

# P-GLYCOPROTEIN EXPRESSION IN HTLV-III CELLS AFTER TREATMENT WITH HIV-1 PROTEASE INHIBITORS

**Introduction:** P-glycoprotein (P-gp), a membrane protein that pumps drugs out of cells, affects the availability and effectiveness of drugs and their extrusion from cells. HIV-1 protease inhibitors (PIs), part of the antiretroviral treatment known as highly active antiretroviral treatment, are substrates and possibly inhibitors of the P-gp pump. Their interaction may represent a potential effect on treatment efficiency. Our objective is to evaluate how the P-gp/PI interaction limits drug effectiveness.

**Methods:** HTLV IIIcc cell cultures were exposed to ritonavir and saquinavir for 24 hours. Supernatant solution was recovered for viral load assessment. Cells were labeled with monoclonal antibody against P-gp and analyzed by flow cytometry.

**Results:** Upregulation in P-gp expression from 1% to 7% was observed when cells were exposed to PIs, compared with cells not exposed to PIs ( $P=.05$ ). Ritonavir 10  $\mu\text{g}/\text{mL}$  caused a similar P-gp increment as did 20  $\mu\text{g}/\text{mL}$  saquinavir. To evaluate P-gp functionality, cells were exposed to rhodamine-123, a fluorescent dye that is also a P-gp substrate. Its accumulation was measured by flow cytometry. Slightly more rhodamine was observed in cells treated with higher PI concentration ( $P=.05$ ). Higher viral load was obtained in suspension of cells with upregulated P-gp. Statistically significant decreased viral load was obtained in supernatants of cells expressing less P-gp ( $P<.04$ ). Ritonavir 20  $\mu\text{g}/\text{mL}$  caused the most marked reduction in viral load.

**Conclusions:** Our results suggest that the use of PIs upregulates the expression of P-glycoprotein on HTLV IIIcc cells, showing slightly inhibited functionality for those treated with higher concentrations. The rapid extrusion of the drug by P-gp seems to limit its action. Decreased viral load in suspensions with ritonavir 20  $\mu\text{g}/\text{mL}$  may represent the inactivation of the transport pump, allowing the drug to work more efficiently. (*Ethn Dis.* 2008;18[Suppl 2]:S2-60–S2-64)

**Key Words:** P-glycoprotein, HIV-1, HIV Protease Inhibitors, H9/HTLV IIIcc Cell Line

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## INTRODUCTION

Multidrug resistance proteins (MDRs) are a family of drug transporters encoded by the MDR1 gene.<sup>1,2</sup> Cells with the MDR 1 gene express an energy-dependent transport pump on their membranes. The transmembrane protein P-glycoprotein (P-gp) is an efflux pump that belongs to the MDR family. It is highly expressed in gut, kidneys, brain, and other organs, where it protects cells and organs from harmful substances.<sup>2,3</sup> Molecules transported by P-gp are classified as substrates, and those that inactivate the transport function of the protein are antagonists.<sup>4</sup> Upregulation or downregulation of P-gp transporters on cell membranes affects the availability of those P-gp substrates inside the cell and their disposition from the cell.<sup>5</sup> At the same time, the effectiveness of that drug is affected, resulting in resistance to treatment when the drug is expelled too soon from the cell, or the cell can be damaged if higher concentrations of the drug remain too long inside the cell. P-gp is also present on membranes of T cells and monocytes, where it may play another role besides drug efflux.<sup>6</sup> The transport of cytokines by P-gp has been investigated by some researchers who study the importance of the transporter for efficient cytokine secretion during an immune response.<sup>7</sup> The potential effect or interference of P-gp on the activation of an efficient immune response is currently a topic of intense discussion.

HIV-1 protease inhibitors (PIs: ritonavir, saquinavir, indinavir, nelfinavir) are part of highly active antiretroviral treatment.<sup>8</sup> They rapidly reduce viral load by suppressing viral replication.<sup>2</sup> The continuous and uninterrupted drug regimen is successful in viral suppression for patients who are highly compliant to the treatment.<sup>2</sup> These PIs are substrates and possibly inhibitors of the P-gp pump.<sup>9,10</sup> It is very important to understand the mechanism involved in that interaction and the potential impact on treatment efficacy.<sup>10</sup> Limited transport capacity may have a direct effect on treatment effectiveness. Treatment effectiveness can be determined by measuring viral loads on supernatants of cultures after exposure to the drug.<sup>10</sup> If the interaction of P-gp with PIs results in decreased PI activity, a subtherapeutic drug level may cause inadequate viral suppression. For HIV-1 patients, that interaction may represent disease progression to AIDS. If it results in increased PI activity, the cells could be damaged by toxicity. Some therapeutic strategies may be developed if the mechanisms of the P-gp/PI interaction and the consequences over therapy effectiveness are elucidated. Enhancement (boosting) of antiretroviral therapy with other drugs or the inactivation of the transport pump with P-gp inhibitors are possible alternatives.<sup>2,4</sup> It is particularly important to increase drug effectiveness in sanctuary sites, like the brain and genitalia, where HIV-1 therapy does not have easy access and viral particles remain as a residual population.<sup>4,11,12</sup> Thus, antiretroviral therapy is designed not only to suppress viral replication but also to avoid the risk of toxicity or the development of drug resistance, to restore and preserve the patients' immunologic functions,

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and to reduce HIV/AIDS morbidity and mortality.<sup>2</sup> The objective of the present study was to determine how the use of PIs in HIV-1 therapy affects the expression and the functionality of P-gp pump on human T-lymphotropic virus (HTLV) IIIcc cells and if the transport capacity is limited after exposure to the drug.<sup>8</sup>

## METHODS

### Cell Line

The H9/HTLV IIIcc NIH 1983 cell line was kindly provided by the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Germantown, Maryland. This cell line originates from the peripheral blood of a patient with dual HTLV-1 and HIV-1 infection.<sup>13</sup> The cells express P-gp on their membranes. Expression was artificially increased by activation of the cells in order to obtain sufficient P-gp expression to be measured by flow cytometry.<sup>13</sup>

### Reagents

H9/HTLV IIIcc cells were treated for 24 hours with two different PIs provided by the NIH AIDS Reagent Program—saquinavir and zidovudine.<sup>14</sup> Dehydrated PIs were reconstituted with ethanol and dimethyl sulphoxide (Sigma-Aldrich, Saint Louis, Mo), respectively.<sup>13-15</sup> Final doses of 10 µg/mL and 20 µg/mL (15 or 20 µM), which are close to clinical plasma levels, were added to the cells cultured in RPMI 1640 media (Sigma-Aldrich) with 10% fetal bovine serum (HyClone Laboratories, Inc. Logan, Utah).<sup>10,14,16</sup>

### Cell Activation and Labeling

Cells were activated with 10 µL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 10 µL ionomycin (Sigma-Aldrich), final concentration of 10 nM and .4 mM, respectively, and incubated at 37°C (98.6°F) in a 5% CO<sub>2</sub> humid environment and harvested

after 48 hours.<sup>7</sup> The supernatant solutions were recovered and frozen for further viral load assessment. After washing the cells with staining buffer, they were labeled with monoclonal antibody against P-gp conjugated to fluorescein isothiocyanate (P-gp-FITC, BD Pharmingen, San Jose, Calif) and fixed with .5% paraformaldehyde.

### P-gp Expression Analysis

One hour after fixation, cells were analyzed by flow cytometry using the four-color BD FACSCalibur cytometer (Beckton-Dickinson Immunocytometry Systems, San Jose, Calif). A total of 10,000 events were acquired for each sample. Forward scatter and side scatter were first accessed to select, by electronic gating, the HTLV IIIcc population to be analyzed.<sup>14</sup> P-gp expression was observed in the FL-1/FITC fluorescence channel and the P-gp-positive cells were identified by comparison with negative control sample labeled with γ1 isotype.<sup>14</sup> The CELLQuest Software (Beckton-Dickinson) provided the tools to analyze the HTLV IIIcc cells' expression of P-gp when each sample was observed in a dot plot on a logarithmic scale. Statistics of every quadrant area in the plot allowed evaluating the percentage of cells expressing P-gp on their membranes and the intensity of that expression.

### P-gp Functionality Analysis

To evaluate P-gp functionality, HTLV IIIcc cells treated and untreated with PIs and previously labeled with allophycocyanin-conjugated monoclonal antibody against P-gp (P-gp-APC, BD Pharmingen, San Jose, Calif) were exposed, for 30 minutes, at 37°C (98.6°F), to rhodamine-123 (Molecular Probes, Eugene, Ore), a fluorescent dye which is also a P-gp substrate.<sup>15,17</sup> The use of P-gp monoclonal antibody conjugated to APC allows the evaluation, in the same plot, of both P-gp expression and rhodamine accumulation by HTLV IIIcc cells. Both parameters were mea-

sured by flow cytometry after 30 minutes of exposure to the dye and after three hours efflux period.

### Viral RNA Extraction

Viral RNA was recovered from cultures supernatants after the 24-hour periods exposed to zidovudine and saquinavir. The QIAamp viral RNA Mini Kit (QIAGEN Inc., Valencia, Calif) was used for that purpose, according to the manufacturer's instructions. The extraction product was then quantified to determine the RNA concentration and frozen for further viral load assessment.<sup>18</sup>

### Viral Load Quantitation

The virus copy number was quantified in culture supernatants of cells exposed to zidovudine and saquinavir and compared to the original viral load in supernatants not exposed to PIs. The real-time reverse transcription PCR (RT-PCR) technique with the QuantiTect Probe RT-PCR kit (QIAGEN Inc.) was applied for the quantitation. PCR primers and probes were selected by using the Primer Quest (Integrated DNA Technologies, Inc, Coralville, Iowa). They were forward gag 6F- 5' CATGTTTTTCAGCATTATCA-GAAGGA-3', reverse gag 84R- 5' TGCTTGATGTCCCCCACT-3', and gag6F84R probe- 5'/56-FAM/CCACCCACAAGATTTAAACAC-CATGCTAA/3BHQ\_1/-3'.<sup>18</sup> Lyophilized content was reconstituted with water to a 50-nM concentration. Reaction mixture was prepared with 12.5 µL 2x QuantiTect master mix (which included the Hot Start Taq DNA polymerase, the dNTP mix, and MgCl<sub>2</sub>), .2 µL each primer, the probe and reverse transcriptase, 8.7 µL RNAase-free water, and 3.0 µL template (samples, controls, and standards) for a final volume of 25 µL. For cycling we used the the BIO-RAD iCycler Thermal cycler (BIO-RAD Laboratories, Inc., Hercules, Calif). Cycler conditions included a 15-minute reverse transcrip-

**Table 1. Mean percentage of cells expressing P-glycoprotein (%)**

	Mean ±SD (%)	t test
PMA/ionomycin (St)	.48±.28	
Saquinavir 10 µg/mL/St	1.38±1.28	.20
Saquinavir 20 µg/mL/St	6.26±1.22	.01*
Ritonavir 10 µg/mL/St	6.17±1.79	.02*
Ritonavir 20 µg/mL/St	2.31±.49	.02*

SD = standard deviation, PMA = phorbol 12-myristate 13-acetate.  
\* Statistically significant at  $P<.05$ .

tion cycle at 55°C (131°F), a polymerase activation cycle of 10 minutes at 95°C (203°F), and 45 cycles of 15 seconds at 95°C (203°F) and 1 minute at 60°C (140°F). Quantification of virus copy number was obtained for all culture supernatants, both exposed and not exposed to PIs.<sup>18</sup>

**Statistical Analysis**

The results analyzed were obtained from five repetitions of the PI treatment protocol. Sample mean, standard deviation, and standard error were calculated

for each set of results and used to create charts and establish comparison among treated and untreated groups. The Microsoft Office Excel 2003 program (Microsoft Corp., Redmond, Wash) was used to determine the central tendency and dispersion statistics and hypothesis testing. Statistical significance of the difference among groups was determined by using a one-tailed Student *t* test. A value of  $P<.05$  was considered significant.<sup>10,19</sup>

The expression of P-gp was evaluated by using Cell Quest Software

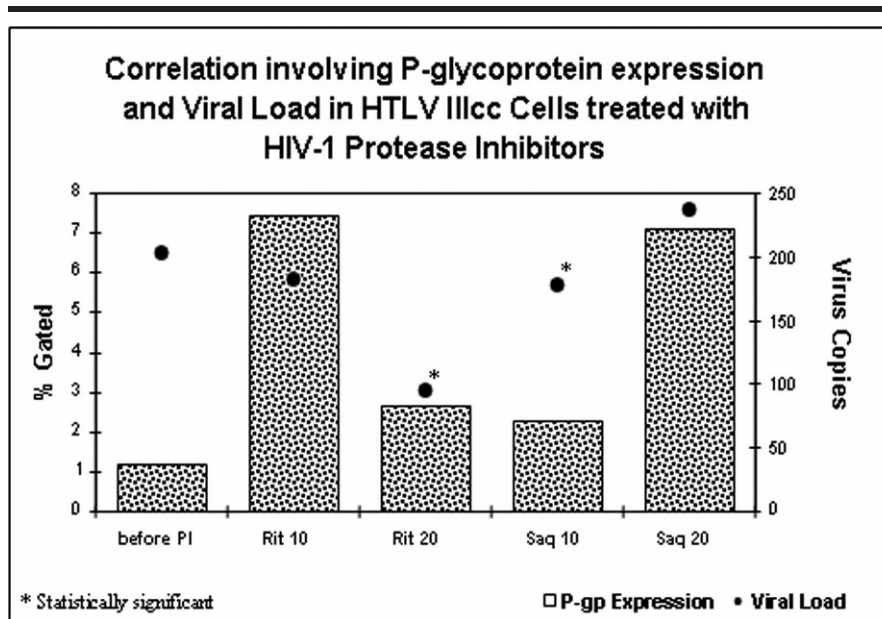
(Beckton-Dickinson). Dot plots were created to analyze P-gp expression of HTLV IIIcc cells after exposure to ritonavir and saquinavir and activation period. The P-gp-FITC fluorescence was observed in the lower right quadrant. The Cell Quest Software provides statistical analysis for each quadrant in the plot. They provide the percentage of cells gated in each quadrant as well as the geometric mean of the fluorescence intensity. This expression intensity represents the amount of transport proteins on each cell membrane. A mean, standard deviation, and standard error of P-gp expression were calculated for all five protocol repetitions.

The one-tailed Student *t* test was used to determine statistical significance ( $P<.05$ ) among mean values (Table 1).

**RESULTS**

Our results show 1%–7% upregulation in P-gp expression when cells were exposed to PIs and activated, compared with cells activated with PMA/ionomycin but not exposed to PIs (Figure 1). For cell cultures exposed to 20 µg/mL saquinavir, more cells expressed P-gp on their membranes than did cells that were merely activated (Table 1). For ritonavir and 10 µg/mL saquinavir, no difference was observed. The mean viral load in supernatants of PI-treated cells was significantly lower for 10 µg/mL saquinavir and 20 µg/mL ritonavir but greater for 20 µg/mL saquinavir compared with those without PI treatment (Table 2). Viral load did not differ from control in suspension of cells showing upregulated P-gp (Figure 1), whereas it fell in cell suspension for downregulated P-gp.

Efficient efflux of rhodamine was observed after three hours from exposure to the dye; however, cells exposed to different PIs and different concentrations showed a different efflux rate. Figure 2 shows rhodamine accumulation inside cells, representing an indirect



**Fig 1. The use of protease inhibitors trigger the upregulation of P-gp on human T-lymphotropic virus IIIcc cells, probably resulting in rapid extrusion and limited action of the protease inhibitors. Viral loads similar to that of supernatant without protease inhibitor treatment may be evidence of a limited action of the drug.**

**Table 2. Mean viral load in human T lymphotropic virus IIIcc cells**

	Mean ±SD (copies/mL)	t test
PMA/ionomycin (St)	198.5±6.36	
Saquinavir 10 µg/mL/St	176.5±.71	.02*
Saquinavir 20 µg/mL/St	236±1.41	.01*
Ritonavir 10 µg/mL/St	160.5±30.41	.11
Ritonavir 20 µg/mL/St	116.5±31.82	.03*

SD = standard deviation, PMA = phorbol 12-myristate 13-acetate.

\* Statistically significant at *P*<.05.

efflux measurement.<sup>20</sup> Less rhodamine efflux was observed in cells exposed to higher PI concentrations.

## DISCUSSION

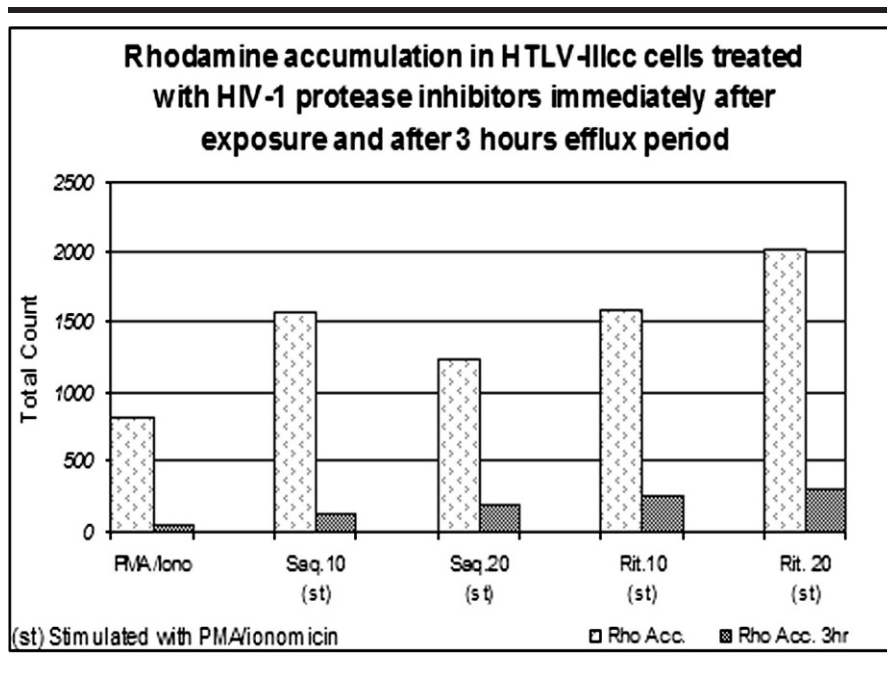
The expression of P-gp on HTLV IIIcc cell membranes increased after the use of PIs. As substrates of P-gp, PIs interact with the transport pump, affecting its expression as well as functionality. We consider a precise P-gp expression to be the product of the percentage of cells expressing the pro-

tein by the geometric mean of the expression intensity. The total count calculation gives a more faithful estimate for the parameter evaluated. On that basis, the upregulation of P-gp expression is clearly observed in this study, but a dose-dependent trend could not be established because lower doses (10 µg/mL) of ritonavir caused greater upregulation of P-gp than did 20 µg/mL ritonavir on HTLV IIIcc cells. Our results are in agreement with other studies, which report ritonavir to have more affinity for P-gp than saquinavir, causing greater P-gp upregulation.<sup>14</sup>

Our results do not completely confirm that hierarchy, but they do establish a slightly reduced transport capacity, particularly with the use of ritonavir. After exposure to the drug, the P-gp pump seems to be somewhat inhibited, limiting rhodamine efflux.<sup>10</sup>

A lower dose of ritonavir (10 µg/mL) was required to stimulate the upregulation of P-gp to a level similar to what was obtained with 20 µg/mL saquinavir; however, only the 20-µg/mL dose of ritonavir was able to decrease the viral load in the supernatant solution.<sup>14</sup> We observed a decreased PI effectiveness in suppressing viral replication and reducing the viral load, as a consequence of the P-gp/PI interaction. Samples with upregulated P-gp expression had viral loads similar to those of the untreated samples. The rapid expulsion of the drug, mediated by increased expression of P-gp, could limit its action. This means that cells might be exposed to subtherapeutic drug levels.<sup>2</sup> Cell suspensions treated with 20 µg/mL ritonavir showed the most noticeable decrease in viral load, which suggests that the inactivation of the transport pump allowed the drug to work more efficiently.

The interaction of PIs with the P-gp pump have triggered the upregulation of the transport pump. This represents a direct effect over the intracellular PI accumulation and its antiviral potency.<sup>2,10</sup> The data obtained from P-gp expression, the functionality analysis, and from viral load quantification show that, even when the transport capacity of P-gp was restricted by the action of PIs, the efflux was just as efficient as in untreated cells. The most relevant result was the limited capacity of PIs to suppress viral replication as measured in supernatants.<sup>14</sup> Based on the results reported here, we can conclude that the most probable consequence of the interaction of P-gp with PIs is the rapid expulsion of the drug and the possibility of a subtherapeutic drug level, not toxicity. The implications of these



**Fig 2. Changes in the accumulation and efflux of rhodamine in cells exposed to protease inhibitors, which may indicate alterations caused by the protease inhibitor to the functionality of this transport protein.**

results can guide the development of approaches and strategies to avoid the negative consequences of rapid drug expulsion.<sup>4</sup>

ACKNOWLEDGMENTS

This study was supported by PSM-RCMI Grant G12 RR03050 and the AIDS Research Program, PSM. Special thanks to the AIDS Research Program Data Core for their assistance with data analysis and figure formatting.

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