes exposed to 0.1nM Tat for 24 hours, although this finding was not statistically significant (Figure 11).

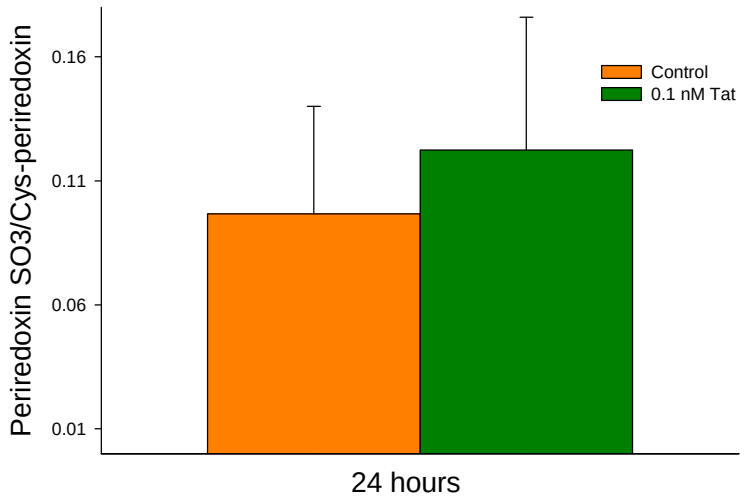

Figure 11: <u>Oxidative Stress in Tat-treated Primary Lymphocytes</u> Graph shows a non-significant increase in levels of oxidative stress in Tat-exposed resting lymphocytes. P-glycoprotein expression was normalized to β-actin and peroxiredoxin sulfate, a marker of oxidative stress, expression normalized to cys-peroxiredoxin. Graphs represent data from 3 separate experiments (each performed in triplicate).

Discussion

Conclusions

These data demonstrate an increase in P-glycoprotein and BCRP expression in HIV infected primary lymphocytes with little effect on the expression of these two efflux pumps in Caco-2 cells (enterocytes), in a human intestine model (summarized in Table 30). Increased drug efflux protein expression in primary lymphocytes is an important discovery as this may result in decreased antiretroviral accumulation within these cells and thereby limits their effectiveness in GI lymphoid tissue.

P-glycoprotein upregulation in response to HIV infection was observed in both activated and resting lymphocytes (Figure 4), which are both important in HIV persistence. Activated

lymphocytes, specifically in the lamina propria of the GI tract, are hosts to HIV-infection and viral replication.[23, 24] Additionally, activated lymphocytes have been shown to be infected with higher levels of HIV, when compared with resting lymphocytes in peripheral blood and GI lymphoid tissue. [3, 25] Resting lymphocytes are also an important potential contributor to HIV persistence as these cells may be a site of residual viral replication[26] and/or may allow unintegrated viral DNA to persist.[27] Despite the lack of integration into the host DNA, unintegrated DNA (a 2-long terminal repeat (LTR) circle) is capable of serving as a transcriptional template for HIV specific proteins, including Tat, which can instigate cell activation, cytokine secretion, and HIV infection[27]. Increased expression of the drug transport protein P-glycoprotein in activated and/or resting lymphocytes could limit the ability of antiretroviral drugs to achieve therapeutic concentrations in these cells and therefore limit their effectiveness.

BCRP expression also was increased in HIV infected activated lymphocytes compared to uninfected controls (Figure 5, Table 3). (Studies of BCRP expression in resting lymphocytes and in Caco2 cells grown in co-culture with resting lymphocytes are ongoing.) Increased expression of BCRP could also contribute to decreased accumulation of antiretroviral therapies within the lymphoid tissues in the GI tract. Our finding of increased expression of BCRP in HIV infected lymphocytes is consistent with findings in other drug resistant pathologies, including several types of leukemias and solid tumors[28].

Although we did not anticipate an increase in P-glycoprotein expression within Caco-2 cells, we did observe increases in P-glycoprotein expression in both Caco2 cells grown in co-culture with both resting and activated lymphocytes (Figure 7), although expression from these samples had much higher variability than lymphocyte samples. We did not observe a substantial

increase in BCRP expression in Caco2 cells co-cultured with activated lymphocytes (Figure 8). We did not anticipate any change in the expression of P-glycoprotein or BCRP within the enterocytes as any alterations of drug efflux proteins within these cells could alter systemic, not localized, antiretroviral exposure. Given the variability of these samples it is difficult to draw definitive conclusions.

Additional investigation revealed that HIV-induced changes in the expression of P-glycoprotein may be mediated by the HIV protein Tat (Figure 9). Our finding of increased P-glycoprotein in lymphocytes in response to treatment with 0.1nM Tat is consistent with Tat's effects on P-glycoprotein in other viral reservoirs. Tat has been shown to increase promoter activity of P-glycoprotein, leading to an increase in the protein's expression in the endothelium of the blood-brain barrier[29]. Hayashi *et al.* (2005) found that exposure to Tat resulted in an increase in the expression of P-glycoprotein in an *in vitro* model of brain microvascular endothelial cells[10]. The upregulation of P-glycoprotein in the blood brain barrier could contribute to lack antiretroviral penetration into the reservoir of the central nervous system.

However, we did not find an increase in the expression of BCRP in lymphocytes treated with Tat compared to controls (Figure 10). These results suggest that the mechanism for increased P-glycoprotein expression in response to HIV infection involves Tat but this mechanism does not contribute to the observed upregulation of BCRP in lymphocytes.

In these experiments, lymphocytes exposed to Tat had higher levels of oxidative stress than controls, as indicated by perioxiredoxin sulfate/2-cys-peroxiredoxin ratios (Figure 11). Tat is known to activate inflammatory pathways within cells and increase levels of oxidative stress within exposed cells [11], therefore we anticipated an increase in oxidative stress in Tat treated

cells. Our experiments resulted in a 27% increase in perioxiredoxin sulfate/2-cys-peroxiredoxin ratio in Tat-treated lymphocytes, however, this difference was not statistically significant. Therefore it is likely that additional mechanisms are contributing to the upregulation of P-glycoprotein we observed in response to Tat exposure.

Our study provides new insight and information about the expression of drug efflux proteins in Caco2 cells and lymphocytes grown in a co-culture model of the human intestine, but it did have some limitations. Although our data demonstrates an important trend toward an increased expression of P-glycoprotein in response to HIV infection in lymphocytes, not all results were statistically significant. To further investigate the implication of our observed trend, functional studies should be performed to determine the consequences of altered protein expression, specifically if the increases in P-glycoprotein and BCRP expression result in increased activity of these proteins and decreased accumulation of antiretrovirals into lymphocytes. Many antiretrovirals, especially protease inhibitors, are known to alter the expression of drug transport and metabolism proteins (Table 1). Therefore HIV effects on the expression of drug efflux proteins *in vivo* may be compounded with changes induced by the antiretroviral drugs themselves. Finally, although many antiretroviral drugs are substrates for P-glycoprotein or BCRP, these two proteins are not exclusively responsible for the uptake and efflux of drugs used in the treatment of HIV. Therefore the upregulation of these P-glycoprotein and BCRP may not fully explain the potential decrease in penetration of selective antiretroviral drugs to reservoir tissues. Future studies should investigate the effects of HIV on other transport proteins, including proteins involved in both uptake and efflux of antiretroviral drugs.

Summary

The purpose of this study was to investigate the effects of HIV infection on drug efflux protein expression, specifically P-glycoprotein and BCRP, in an *in vitro* co-culture model of the human intestine. Our study found an increase in P-glycoprotein and BCRP expression in HIV-infected lymphocytes compared to uninfected controls (Figures 4 & 5). Additionally we observed an increase in the expression of P-glycoprotein but no change in the expression of BCRP in response to the HIV-1 protein Tat (Figure 9 & 10). Tat exposure was accompanied by increased levels of oxidative stress (Figure 11). Collectively these data represent important observations about HIV effects on lymphocytes in a human intestine model. These results suggest a mechanism to explain the decreased penetration into lymphoid rich regions of the GI tract, an important viral reservoir. These changes may contribute to the persistence of HIV despite treatment with antiretroviral therapy and represent a critical finding in the effort to modulate and optimize HIV therapy.

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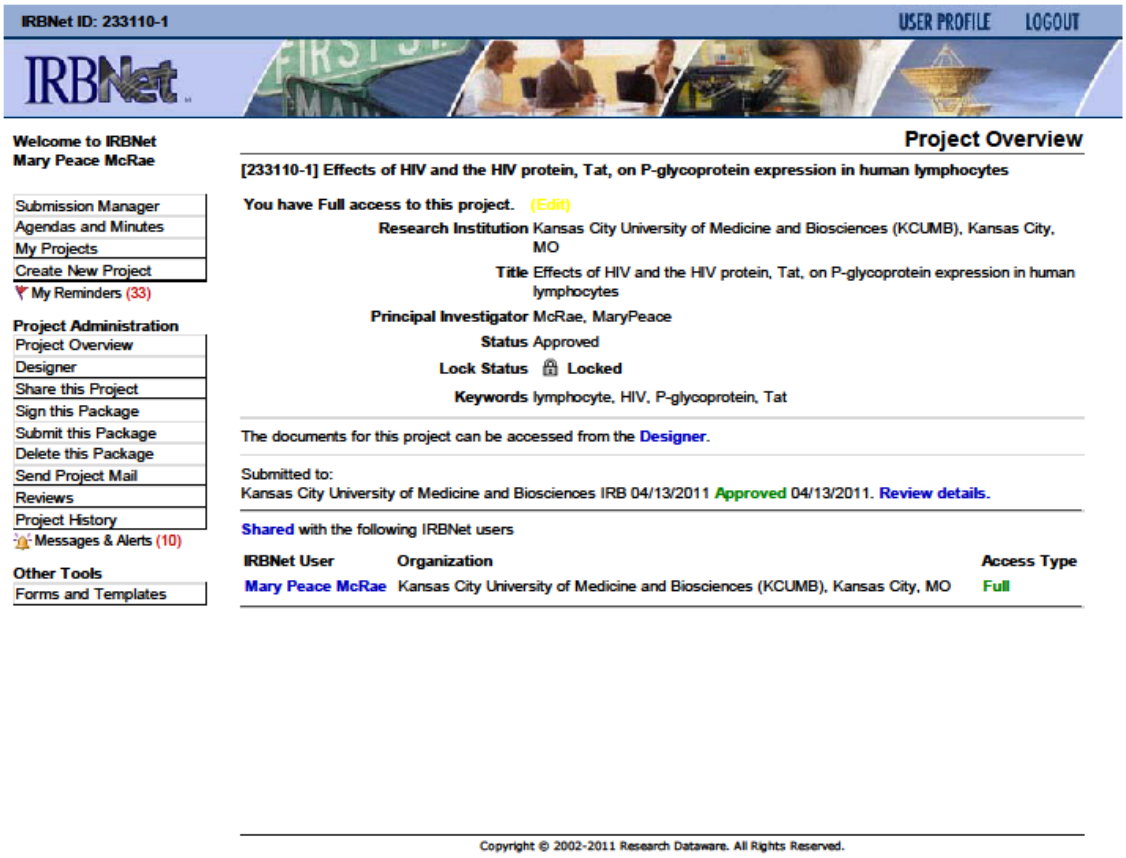
Appendix A: IRB Approval Form



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Project Overview

[233110-1] Effects of HIV and the HIV protein, Tat, on P-glycoprotein expression in human lymphocytes

You have Full access to this project. (Edit)

Research Institution: Kansas City University of Medicine and Biosciences (KCUMB), Kansas City, MO
Title: Effects of HIV and the HIV protein, Tat, on P-glycoprotein expression in human lymphocytes
Principal Investigator: McRae, MaryPeace
Status: Approved
Lock Status: Locked
Keywords: lymphocyte, HIV, P-glycoprotein, Tat

The documents for this project can be accessed from the Designer.

Submitted to:
Kansas City University of Medicine and Biosciences IRB 04/13/2011 Approved 04/13/2011. Review details.

Shared with the following IRBNet users

IRBNet User	Organization	Access Type
Mary Peace McRae	Kansas City University of Medicine and Biosciences (KCUMB), Kansas City, MO	Full

Appendix B: Further Background on P-glycoprotein, Breast Cancer Resistance Protein, HIV-1 Tat, and Oxidative Stress

P-glycoprotein

P-glycoprotein, also called ATP-Binding Cassette B1 (ABCB1), is a transmembrane protein encoded for in humans by MDR1 gene. P-glycoprotein is normally expressed in a variety of healthy tissues, including hepatocytes, renal tubular cells, intestinal epithelial cells, and lymphocytes, where it serves as an ATP-dependent transporter of toxins, drugs and xenobiotics from inside to outside cells, serving a protective function[1]. This protective function is demonstrated in the placenta where P-glycoprotein and other drug transporters, protect the fetus from accumulating toxic agents ingested by the mother during gestation[2, 3] and in the blood brain barrier where P-glycoprotein prevents drugs from penetrating into the central nervous system (CNS).[4]

In addition to this protective function, P-glycoprotein can also prevent beneficial therapies from penetrating into specific tissues, thus promoting drug resistance and treatment failure in a variety of conditions. P-glycoprotein was first discovered in tumors displaying drug resistant phenotype[5] Previous literature has found that P-glycoprotein expression is increased in cells that display multidrug resistance phenotypes including solid tumor.[5] Drug resistance has been linked to high levels of P-glycoprotein expression in neuroblastoma[6] and acute lymphoblastic leukemia.[7] Overexpression of P-glycoprotein has also been hypothesized to contribute to drug resistance in epilepsy.[8] The wide variety of pathologies influenced by P-glycoproetin expression is likely due to the vast number of drugs that are substrates for P-glycoprotein including anticancer drugs, steroids, immunosuppressants, antibiotics, beta-

blockers, and many more.[1] It is important to note that in addition to serving as substrates for P-glycoprotein, many drugs including calcium-channel blockers, steroids, quinolones, refampicin, and immunosuppressive drugs can affect function by inducing and inhibiting P-glycoprotein.[5] In addition to alterations in function caused by certain drugs, the expression of P-glycoprotein can also be influenced by other factors including environmental stress and hormones.

Specifically of interest for HIV, many of the drugs used to treat HIV (protease inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs) and entry inhibitors) are substrates for P-glycoprotein). Overexpression of P-glycoprotein in the microvascular endothelium is associated with decreased penetration of protease inhibitors and nucleoside reverse transcriptase inhibitors into the CNS.[9, 10] Additionally upregulation of P-glycoprotein expression has been shown to limit the accumulation of protease inhibitors in lymphocytic cell lines.[11] Some antiretrovirals are also inhibitors and inducers of P-glycoprotein as well,[12] complicating the clinical treatment and management of HIV infected patients.

Breast Cancer Resistence Protein (BCRP/ABCG2)

BCRP, also known as ATP-Binding Cassette G2 (ABCG2), is another drug efflux pump and has a similar function to P-glycoprotein. BCRP is also expressed in healthy tissues including gastrointestinal endothelium, placenta, liver, and peripheral blood mononuclear cells (PBMCs) including lymphocytes.[13] In these tissues, BCRP has a similar role to other drug efflux pumps in protecting cells from potentially toxic substances including pharmacological therapies. BCRP has also been found to be overexpressed in many resistant cancer lines.[14,15] Elevated expression of BCRP, in addition to other drug transporters, has been found in a variety of hematologic cancers and solid tumors, but BCRP is believed to be uniquely associated with leukemia

resistance to flavopirodol and chemotherapy failure in non-small cell lung cancer.[15] Although BCRP was original discovered in the breast cancer cell line MCF7/AdrVp, multiple studies have failed to attribute BCRP expression to drug resistance in breast carcinoma.[16, 17]

The specific role of BCRP in HIV therapy is still being investigated, as whether many antiretroviral drugs are substrates for BCRP has yet to be studied. To date, it is known that several nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors are substrates for BCRP (Table 1[12, 18]). Additional BCRP substrates include chemotherapy agents, specifically mitoxantrone and camptothecin derivates, and organic anions.[15] BCRP's substrate specificity overlaps to some extent with that of P-glycoprotein.

Given the recent discovery by Fletcher *et. al* that there is decreased antiretroviral penetration into the reservoir tissues of the GI tract, it is possible that HIV infection causes an upregulation of drug efflux pumps thus preventing antiretroviral therapies from penetrating into HIV reservoir tissues. Studying the relationship between HIV and the drug efflux pumps P-glycoprotein and BCRP in lymphoid cells of gastrointestinal tract will contribute to better understanding of the distribution and penetration of antiretroviral drugs into this important reservoir.

Transactivator of Transcription (Tat)

The Transactivator of Trascription (Tat) protein is a HIV-1 specific protein that enhances transcription of the virus. Typically viral proteins can only be transcribed and produced after infection, which required viral DNA to be integrated into the host DNA, but Tat has the unique capability of being transcribed both prior to and after DNA integration.[19] Additionally HIV-infected cells can secrete Tat in an unconventional method that does not require cell lysis.[20]

These properties make Tat able to affect both infected and uninfected cells. Exogenous Tat has been shown to cause numerous bystander effects including activation and apoptosis of uninfected T-cells, upregulation of cytokine expression, and chemotaxis of monocytes. [21] Additionally, Tat can be detected in the sera of HIV-infected individuals,[22] implying biological activity of this protein individually and in cooperation with HIV.

Previous research on Tat and drug efflux pumps has found that Tat exposure can induce P-glycoprotein expression in the microvascular endothelial cells that comprise the blood brain barrier[23]. Whether or not Tat can affect the expression of BCRP has not previously been studied.

Tat and Intracellular Oxidative Stress

Oxidative stress is involved the pathogenesis of many conditions including cerebral ischemia,[24] cancer, inflammation, and HIV. [25, 26] HIV-1 Tat has been implicated as part of this process, although the mechanisms through which Tat can cause oxidative stress is not entirely understood. Tat has been shown to increase reactive oxygen species (ROS) levels in neurons[25] and enterocytes[27] and it is known that Tat blocks transcription of manganese superoxide dismutase and glucose-6-phosphate dehydrogrenase, both enzymes that are involved in pathways that maintain intracellular oxidative balance.[27, 28] Additionally, Tat has been shown to activate oxidative pathways involving nuclear factor-κB (NF- κB),[29, 30] which has binding sequences in the promoter region of the gene that codes for P-glycoprotein.[25] Based on this evidence, it is likely that oxidative stress is associated with Tat mediated effects on drug efflux proteins.

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Appendix C: Overcoming the Obstacles of HIV/AIDS: Emerging Trends in Prevention and Barriers to a Cure.

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Abstract

Although current therapies for HIV have revolutionized the fight against AIDS, there are still many obstacles to overcome. Current HIV research efforts are focused on developing an HIV vaccine, improving pre-exposure prophylaxis, and overcoming barriers to a cure. This article offers a review of recent findings in each of these areas and discusses current HIV research at Kansas City University of Medicine and Biosciences.

Introduction

Human Immunodeficiency Virus (HIV-1) infection continues to be a global health crisis with no cure. Over 60 million people have been infected with HIV and 25 million have died from HIV-related causes since the beginning of this epidemic 31 years ago.[1] In Missouri over 10,000 individuals are living with HIV.[2] The majority of these cases are located in the state's two major metropolitan areas: Kansas City and St. Louis. The introduction of highly active antiretroviral therapy (HAART) revolutionized the fight against HIV/AIDS. These interventions have significantly decreased the mortality due to HIV infection from the leading cause of death (in 25-44 year olds) in 1994 to the sixth leading cause of death in the same age group in 2007.[3] Additionally HAART has extended the life expectancy of HIV-positive individuals (diagnosed at age 20) from 36 years in the pre-HAART era to nearly 50 years after implementation of HAART.[4] HAART improves the markers of HIV disease such as decreasing viral load and

increasing CD4+ counts. Because of the biological efficacy and the epidemiological benefits, HAART has been transformative in the HIV/AIDS epidemic. However, patients must remain on HAART for the duration of their lives because there is currently no cure for the HIV. HAART treatment is also associated with negative health and financial implications. The drugs used in the combination therapy can have immediate adverse side-effects such as hypercoaguability and hepatotoxicity.[5] Additionally, long-term therapy and the extended duration of the disease result in a series of debilitating sequelae. These include the development of diabetes mellitus, hyperlipidemia, cardiovascular disease, and liver failure.[6] Collectively, the adverse effects of the drugs and long-term sequelae of the disease can compromise disease management and significantly decrease patients' quality of life. In addition to the adverse health effects from HIV infection and therapy, the cost associated with living with this disease can range from $400-$3000 per month.[7] The majority of expenses are due to high cost of drugs and medical bills. These factors result in a significant burden on both individuals with this disease and the health care system as a whole and create a need for better solutions for the prevention, treatment, and eradication of HIV/AIDS.

To address this problem, research efforts in the HIV/AIDS field have taken many different approaches. Three important areas of focus are: vaccines, pre-exposure prophylaxis, and overcoming barriers to a cure. Research on vaccines and pre-exposure prophylaxis is attempting to reduce the number of new infections while barriers to a cure research focuses on eliminating HIV from individuals already living with the disease. This article will briefly present and discuss recent developments in each of these three areas of HIV research.

HIV Vaccine

Creating a HIV vaccine could potentially be the most economical and effective way to decrease new infections and make progress towards the ultimate goal of eradicating the virus. Vaccination programs have been successful in limiting viral epidemics such as measles and polio and have even eliminated viruses like smallpox. Vaccines depend on stable viral antigens to stimulate either a humoral or cell-mediated immune response from the host's immune system. Unfortunately, thirty years of work toward creating a viable vaccine for HIV have been largely unsuccessful due to the rapid evolution of the error-prone HIV genome. HIV adapts under selective pressure from the hosts' immune systems and quickly becomes resistant to CD8+ T-cells and humoral immune defenses. Most humoral and cell-mediated approaches to HIV vaccines have failed in the early stages of development. Table 1 summarizes a few of the largest and most notable trials. Recently, a string of new successes have brought hope back to the search for a HIV vaccine. In 2009, the RV144 HIV Vaccine Trial in Thailand used a combination of methods to stimulate both protective antibody responses and T-cell mediated response with a resulting 31% reduction of being infected with HIV in the intention to treat group.[8] Although these results were far better than any HIV vaccine result to that point, the reduction in risk was significantly lower than seen with other similar vaccines such as the HPV quadrivalent vaccine, which reduced the intention-to-treat risk of developing HPV genital lesions by 66%.[9] The risk reduction seen in the RV144 study was also transient and protection was highest in groups with the lowest exposure to HIV, suggesting that current HIV vaccines would not be as effective in high-risk groups.[8]

The most important outcome of the RV144 study was a new interest in understanding the specific mechanisms of how HIV vaccines prevent infection. Researchers learned from the successes and limitations of the RV144 trial and are subsequently designing studies that should

be better equipped to investigate the protective mechanisms behind successful HIV vaccines.[10] Once the mechanisms and pharmacokinetics are better understood, the vaccines can be redesigned to increase efficacy and duration and hopefully provide better protection for members of high-risk groups. Unfortunately, there are still many unanswered questions and years of research left before vaccines can become a reliable option for prevention of HIV infection.

Pre-exposure Prophylaxis

Although recent successes in HIV vaccine research have been promising, Pre-exposure Prophylaxis (PrEP) has proven to be a more effective method to reduce the risk of HIV infection in high-risk groups. The rationale of PrEP is that the medications used in HAART are very effective in decreasing viral loads could be used to prevent the virus from replicating and establishing an infection in healthy individuals, preventing transmission in the event of HIV exposure. Many of the HAART drugs have been shown to have higher concentrations in genital tissues where most initial exposure to the virus occurs, thus making them ideal candidates for PrEP.[11]

Four major PrEP studies have been published with large samples and two more articles are in progress (see Table 2). In the Preexposure Prophylaxis Initiative (iPrEx), a combination oral dose of the Nucleoside/tide Reverse Transcriptase Inhibitors (NRTI's) tenofovir/emtricitabine produced a 44% reduction in HIV infection risk in a high-risk men-who-have-sex-with-men (MSM) population.[12] The TDF2 and PartnersPrEP studies used tenofovir/emtricitabine in heterosexual men, women and discordant couples with a resulting 62% reduced risk in the TDF2 study and 73% reduced risk in the PartnersPrEP study.[13, 14] Topical tenofovir has also shown promising results with a 39% to 54% reduction in HIV infection risk in women using a vaginal gel, with success dependent on adherence rate.[15] The lack of effectiveness demonstrated in these

studies has led many researchers to argume that HIV vaccination research be abandoned and efforts refocused on improving PrEP.

Unfortunately, PrEP faces a number of obstacles to practical implementation. The medications used in PrEP (mainly NRTI's such as tenofovir and emtricitabine) are expensive and the cost of using them on a population-wide scale is unrealistic. Also, the side effects (such as renal failure, bone demineralization, hepatic failure, and lactic acidosis) of long-term NRTI therapy in healthy individuals could outweigh benefits and decrease compliance. In addition, patients who seroconvert while taking antiretroviral treatment for PrEP could produce drug resistant strains.[11] Finally, topical tenofovir regimens lower the possibility of side effects and viral resistance and have proven successful in women using a vaginal gel, but a large-scale program using topical antiretroviral drugs would be expensive and would depend on high rates of adherence among participants.

The challenges to both HIV vaccine research and PrEP implementation have led to the need for a combined approach to preventing initial infection with HIV. Although HIV vaccines have not been effective for protecting high risk groups, the cost is low and can easily be implemented across a population. Combining a vaccine with only modest benefit with a PrEP protocol that has been proven successful in high-risk groups, could compensate for low PrEP adherence rates and provide the best overall outcomes in preventing initial infection with HIV. Although significant progress has been made in both the HIV vaccine and PrEP fields, much more research is needed before these strategies will significantly decrease the number of new HIV infections.

Barriers to a Cure

As previously discussed, HIV currently cannot be cured. Antiretroviral therapy can reduce viral levels in blood plasma below detection by standard assays. This is called a "functional"

cure or remission. However, if therapy is halted, viral levels quickly rebound and the disease progression continues. The goal of much HIV research is to find a "sterilizing" cure, an intervention that would result in complete elimination of the virus from the body. Some known barriers to finding a cure for HIV are viral latency in T-cells, residual viral replication and anatomical barriers (also called reservoirs).[16]

HIV latency occurs when viral DNA becomes integrated into resting T-cells. When this happens, the virus is able to persist for the life of the cell without actively replicating. Antiretroviral therapies target the replication and production of the virus, and therefore, are not effective on latent virus. Latency can be the result of HIV-infected activated T-cells returning to the resting state or from the direct infection of resting T-cells.[17] Latency is maintained, in part, because the cells that become infected have a very long life cycle. Additionally, Smith, Wightman and Lewin have proposed that certain biological proteins and substances can negatively regulate T-cell activation, thus prolonging the duration of the resting state and promoting latency of the virus.[18] Activating resting T cells may be potentially beneficial, thus restarting viral replication and allowing the drugs to act on the no-longer-latent HIV. Recent studies are using drugs with a variety of mechanisms such as vorinostat and disulfram[16] to test this theory, but this testing is still in the early stages, and will require further study before conclusions can be drawn.

In addition to latent virus throughout the body, a subset of virus undergoes low level replication (residual replication), which occurs even during "suppressive therapy" of HAART. One theory suggests that this low level residual replication occurs in tissues, although that theory remains controversial.[19] Opponents of the residual replication hypothesis argue that continual replication, even at low levels would likely result in the evolution of HIV DNA or RNA, or the

development of drug resistance, which has not been conclusively demonstrated to date. Also, low level replication would likely respond to treatment intensification (adding additional antiretroviral to an already suppressive regimen). However, recent intensification trials have failed to demonstrate any clinical benefit to increased antiretroviral therapies.[19,20] Conversely the arguments in favor of residual replication have been supported by recent studies where viral concentrations in tissues have shown to be much higher than plasma concentrations.[21] Additionally, the HIV tissue concentrations correlate with immune activation despite treatment with HAART.[20] Other evidence in support of residual replication is that the virus can be spread cell-to-cell without the release of viral particles. Because this type of infection does not involve the viral replication cycle, cell-to-cell infection is not targeted by HAART, which acts on the replication and release of viral particles implicated in cell-free infection.[22]

The last major barrier to an HIV cure is anatomical reservoirs including the gastrointestinal (GI) tract, brain, genital tract, and lymph nodes.[23] These tissues remain infected with HIV despite therapy. Although the mechanism of reservoir maintenance is not well understood, one main theory is decreased antiretroviral penetration into reservoir tissues. This could prevent therapeutic concentrations from being achieved at these sites, thus preventing the drugs from being effective. Active research in this area is focusing on studying potential mechanisms for decreased antiretroviral penetration into reservoir tissues. Recent evidence has found discrepancies in antiretroviral penetration between blood plasma and reservoir tissues.[24] There is currently a study underway that is examining penetration of antiretroviral agents into lymph tissues with results pending.[21]

HIV Research at KCUMB

At the Kansas City University of Medicine and Biosciences (KCUMB), we are looking at mechanisms contributing to decreased antiretroviral penetration into the gastrointestinal reservoir. Many antiretroviral drugs are transported into and out of cells by various drug transport proteins. The ATP-binding cassette protein P-glycoprotein is responsible for limiting drug accumulation within a variety of cell types including lymphocytes, intestinal epithelial cells, hepatocytes, and renal tubular cells.

Current HIV research at KCUMB is centered around the effects of HIV infection on the expression of P-glycoprotein within an *in vitro* model of the lymphocyte rich reservoir region of the gastrointestinal tract. Many antiretroviral drugs are substrates for P-glycoprotein. Therefore, dysregulation of this protein could affect the distribution and penetration of these agents into certain tissues. Specifically we are looking at P-glycoprotein expression in response to HIV infection within enterocytes and lymphocytes. Preliminary results suggest an upregulation of P-glycoprotein in HIV infected lymphocytes, which could therefore serve to limit drug penetration into the gut reservoir tissue and allow HIV to persist. These initial results warrant further investigation and contribute to our understanding of a potential mechanism that contributes to HIV reservoir maintenance. Our future studies will compare antiretroviral concentrations in the gastrointestinal tract of HIV infected individuals with plasma concentrations of these drugs to determine if a discrepancy exists.

Conclusion

Although the field of HIV/AIDS research is making strides to prevent new HIV infections and eradicate the virus, HIV/AIDS continues to be a devastating pandemic. More research needs to be done to discover effective methods to eliminate HIV/AIDS. A number of advancements have been made to develop HIV vaccines, improve pre-exposure prophylaxis, and overcome

other barriers to curing HIV. Specifically at KCUMB, we are focused on better understanding the viral reservoir of the gastrointestinal tract, and the role that drug transport proteins and drug distribution may play in the maintenance of this reservoir. With a multi-faceted approach to HIV/AIDS research, prevention, and treatment, the research community can continue to make small steps toward making HIV/AIDS a disease of the past.

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Appendix D: Data

Activated Lymphocytes Probed for P-glycoprotein

Experiment #1

	Pgp Area	Beta-actin Area	Relative area
6-	33575.078	44858.241	0.7484707
6+	43593.2	46434.413	0.9388123
18-	33765.3	39156.22	0.8623228
18+	50393.22	37600.099	1.3402417
24-	32468.886	29984.321	1.0828621
24+	46875.806	33207.149	1.4116179
48-	34068.2	41961.22	0.8118973
48+	44710.806	45789.756	0.9764369
96-	33583.2	32595.271	1.030309
96+	38570.321	32836.463	1.1746186

Experiment #2

	Pgp Area	Beta-actin Area	Relative area
6-	16390.38	21898.329	0.748476288
6+	18923.915	21761.572	0.869602389
18-	10624.48	21891.016	0.485335171
18+	12146.723	23429.966	0.51842683
24-	26156.815	27809.401	0.940574556
24+	12266.43	24518.037	0.500302288

Experiment #3

	Pgp Area	Beta-actin Area	Relative area
6-	15508.886	54213.978	0.286068
6+	15987.865	52109.028	0.3068156
18-	25621.664	56512.978	0.4533766
18+	35705.563	54687.149	0.6529059
24-	31188.028	56210.685	0.5548416
24+	36804.128	56839.291	0.6475121
48-	11722.359	58220.291	0.2013449
48+	35008.472	57828.927	0.6053799
96-	22133.522	57554.907	0.3845636
96+	48176.421	54860.492	0.8781624
168-	17582.815	55824.806	0.3149642
168+	29072.856	56730.099	0.5124767

Experiment #4

	Pgp Area	Beta-actin Area	Relative area
6-	8589.652	47128.099	0.182261797
6+	23411.007	43026.492	0.544106803
18-	21061.229	48013.534	0.438651923
18+	38563.563	44920.614	0.858482544
24-	39145.593	47172.735	0.829835137
24+	29326.007	44401.907	0.660467286
48-	34584.078	49969.969	0.692097247
48+	34126.25	54633.806	0.624636146
96-	17786.714	64558.848	0.275511639
96+	32070.886	49616.028	0.646381568
168-	13135.329	46233.584	0.284107955
168+	39646.593	48437.099	0.818517083

Experiment #5

	Pgp Area	Beta-actin Area	Relative area
6-	2008.619	35396.865	0.0567457
6+	4443.246	42488.543	0.1045752
18-	29292.35	39401.522	0.7434319
18+	25516.622	43802.957	0.582532
24-	2074.912	37099.643	0.0559281
24+	28544.087	41072.986	0.6949601
48-	2802.033	39282.472	0.0713304
48+	13428.187	42364.643	0.3169668
96-	1600.669	46503.472	0.0344204
96+	39500.007	48671.765	0.811559
168-	33496.936	46201.128	0.7250242
168+	33393.057	45453.421	0.7346654

Appendix D: Data

Caco2 Cells Grown in Co-culture with Resting Lymphocytes Probed for P-glycoprotein

Experiment #1

	Pgp Area	Beta-actin Area	Relative area
48-	5775.56	36638.815	0.157635
48+	16493.773	32447.572	0.5083207
96-	17925.48	45218.614	0.3964182
96+	26392.43	47190.856	0.55927

Experiment #2

	Pgp Area	Beta-actin Area	Relative area
48-	37627.64	37473.47	1.00411414
48+	38984.37	36877.69	1.05712608
96-	14199.5	33315.74	0.42620993
96+	34352.77	32645.33	1.05230261

Experiment #3

	Pgp Area	Beta-actin Area	Relative area
48-	37330.59	32109.99	1.162585
48+	27285.26	34103.33	0.800076
96-	27870.62	31695.04	0.879337
96+	37130.16	36308.77	1.022622

Experiment #4

	Pgp Area	Beta-actin Area	Relative area
48-	51814.51	35204.35	1.47182132
48+	46313.76	39361.74	1.1766185
96-	39765.49	33645.92	1.18188172
96+	42492.53	32239.67	1.31802001

Experiment #5

	Pgp Area	Beta-actin Area	Relative area
6-	5563.267	18599.409	0.2991099
6+	1344.163	20400.187	0.0658897
18-	12353.137	24279.066	0.5087979
18+	12333.137	22274.409	0.5536909
24-	2138.376	21941.702	0.0974572
24+	14416.794	25743.238	0.5600226
48-	23582.057	24428.238	0.9653605
48+	18390.572	25998.238	0.7073776
96-	14186.501	23670.409	0.5993348
96+	26455.179	28581.723	0.9255978
168-	11413.894	21842.167	0.5225623
168+	21313.472	20651.167	1.0320711

Experiment #6

	Pgp Area	Beta-actin Area	Relative area
6-	10901.38	17253.217	0.631846223
6+	10443.48	18196.217	0.573936879
18-	11593.258	15135.853	0.765946789
18+	23991.957	19767.217	1.213724572
24-	5922.217	18351.095	0.322717364
24+	18170.451	20868.167	0.870725781
48 -	16330.986	20199.53	0.808483465
48 +	16921.279	19441.995	0.870346845
96-	28408.907	16366.581	1.735787517
96+	10905.208	19920.681	0.547431486
168-	20061.643	18647.146	1.075855951
168+	38201.664	15251.146	2.504838915

Experiment #7

	Pgp Area	Beta-actin Area	Relative area
6-	28784.279	16638.388	1.7299921
6+	19682.38	19079.874	1.0315781
18-	23547.179	18576.752	1.2675617
18+	15852.551	20681.652	0.7665031
24-	25548.35	19723.874	1.2953008
24+	21520.401	21818.995	0.986315
48-	18582.501	20342.045	0.9135021
48+	20756.622	23029.288	0.9013141
96-	20193.108	19776.045	1.0210893
96+	22219.501	19588.045	1.1343399
168-	20977.037	15399.024	1.3622316
168+	23529.744	16142.267	1.457648

Experiment #8

	Pgp Area	Beta-actin Area	Relative area
24-	35376.555	26888.815	1.315660619
24+	37809.99	31063.593	1.217180189
24-	25944.827	31675.957	0.819070028
24+	39321.697	37782.907	1.040727147
24-	24355.806	45087.685	0.540187548
24+	28026.777	35168.836	0.796920802

Appendix D: Data

Activated Lymphocytes Probed for BCRP

eriment #1

	Bxp-21 Area	Beta-actin Area	Relative area
6-	6830.903	44858.241	0.1522775
6+	9828.803	46434.413	0.2116707
18-	8399.439	39156.22	0.214511
18+	20281.986	37600.099	0.5394131
24-	4228.125	29984.321	0.1410112
24+	10992.167	33207.149	0.3310181
48-	10941.258	41961.22	0.2607469
48+	15483.501	45789.756	0.3381433
96-	7770.995	32595.271	0.2384087
96+	13441.108	32836.463	0.4093348

Experiment #2

	BXP-21 Area	Beta-actin Area	Relative area
6-	4657.681	21898.33	0.21269573
6+	5430.167	21761.57	0.24953009
18-	4457.903	21891.02	0.20364075
18+	12377.09	23429.97	0.52825885
24-	7107.238	27809.4	0.25556962
24+	9193.43	24518.04	0.37496599

Experiment #3

	BXP-21 Area	Beta-actin Area	Relative area
6-	49307.06	14402.65	3.423471
6+	51997.35	16968.19	3.064402
18-	50586.11	22238.92	2.274666
18+	54839.94	20658.33	2.654616
24-	40239.43	15708.89	2.56157
24+	47001.82	14921.36	3.149969
48-	46647.55	20885.5	2.233489
48+	37965.18	20157.38	1.883438
96-	35905.4	18562.97	1.934249
96+	52403.13	23576.45	2.22269
168-	38542.97	17877.65	2.15593
168+	56003.63	19753.84	2.835075

Experiment #4

	BXP-21 Area	Beta-actin Area	Relative area
6-	1209.426	35396.87	0.0341676
6+	3100.175	42488.54	0.07296496
18-	9164.238	39401.52	0.23258589
18+	11464.36	43802.96	0.26172569
24-	7025.995	37099.64	0.18938174
24+	13238.14	41072.99	0.32230764
48-	6233.409	39282.47	0.15868169
48+	11930.57	42364.64	0.28161625
96-	6285.752	46503.47	0.13516737
96+	13978.87	48671.77	0.28720686
168-	7296.409	46201.13	0.15792707
168+	7640.995	45453.42	0.16810605

Appendix D: Data

Caco2 Cells Grown in Co-culture with Activated Lymphocytes Probed for P-glycoprotein

Experiment #1

	Pgp Area	Beta-actin Area	Relative area
6-	15353.229	20699.409	0.741723061
6+	31268.22	22579.359	1.384814334
18-	31907.392	20593.187	1.549414959
18+	38065.291	23796.773	1.599598862
24-	25046.371	21500.066	1.164943912
24+	23112.128	22648.773	1.020458283
48-	10683.258	24739.309	0.431833322
48+	16938.279	19766.48	0.85691934
96-	26394.735	22499.673	1.173116383
96+	13306.794	20279.43	0.656171993

Experiment #2

	Pgp Area	Beta-actin Area	Relative area
6-	8133.602	8324.974	0.977012301
6+	24817.79	9079.803	2.733295535
18-	11321.92	10482.56	1.080071566
18+	28340.22	15950.995	1.776705466
24-	20383.08	14793.995	1.377794031
24+	22421.15	14674.652	1.527882842
96-	24717.32	14388.217	1.717886309
96+	26865.98	10005.974	2.684993785

Experiment #3

	Pgp Area	Beta-actin Area	Relative area
6-	8853.945	27294.43	0.324386514
6+	21505.664	31635.794	0.679788976
18-	21475.421	31377.43	0.684422561
18+	28946.664	32180.208	0.899517617
24-	26563.25	35868.622	0.740570686
24+	31855.472	35195.794	0.905093148
48-	6220.974	24826.652	0.250576437
48+	25036.158	22139.874	1.130817547
96-	13473.48	19030.217	0.708004538
96+	2197.79	22351.217	0.098329769
168-	11103.48	19473.681	0.570178797
168+	31109.572	15415.024	2.018133219

Experiment #4

	Pgp Area	Beta-actin Area	Relative area
6-	25664.17	14402.652	1.78190586
6+	37156.89	16968.187	2.189797236
18-	41215.04	22238.915	1.853284659
18+	40956.12	20658.329	1.982547717
24-	46408.48	15708.894	2.954280804
24+	48737.07	14921.359	3.266262141
48-	31009.05	20885.501	1.48471655
48+	37481.88	20157.38	1.859461746
96-	34789.51	18562.966	1.87413547
96+	48198.84	23576.451	2.044363632
168-	40587.24	17877.652	2.270278054
168+	49613.43	19753.844	2.511583315

Experiment #5

	Pgp Area	Beta-actin Area	Relative area
6-	11543.016	16583.309	0.696062288
6+	39728.057	19264.794	2.06221032
18-	30748.673	19160.208	1.604819374
18+	38573.007	20855.401	1.849545209
24-	45717.957	23001.329	1.987622411
24+	49399.463	21417.279	2.306523765
48-	23791.057	16112.501	1.476558915
48+	41079.401	17657.037	2.326517241
96-	18226.229	19718.158	0.924337304
96+	41352.765	23491.936	1.760296171
168-	26615.451	22086.744	1.205041857
168+	23588.936	22857.865	1.031983346

Appendix D: Data

Resting Lymphocytes Probed for P-glycoprotein

Experiment #1

	Pgp Area	Beta-actin Area	Relative area
48-	2682.912	18219.49	0.14725506
48+	14052.02	24183.22	0.58106512
96-	8547.054	20359.32	0.41981045
96+	11971.61	19697.61	0.60776967

Experiment #2

	Pgp Area	Beta-actin Area	Relative area
48-	446.749	41656.56	0.01072458
48+	463.849	48735.74	0.00951764
96-	2019.21	53208.32	0.03794905
96+	1220.48	49190.15	0.02481141

Experiment #3

	Pgp Area	Beta-actin Area	Relative area
48-	177.021	40351.42	0.00438698
48+	1458.527	39332.94	0.03708157
96-	10543.12	44115.96	0.23898645
96+	1542.598	40589.4	0.03800495

Experiment #4

	Pgp Area	Beta-actin Area	Relative area
48-	10693.5	44674.25	0.2393663
48+	17668.6	47384.49	0.37287731
96-	4178.83	47272.91	0.08839803
96+	9210.02	50870.37	0.18104889

Experiment #5

	Pgp Area	Beta-actin Area	Relative area
6-	100.95	17579.65	0.00574243
6+	4918.56	17720.14	0.27756896
18-	332.092	19779.48	0.01678972
18+	569.506	23729.26	0.02400016
24-	8450.116	27838.92	0.30353611
24+	29654.17	26109.57	1.13575856
48-	26589.13	34920.35	0.76142215
48+	19033.52	26426.79	0.72023576
96-	22534.77	28082.45	0.80245008
96+	28560.01	29472.57	0.9690368
168-	478.92	13069.22	0.03664489
168+	3995.276	6970.761	0.57314775

Experiment #6

	Pgp Area	Beta-actin Area	Relative area
6-	28.95	17611.82	0.00164378
6+	28.95	23716.97	0.00122065
18-	565.092	26798.92	0.02108638
18+	1445.06	30625.69	0.04718463
24-	1111.41	28935.67	0.03840954
24+	4358.78	28271.74	0.1541745
48 -	652.335	24205.16	0.02695025
48 +	8361.95	32478.06	0.25746445
96-	20929.3	31134.99	0.67221164
96+	6940.82	28030.55	0.24761636
168-	1133.13	16893.17	0.06707647
168+	1419.06	6276.711	0.22608369

Experiment #7

	Pgp Area	Beta-actin Area	Relative area
48 -	1228.891	35648.59	0.03447236
48 +	10615.62	41198.06	0.25767288
96-	24398.32	40486.84	0.60262355
96+	10291.17	41977.25	0.24516058
168-	18330.67	40008.49	0.45816956
168+	18370.11	36824.42	0.49885667

Experiment #8

	Pgp Area	Beta-actin Area	Relative area
24-	35376.6	26888.82	1.31566062
24+	37810	31063.59	1.21718019
24-	25944.8	31675.96	0.81907003
24+	39321.7	37782.91	1.04072715
24-	24355.8	45087.69	0.54018755
24+	28026.8	35168.84	0.7969208

Appendix D: Data

Caco2 Cells Grown in Co-culture with Activated Lymphocytes Probed for BCRP

Experiment #1

	BXP-21 Area	Beta-actin Area	Relative area
6-	13212.77	8324.974	1.58712388
6+	25035.81	9079.803	2.75730718
18-	10654.21	10482.56	1.01637463
18+	33844.31	15951	2.12176808
24-	7658.995	14794	0.51770972
24+	12568.77	14674.65	0.85649493
96-	39199.9	14388.22	2.72444445
96+	25070.46	10005.97	2.50554948

Experiment #2

	BXP-21 Area	Beta-actin Area	Relative area
6-	49307.1	14402.65	3.42347097
6+	51997.4	16968.19	3.06440246
18-	50586.1	22238.92	2.27466632
18+	54839.9	20658.33	2.65461645
24-	40239.4	15708.89	2.56157015
24+	47001.8	14921.36	3.14996898
48-	46647.5	20885.5	2.23348944
48+	37965.2	20157.38	1.88343832
96-	35905.4	18562.97	1.9342493
96+	52403.1	23576.45	2.22268958
168-	38543	17877.65	2.15593015
168+	56003.6	19753.84	2.83507483

Experiment #3

	BXP-21 Area	Beta-actin Area	Relative area
6-	8036.773	16583.31	0.48463024
6+	33963.39	19264.79	1.76297717
18-	36390.56	19160.21	1.89927808
18+	41884.41	20855.4	2.00832451
24-	36702.39	23001.33	1.59566397
24+	45282.83	21417.28	2.11431279
48-	48634.82	16112.5	3.01845244
48+	52444.25	17657.04	2.97016158
96-	34442.61	19718.16	1.74674556
96+	47511.87	23491.94	2.02247567
168-	40722.43	22086.74	1.84374999
168+	54544.27	22857.87	2.3862366

Appendix D: Data

Resting Lymphocytes Treated with Tat for 24 hours Probed for P-glycoprotein

Experiment #1

	Pgp Area	Beta-actin Area	Relative area
0nM	9751.815	34352.886	0.283871783
0.1nM	25328.51	43546.149	0.581647415

Experiment #2

	Pgp Area	Beta-actin Area	Relative area
0nM	30974.32	48148.484	0.643308396
0.1nM	44945.6	57705.798	0.778874889

Experiment #3

	Pgp Area	Beta-actin Area	Relative area
0nM	12211.94	37183.12	0.328426878
0.1nM	15060.42	34773.635	0.433098841

Resting Lymphocytes Treated with Tat for 24 hours Probed for BCRP

Experiment #1

	BXP-21 Area	Beta-actin Area	Relative area
0nM	13869.14	25642.271	0.540870229
0.1nM	14831.64	27717.09	0.535108231

Experiment #2

	BXP-21 Area	Beta-actin Area	Relative area
0nM	25902.53	54807.902	0.472605666
0.1nM	28339.79	58269.023	0.486361149

Experiment #3

	BXP-21 Area	Beta-actin Area	Relative area
0nM	17219.74	47144.011	0.365258357
0.1nM	17695.2	37651.647	0.469971473

Resting Lymphocytes Treated with Tat for 24 hours Probed for Peroxiredoxin Sulfate standardized to 2-cys-Peroxiredoxin

Experiment #1

	Prx-SO3 Area	2-Cys-Prx Area	Relative area
0nM	755.335	25704	0.029386
0.1nM	2738.619	27749	0.098694

Experiment #2

	Prx-SO3 Area	2-Cys-Prx Area	Relative area
0nM	7494.894	42193	0.177633
0.1nM	11620.3	51691	0.224802

Experiment #3

	Prx-SO3 Area	2-Cys-Prx Area	Relative area
0nM	2919.589	35159	0.083039
0.1nM	1271.82	29048	0.043783