# Interactions of Pluronic Block Copolymers on P-gp Efflux Activity: Experience With HIV-1 Protease Inhibitors

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## Abstract

The objective was to examine the influence of Pluronic block-copolymers on the interaction between the drug efflux transporter, P-glycoprotein and HIV-1 protease inhibitors (PIs). The ATPase assay determined the effect of various Pluronics on PI-stimulated P-gp ATPase activity. Cellular accumulation studies were conducted using MDCKII and LLC-PK1 cells transfected with human MDR1 to assess Pluronic modulation of PI efflux. Pluronic P85 inhibited both basal and nelfinavir-stimulated P-gp ATPase activity, while Pluronic F127 had no effect. In cell accumulation studies, Pluronic P85 restored the accumulation of nelfinavir in MDCKII-MDR1 cells while Pluronic F127 and F88 had no effect. Pluronic P85 increased saquinavir accumulation in wild-type and MDR1-transfected cells in both the MDCKII and LLC-PK1 cell models, suggesting inhibition of multiple transporters, including MRPs. In conclusion, this study provides evidence that a block-copolymer, Pluronic P85, effectively inhibits the interaction of P-gp with nelfinavir and saquinavir. These data indicate that effective inhibition of HIV-1 PI efflux by Pluronic P85 may influence the distribution of antiretroviral agents to sites protected by efflux mechanisms, such as the blood–brain barrier, and possibly increase the brain exposure of these drugs resulting in suppression of viral replication and reduction in the incidence of drug resistant mutants.

### Keywords

efflux pumps; HIV-1 protease inhibitors; pluronics; polymers; ATPase P-glycoprotein; blood–brain barrier

## INTRODUCTION

Protease inhibitors (PIs) are drugs used in the treatment of HIV-1 infection and are an integral part of active retroviral therapy (ART), which involves the use of a combination of different classes of antiretroviral drugs. PIs prevent the formation of mature HIV-1 particles by inhibition of the viral protease. By decreasing the morbidity and mortality related to HIV-1 infection and AIDS, PIs have become the mainstay of anti-HIV-1 ART therapy.

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Given the frequent mutations the virus undergoes due to its retro-viral nature, development of resistance to PIs has been reported[1] and these mutants may persist even after PI therapy is stopped.[2] This phenomenon is more pronounced in tissues such as the brain, which has been implicated as a sanctuary site for HIV-1. Proliferation of the virus in brain tissue can lead to such conditions as the AIDS dementia complex and HIV-1 encephalopathy.[3] Many PIs have been shown to have much lower concentrations in CNS compared to plasma,[4,5] resulting in drug levels that provide an ideal environment for the evolution of more virulent forms of the virus.[6]

The low concentration of PIs in brain tissue has been shown to be a result of active efflux of these drugs out of the brain.[7–9] One of the main transport proteins responsible for this active efflux is P-glycoprotein. P-glycoprotein (P-gp, ABCB1) is a 170 kD trans-membrane protein belonging to the ATP binding cassette (ABC) super-family that is expressed in a number of tissues important for the ADME of drugs, such as the gut wall, liver canaliculi and proximal tubules in kidney.[10] In brain, P-gp is expressed at the luminal surface of the blood–brain barrier[11] and uses the energy generated by ATP hydrolysis to efflux drug substrates out of the brain against a concentration gradient, thereby decreasing brain levels. P-gp has been shown to efflux PIs both *in vitro* and *in vivo*,[7,12–15] in addition to a number of structurally diverse compounds. *In vitro* biochemical assays such as the ATPase assay have shown the ability of a number of PIs to stimulate P-gp ATPase activity.[16,17] Also, PIs have been shown to compete with substrates for the P-gp binding site.[9] *In vitro* cell accumulation studies have shown the ability of P-gp to limit cellular accumulation of PIs both in P-gp transfected cell models,[18] as well as primary cultures of brain capillary endothelial cells.[16] Directional flux studies across P-gp transfected cell monolayers have shown the preferential transport of PIs in the direction of P-gp mediated efflux.[8] There is some evidence regarding the ability of PIs to up-regulate P-gp expression.[19] *In vivo* studies in different animal models have shown that the brain-to-plasma ratio for PIs is much lower than unity following intravenous, as well as oral dosing.[8,14,15] Inhibition of P-gp using small molecule inhibitors has been shown to increase cellular accumulation and directional transport of PIs in P-gp over-expressing cells.[7,8] The brain-to-plasma ratio for PIs was significantly increased on treatment with P-gp inhibitors or in transgenic P-gp knockout mouse models.[18,20]

Small molecules have been traditionally employed as inhibitors for P-gp both *in vitro* and *in vivo*. Recent reports have provided evidence that surfactant and polymeric molecules, used primarily as excipients, have biologic activity against P-gp function.[21] Surfactants such as TPGS,[22–24] Cremophor EL,[25] Tween 80[26] and polymers such as Pluronics[27,28] and amphiphilic diblock copolymers[29] have been shown to inhibit P-gp function both *in vitro* and to enhance drug exposure *in vivo*. The effect of excipients on passive drug permeation across the plasma membrane has also been investigated.[30]

Pluronics consist of a combination of polyethylene oxide and polypropylene oxide chains arranged as a tri-block copolymer. Pluronic P85, an amphiphilic block copolymer with two chains of hydrophilic polyethylene oxide groups flanking a hydrophobic polypropylene oxide group, has been shown to inhibit P-gp efflux activity.[28]

The objective of the study was to evaluate the ability of Pluronic P85 to modulate the interaction of HIV-1 PIs with P-glycoprotein using the biochemical ATPase assay and *in vitro* cell accumulation studies.

# MATERIALS AND METHODS

## Chemicals

[$^3$H]-Vinblastine sulfate, [$^3$H]-saquinavir, and [$^{14}$C]-nelfinavir were obtained from Moravek Biochemicals (Brea, CA). Verapamil was obtained from the Sigma Chemical Co. (St. Louis, MO). Pluronics P85, F127, and F88 were a gift from BASF (Florham Park, NJ). Human P-gp expressing membranes were purchased from BD Biosciences (San Jose, CA). All other chemicals used were HPLC or reagent grade.

## Cell Lines

Wild-type (WT) and MDR1-transfected epithelial Madin-Darby canine kidney (MDCKII) cells, as well as wild-type and MDR1-transfected porcine kidney epithelial cells (LLC-PK1 WT and LLC-PK1 MDR1) were obtained from Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The MDCKII cells were maintained in Dulbecco's modified eagle medium (Mediatech, Inc., Herndon, VA) fortified with 10% heat deactivated fetal bovine serum (Sera-Care Life Sciences, Inc., Oceanside, CA) and the LLC-PK1 cells were maintained in Medium 199 (Mediatech, Inc.) and fortified with 3% fetal bovine serum. Both the canine and porcine cell lines were treated with 100 U/mL of penicillin and 100 μg/mL of streptomycin (Sigma–Aldrich, St. Louis, MO) and incubated at 37°C under humidity and 5% CO$_2$ tension. The MDCKII-MDR1 cell growth media additionally contained 80 ng/mL of colchicine to maintain positive selection pressure for P-gp expression. Cells between passages 5 and 15 were used in all experiments.

## P-gp ATPase Assay

P-gp ATPase activity was evaluated using a suspension of human P-gp over-expressing membranes obtained from baculovirus-infected insect cells (High Five, BTI-TN5B1-4) in TMEP buffer. Care was taken to ensure that all the membranes had the same lot number to minimize lot to lot variability. Upon arrival, the membranes from different vials (0.5 mL/vial at 5 mg/mL of protein) were pooled together. Two hundred microliter aliquots in individual tubes were stored at −80°C for future use. This was done in order to minimize repeated freeze-thaw denaturation of the membranes.

Briefly, a suspension of P-gp over-expressing membranes was incubated at 37°C for 20 min in presence of 4 mM ATP with and without different concentrations of the test compound. An identical reaction mixture containing 100 μM sodium orthovanadate was assayed in parallel. Orthovanadate inhibits P-gp by trapping Mg2+ ADP in the nucleotide-binding site. Thus, the ATPase activity measured in the presence of orthovanadate represents non-P-gp ATPase activity and was subtracted from the total activity measured in the nonorthovanadate treated case to yield the P-gp mediated ATPase activity. The reaction was stopped by the addition of 10% SDS and the liberated inorganic phosphate was detected by a colorimetric reaction of inorganic phosphate with a solution of ascorbic acid and ammonium molybdate

at 650 nm. The nanomoles of phosphate released were determined from the absorbance values, using a standard curve generated from inorganic potassium phosphate standards (0– 60 nM range). The same procedure was followed for control membranes (also obtained from BD Biosciences) that do not express P-gp.

Verapamil (20 μM) was used as a positive control for P-gp ATPase stimulatory activity. Initially, the Pluronic P85 was evaluated in the verapamil stimulated P-gp ATPase system for its ability to inhibit P-gp ATPase activity. Subsequently, the kinetics of P-gp ATPase activity was determined in isolated membranes using up to 50 μM concentrations of the model P-gp substrate verapamil with and without 0.01% (w/v) P85. Finally, nelfinavir was used in the P-gp ATPase assay at a concentration of 20 μM and Pluronics used at 0.01% (w/w) P85 (22 μM), 0.01% (w/w) F127 (7 μM), and 0.01% (w/w) F88 (9 μM), were evaluated for their ability to inhibit nelfinavir stimulated P-gp ATPase activity.

### Cellular Accumulation Studies in MDCKII and LLC-PK1 Cells

For the accumulation experiments, cells were seeded in clear polyester 12-well plates (TPP$^©$ cell culture plate) at a seeding density of $2 \times 10^5$ cells/well. The media were changed every other day and the cells formed confluent monolayers in 4 days. On the day of the experiment, the media were aspirated and the confluent monolayer was washed twice with prewarmed (37°C) assay buffer. The cells were preincubated for 30 min with 1 mL of assay buffer, after which the buffer was aspirated and the cells were exposed to a tracer solution of radiolabeled drug in 1 mL assay buffer per well. The plates were continuously agitated at 60 rpm in an orbital shaker and maintained at 37°C for the duration of the experiment. Following a 3-h accumulation period the supernatant was aspirated and the cells were first washed three times with 2 mL of ice cold phosphate buffered saline (PBS) and then solubilized using 1 mL of mammalian protein extraction reagent (MPER; Pierce Biotechnology, Inc., Rockford, IL). A 200 μL sample was drawn from each well in triplicate and 4 mL of scintillation fluid (ScintiSafe Econo1 cocktail; Fisher Scientific Co., Fair Lawn, NJ) was added to each sample and counted using liquid scintillation counting (LS-6500, Beckman Coulter, Inc., Fullerton, CA) to determine the radioactivity associated with the cells. Accumulated drug was normalized to cellular protein using the BCA protein assay (Pierce Biotechnology, Inc.).

In the case of inhibition studies, the cells were pretreated with 0.01% (w/w) Pluronic for 30 min followed by incubation with both test drug and inhibitor for the duration of the accumulation. Drug accumulation was expressed as a percentage of the accumulation in WT cells with the WT cells being designated as 100. All the stocks were diluted to working concentrations using assay buffer (NaCl, 122 mM; NaHCO$_3$, 25 mM; Glucose, 10 mM; HEPES, 10 mM; KCl, 3 mM; MgSO$_4$·7 H$_2$0, 1.2 mM; CaCl$_2$·H$_2$0, 1.4 mM; K$_2$HPO$_4$, 0.4 mM; pH of 7.4).

### Model Validation

Confluent monolayers of MDCKII WT and MDR1 cells were trypsinized and the cells washed with cold PBS to remove trypsin. Four to five million cells were used for total RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration, as well as

purity of the extracted RNA, was determined by the use of UV spectrophotometry (Beckman Coulter, DU 530 UV/Visible spectrophotometer). Equal amounts of total RNA for each cell line was immediately reverse transcribed to obtain stable cDNA using the Omni script RT kit (Qiagen). Quantitative RT-PCR was performed for each cell line and the reaction tube contained QPCR master mix (Brilliant SYBR green qPCR master mix, Stratagene, La Jolla, CA), template (cDNA), primers synthesized for the gene of interest, and SYBR green dye. Expression of the gene of interest was always normalized to the housekeeping gene β-actin. Quantitative real time RT-PCR was conducted using a Stratagene Mx3000P qRT-PCR system. The machine was set to run for 40 cycles with a thermal profile made up of heating at 95°C for 30 s followed by 1 min at 55°C and 30 s at 72°C. The results were evaluated using the Mx3000P software to obtain the threshold cycle ($C_t$) values for each gene and the relative expression of the MDR1 gene signal in the P-gp over expressing MDCKII cells compared to the MDCKII WT cells was computed. The nontemplate controls lacking the cDNA template required for signal amplification were also used in the experiment.

For evaluation of P-gp function [3H]-vinblastine was used as a model P-gp substrate and its cellular accumulation was used to validate increased P-gp function in the MDCKII-MDR1 cell model compared to MDCKII WT cells.

### Cellular Accumulation Studies With Protease Inhibitors

[14C]-Nelfinavir cellular accumulation was studied in MDCKII WT and MDR1-transfected cell lines to determine the interaction between nelfinavir and P-gp. Subsequently, the ability of three different Pluronics (P85, F127, and F88) to modulate the interaction between nelfinavir and P-gp was evaluated. [3H]-Saquinavir cellular accumulation was studied in both MDCKII and LLC-PK1 cell models and the effect of Pluronic P85 on the interaction between saquinavir and P-gp was evaluated.

### Statistical and Data Analysis

The Michaelis–Menten model for saturable enzyme kinetics was fit to the data for P-gp ATPase activity with increasing concentrations of the substrate verapamil. The enzyme kinetics module in Sigma Plot (version 9.0.1, SYSTAT software) with nonlinear regression was used to fit the Michaelis–Menten model to the velocity data and estimates of the kinetic parameters, $V_{max}$ and $K_m$ were obtained. For the rate versus substrate concentration plot in presence of the inhibitor P85, the data were fit by linear regression to the equation of a straight line with intercept and the ratio of $V_{max}/K_m$ was determined as the slope of the line.

Statistical comparisons between two groups were made using the two sample $t$-test at a $p < 0.05$ level of significance. Multiple groups were compared using ANOVA with Holm–Sidak post-hoc test at a $p < 0.05$ level of significance.

## RESULTS

### P-gp ATPase Assay

**Verapamil P-gp ATPase Assay**—The P-gp ATPase assay was initially validated using verapamil in P-gp over-expressing membranes. Treatment with 20 μM verapamil stimulated

the P-gp ATPase activity and resulted in a 10-fold increase in the amount of inorganic phosphate generated compared to the control case (Fig. 1). Treatment with 0.01% (w/w) of Pluronics P85 and F127 resulted in significant inhibition of verapamil-stimulated P-gp ATPase activity ($p < 0.05$; Fig. 1). The basal P-gp ATPase activity was completely abolished by Pluronic P85 but not by F127 ($p < 0.05$; Fig. 2).

The interaction of P-gp ATPase with verapamil showed concentration dependence and was saturable (Fig. 3). The $V_{max}$ and $K_m$ (mean ± SE) were estimated to be 40.6 ± 1.7 nmol phosphate/mg protein/min and 3.4 ± 0.7 μM, respectively. The $R^2$ value for the fit of the model to the data was 0.91. For the experiments with 0.01% (w/w) P85, there was significant inhibition of ATPase activity at all concentrations of verapamil and the maximal rate obtainable was no more than 6–8 nmol phosphate/mg protein/min at 50 μM verapamil (Fig. 3). The $V_{max}/K_m$ was determined to be 0.072 ± 0.02 mL/min/mg protein from the fit of the equation for a straight line to the data. The linear regression was significant with a $p$-value of 0.0113.

**Nelfinavir P-gp ATPase Assay**—Nelfinavir, used at a concentration of 20 μM, significantly stimulated the P-gp ATPase activity and Pluronic P85 completely abolished the nelfinavir mediated P-gp ATPase stimulation ($p < 0.05$). As seen in Figure 4, Pluronic F127 and F88 did not significantly inhibit nelfinavir stimulated P-gp ATPase activity ($p < 0.05$).

## Cell Accumulation Studies in MDCKII Cells

### MDCKII Model Validation

**P-gp Gene Expression:** Figure 5a shows that the $C_t$ value for P-gp expression in the MDR1 transfects was much lower than the WT (17.16 vs. 23.79) indicating a much higher level of expression of the MDR1 gene in the human P-gp over-expressing cells. No difference was observed in the $C_t$ values for the housekeeping gene in both the WT and MDR1-transfected MDCKII cells (14.14 and 14.73). The nontemplate controls for MDR1 and β-actin did not undergo amplification.

**P-gp Function:** The accumulation of vinblastine in the P-gp over-expressing MDCKII-MDR1 cells was significantly ($p < 0.05$) lower than in the wild-type cells. Vinblastine accumulation in the MDR1 cells was only ~20% of that observed in the wild-type MDCKII cells (data not shown).

## Cellular Accumulation Studies With Protease Inhibitors

### Nelfinavir Accumulation in Wild Type (WT) and P-gp Transfected MDCKII-MDR1 Cells—Nelfinavir accumulation in MDCKII-MDR1 cells was significantly lower than in the WT cells ($p < 0.05$; Fig. 6). There was ~85% lower accumulation of nelfinavir in the MDR1 cells compared to the WT cells indicating that P-gp efflux is responsible for lower accumulation in the MDR1 cells. Pluronic P85 at a concentration of 0.01% (w/w) significantly enhanced the accumulation of nelfinavir in MDR1 cells such that it was comparable to the level in WT cells ($p < 0.05$). F88 and F127 did not show any increase in accumulation in the MDR1 cells compared to the control case. Treatment of WT cells with Pluronic P85 resulted in a slight but not significant increase in nelfinavir accumulation ($p <$

0.05). Pluronics F127 and F88 did not show any effect on nelfinavir accumulation in the WT cells.

**Saquinavir Accumulation in Wild Type (WT) and P-gp Transfected MDCKII-MDR1 Cells**—Saquinavir accumulation in the MDCKII-MDR1 cells was ~40% of that seen in the WT cells ($p < 0.05$; Fig. 7). Treatment with 0.01% (w/w) P85 resulted in a twofold increase in saquinavir accumulation in MDR1 cells. Interestingly, the WT cells showed a greater increase (approximately sixfold) in accumulation on treatment with 0.01% (w/w) P85. The positive control used in this study, vinblastine, also showed a fourfold increase in accumulation in the WT cells but a greater (sevenfold) increase in accumulation in the MDR1 cells on treatment with P85.

**Saquinavir Accumulation in Wild Type (WT) and P-gp Transfected LLC-PK1 MDR1 Cells**—In the LLC-PK1 cell model, saquinavir accumulation in the MDR1 cells was only ~20% of that seen in LLC-PK1 WT cells (Fig. 8). Treatment with 0.01% (w/w) P85 significantly increased saquinavir accumulation by about fivefold in the MDR1 cells ($p < 0.05$) while in the WT cells the increase was about 3.5-fold.

## DISCUSSION

P-glycoprotein is an efflux drug transporter that utilizes energy released from the hydrolysis of ATP (ATPase) for its efflux action.[9] Drugs that interact with P-gp are known to affect its ATPase activity. One of the objectives of this study was to evaluate the inhibitory effect of Pluronics on the interaction between a HIV-1 PI and human P-gp ATPase. The ability of Pluronic P85 to inhibit drug-stimulated P-gp ATPase activity has previously been reported.[31] Initially, verapamil, a classic substrate of P-gp[32] was used as a model P-gp substrate. Pluronic P85 showed significant inhibition of P-gp ATPase activity in verapamil-stimulated membranes (Fig. 1), as well as basal P-gp ATPase activity (Fig. 2). This is in agreement with previous reports describing the ability of P85 to significantly inhibit basal, as well as drug-stimulated P-gp ATPase activity in the same membrane system.[31,33]

An interesting observation in this study was the effect of Pluronic F127 on verapamil-stimulated ATPase activity. In a previous study, Batrakova et al.,[33] reported that F127 did not inhibit basal P-gp ATPase activity. A similar observation was made in this study where there was no difference between basal ATPase activity and activity in unstimulated membranes treated with Pluronic F127 (Fig. 2). However, treatment with Pluronic F127 in the presence of verapamil resulted in significant inhibition of the verapamil-stimulated P-gp ATPase activity (Fig. 1), suggesting that P-gp interaction with a ligand may be required for Pluronic F127 to show its inhibitory effect.

The interaction of P-gp ATPase with verapamil in human P-gp expressing membranes followed Michealis–Menten kinetics allowing for the estimation of $V_{max}$ and $K_m$ (Fig. 3). For the hydrolysis of ATP the $V_{max}$ and $K_m$ values ($V_{max}$—40.6 nmol/mg/min and $K_m$—3.4 μM), were comparable to the values previously observed for verapamil in Sf9 insect cell membranes ($V_{max}$—45 nmol/mg/min, $K_m$—2.5 μM) by Sharom et al.[34] The efficiency of P-gp ATPase in the presence of verapamil was calculated as $V_{max}/K_m$ and was ~11

mL/min/mg protein. A similar value for the efficiency of P-gp ATPase has been reported for the interaction between P-gp membranes and vinblastine.[35] Batrakova et al., reported that the vinblastine-stimulated P-gp ATPase activity followed saturable kinetics with an efficiency of 11 mL/min/mg which was decreased to 0.46 mL/min/mg upon treatment with 0.01% (w/w) Pluronic P85.

Upon treatment with 0.01% (w/w) P85, the rate of formation of inorganic phosphate by P-gp ATPase was much lower when compared to the verapamil only case (Fig. 3). The Micheailis–Menten model was deemed unsuitable for analyzing these data since the velocity of the reaction did not show saturation over the range of verapamil concentrations used and was essentially linear. For this reason the equation of a straight line with intercept was used which would provide a $V_{max}/K_m$ ratio from the slope of the line. The $V_{max}/K_m$ ratio as given by the slope was 0.072 mL/min/mg protein. The ability of Pluronic P85 to dramatically decrease the efficiency of P-gp ATPase activity, stimulated by an excellent substrate of P-gp, shows its ability to strongly inhibit the interaction between drug and P-gp in the ATPase assay possibly by altering both the $V_{max}$ and/or $K_m$.

HIV-1 PIs have been shown to interact with isolated P-gp membranes and stimulate ATPase activity.[9] This activity has been shown to be inhibited by small molecule inhibitors like PSC833.[9] In contrast, the ability of relatively large polymers such as Pluronics to modulate nelfinavir-stimulated P-gp ATPase activity was evaluated in this study. Nelfinavir stimulation of P-gp ATPase activity (Fig. 4) resulted in a rate of hydrolysis similar to what has been previously reported in the same membrane system.[16] Bachmeier et al., reported a $V_{max}$ of $20.8 \pm 6.2$ nmol/mg/min (20 μM nelfinavir), which was similar to the rate of inorganic phosphate generation measured in this study ($22 \pm 5.1$ nmol/mg/min) with 20 μM nelfinavir. The complete inhibition of nelfinavir-mediated P-gp activity by Pluronic P85 provides evidence toward its ability to effectively inhibit the interaction between P-gp and nelfinavir.

Pluronics F88 and F127 did not exhibit significant inhibition of P-gp ATPase activity and these treatments did not result in ATPase activity significantly different from control (Fig. 4). Interestingly, when verapamil was used as the stimulating agent in place of nelfinavir, Pluronic F127 did show significant inhibition ($p < 0.05$) of verapamil-stimulated P-gp ATPase activity (Fig. 1). Pluronic F127-mediated inhibition of verapamil-stimulated but not nelfinavir-stimulated ATPase activity or basal P-gp ATPase activity, suggests that inhibition of P-gp by Pluronic F127 may depend not only on the interaction of P-gp with the ligand, but also possibly on the site of interaction of the ligand with P-gp. Nelfinavir and verapamil could be interacting with P-gp at different sites leading to the differential inhibitory effect of F127. P-gp possesses multiple binding sites[36] and has been shown to interact with different drugs at different binding sites. Orlowski et al.,[37] have reported that the inhibition of basal, as well as verapamil-stimulated, P-gp ATPase activity by various detergents at concentrations below their CMC does not correlate with the amount of detergents incorporated in the membrane, suggesting the existence of specific interactions between P-gp and detergents. These observations support the possibility of specific interaction of Pluronics with the different binding sites on P-gp. It is possible that nelfinavir and verapamil could be interacting with P-gp at different sites on the protein and Pluronic F127 may be

inhibiting the interaction at the site of verapamil interaction but not at the site of nelfinavir interaction with P-gp. This specificity in binding sites may be more apparent in the *in vitro* ATPase assay than in a cell based system where Pluronics may also show inhibitory effect by depletion of ATP and limited access to the cytoplasm could result in lack of inhibitory effect.[31] This is true for Pluronic F127 which did not show any inhibitory effect on P-gp mediated efflux of drugs in cellular accumulation studies, as seen in this and previous works.[33]

Pluronic P85 on the other hand was able to inhibit P-gp ATPase activity under both unstimulated and stimulated conditions[31] (see Figs. 1 and 2). This has been attributed to presence of an intermediate hydrophobic chain length for Pluronic P85 that may allow it to be incorporated into the bi-lipid layer resulting in a decrease in the membrane micro-viscosity. A decrease in micro-viscosity would unfavorably affect the conformation of P-gp resulting in loss of its function.[33]

The P-gp ATPase assay allowed the study of the interaction between the PI, nelfinavir and P-gp, as well as the effect of Pluronic P85 on this interaction in isolated human P-gp membranes without the interfering effects of other transporters that may confound any conclusive results. On the other hand, it must be noted that the ATPase assay can be subject to substantial variability and may therefore provide results that are subject to question. Therefore, the P85 inhibitory effect was evaluated in an intact cell based system which also provides the advantage of a more complete system. Pluronic P85 was evaluated for its ability to enhance cellular accumulation of PIs in the human P-gp over-expressing MDCKII cell model. This model has been routinely employed for evaluation of substrate status and to measure the ability of a molecule to inhibit P-gp-mediated efflux.[38,39]

Quantitative real-time RT-PCR[40] was used to examine the higher expression of the human MDR1 gene in the MDCKII-MDR1 transfects compared to the MDCKII-WT cells (Fig. 5). Higher expression of the MDR1 gene translated to lower accumulation of the model probe [$^3$H]-vinblastine in the MDCKII-MDR1 cells compared to WT cells, indicating greater efflux function of P-gp in the transfects.

The sixfold lower accumulation of nelfinavir in the P-gp over-expressing cells (Fig. 6) demonstrates P-gp mediated efflux of nelfinavir. Treatment with Pluronic P85 significantly ($p < 0.05$) increased the nelfinavir cellular accumulation in the MDCKII-MDR1 cells by about fivefold while there was a 1.2-fold increase in nelfinavir accumulation in the P85 treated WT cells. The increase in accumulation could be attributed to inhibition of endogenous P-gp in the MDCKII WT cells. Neither Pluronic F88 nor F127 enhanced nelfinavir accumulation in MDR1 or WT cells (Fig. 6). This result is in agreement with the lack of effect observed for these Pluronics in the nelfinavir P-gp ATPase assay.

In a recent study, Chandler et al., reported the enhanced accumulation of nelfinavir in wild-type HIV-1 infected MT4 cells, treated with the P-gp inhibitor verapamil, compared to untreated cells. The incidence of drug resistant mutations was significantly reduced in MT4 cells treated with verapamil possibly as a result of higher intracellular exposure to nelfinavir.[41] Similarly, effective inhibition of P-gp at the blood–brain barrier could increase

the exposure of target cells in the brain to nelfinavir resulting in suppression of viral replication and reduction in the incidence of drug resistant mutants. Adequate antiretroviral drug levels in the brain could compromise its status as a sanctuary site for the HIV-1 virus.

Saquinavir accumulation in the MDCKII cell system appears to be a more complex phenomenon than for nelfinavir (Fig. 7). The significant enhancement in saquinavir (sevenfold) and vinblastine (fourfold) accumulation in the WT cells on treatment with Pluronic P85 may be a result of its inhibitory activity against multiple drug transporters. Saquinavir has been shown to be directionally transported both in MDCKII-MRP2 and -MRP1 over-expressing cell lines. Indeed, the MDCKII WT cells were also shown to directionally transport saquinavir.[42,43] Western blot analysis data for MDCKII WT cells indicated the expression of both MRP1 and MRP2 along with P-gp in these cells.[44] *In vivo*, saquinavir brain penetration is limited by P-gp, as well as MRPs in the mouse.[45] Vinblastine has also been shown to be a MRP substrate.[43,44] Moreover, Pluronic P85 can inhibit MRP1 and MRP2 ATPase activity in isolated membranes[35] and MRP1 and MRP2 mediated efflux in MDCKII-MRP1 and MRP2 transfected cells.[44,46] Taken together, all the above studies provide evidence for both P-gp and MRP being responsible for the transport of both saquinavir and vinblastine in the MDCKII cell model.

The LLC-PK1 cell line has been shown, using western blot and functional studies, to have much lower endogenous P-gp compared to the MDCKII cells.[47–49] Consequently, saquinavir cellular accumulation was also evaluated in the porcine kidney cell model (LLC-PK1) using LLC-PK1 WT and LLC-PK1-MDR1 cell lines. The LLC-PK1 cell model has also been used to study the effect of P-gp on cellular accumulation of drugs.[50,51] The cellular accumulation of saquinavir in the LLC-PK1 WT cells was higher than in the MDCKII-WT cells, indicating lower expression of P-gp in these cells and transport of saquinavir by P-gp. In the LLC-PK1 cell system the accumulation of saquinavir in the P-gp over-expressing LLC-PK1-MDR1 cells was sixfold lower than in the WT cells indicating that the LLC-PK1 cell model may be a better system to test the interaction between saquinavir and P-gp (Fig. 8). Pluronic P85 treatment significantly increased saquinavir accumulation in both WT and MDR1 cells, similar to the result seen in the MDCKII cell model. Nonetheless, the fold increase in accumulation of saquinavir in the P85-treated MDR1 cells was greater than in the WT cells (3.5-fold in WT vs. fivefold in MDR1), indicating that the MRPs may be having less of a confounding effect on the results possibly due to lower endogenous expression.[47] Taken together, all the above data for saquinavir demonstrated the ability of Pluronic P85 to effectively inhibit both P-gp and MRPs to enhance the cellular accumulation of saquinavir. Inhibition of multiple efflux transporters may considerably enhance the therapeutic concentrations in hard to reach sites such as the brain.

Given that Pluronic P85 significantly increased the cellular accumulation of PIs, one question that arises is whether or not the P85 treatment itself changed the expression of the efflux proteins. If so, the change in substrate accumulation may be in part a response to differing expression of the transporters. Other lipid excipients have been shown to decrease the P-glycoprotein expression upon very long-term exposure.[52] It is important to note that Pluronic P85 has been implicated in the transcriptional activation of gene expression.[53,54] A recent study evaluated the effect of Pluronic P85, used alone or in conjunction with the

cytotoxic anticancer agent doxorubicin, on expression of the MDR1 gene in the human breast cancer MCF7 cell line.[55] It was shown that a number of MDR1-related genes were up-regulated in cells cultured in medium containing doxorubicin alone, but were not up-regulated in cells cultured in medium containing a mixture of doxorubicin and Pluronic P85. However, cells cultured in medium containing Pluronic P85 alone over 305 days did not show any change in expression of the MDR1 gene compared to drug sensitive parental MCF7 cells.[55] Therefore, the increase in cellular accumulation of drug with the P85 treatment in the current study is unlikely to be due to an effect of Pluronic P85 on MDR1 gene expression.

## CONCLUSION

In summary, this study used biochemical and cellular accumulation assays to determine the inhibitory effect of Pluronics on the interaction between HIV-1 PIs and P-gp. The polymeric inhibitor Pluronic P85 inhibited the interaction between PIs and P-gp both in the ATPase assay, as well as in cell accumulation studies. Pluronic P85 inhibited both basal and drug-stimulated P-gp ATPase activity. This study also exhibited the ability of Pluronic P85 to reduce the efficiency of verapamil-stimulated P-gp ATPase activity. Pluronic F127 on the other hand, did not inhibit basal ATPase activity. F127 required the presence of a ligand to inhibit P-gp ATPase activity and inhibited verapamil-stimulated but not nelfinavir-stimulated, ATPase activity. These data suggested that F127 inhibitory effect may be contingent on P-gp binding a ligand at a specific binding site. Cellular accumulation studies with nelfinavir provided conclusive evidence that P85, but not F127 and F88, inhibits P-gp mediated efflux of nelfinavir demonstrating that P85 inhibitory effect is a specific phenomenon rather than a general membrane effect. Cellular accumulation studies with the PI saquinavir in two different cell lines exhibited the ability of Pluronic P85 to inhibit multiple transporters. In conclusion, this is the first study to provide evidence of the ability of a block copolymer, Pluronic P85, to effectively inhibit the interaction of P-gp and the HIV-1 PIs nelfinavir and saquinavir.

## Acknowledgments

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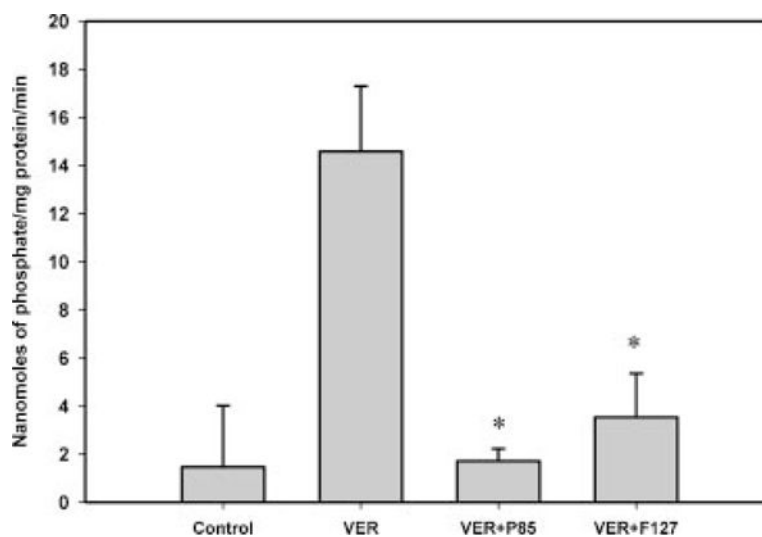
**Figure 1.**

P-gp ATPase activity expressed as nanomoles of phosphate generated/mg protein/min. The positive control verapamil (20 μM) significantly stimulated P-gp ATPase activity. Pluronic P85 and F127 significantly inhibited the verapamil-stimulated P-gp ATPase activity (*$p < 0.05$); $n = 9$.
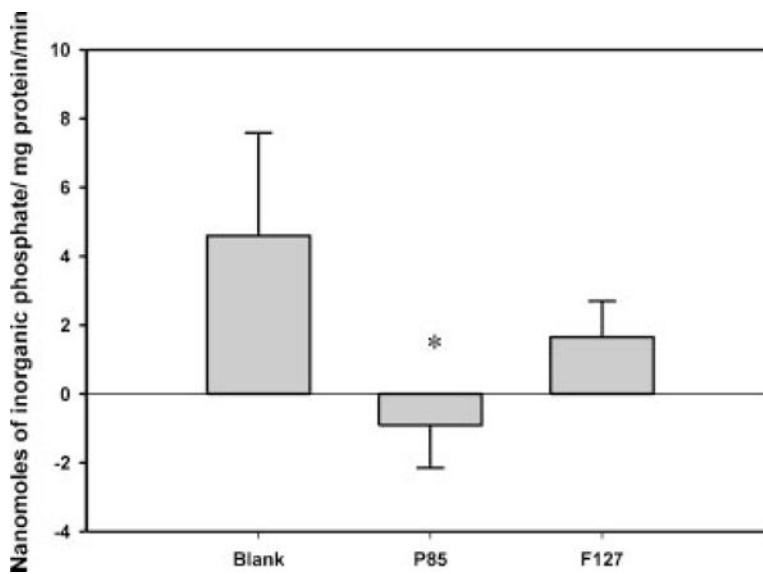
**Figure 2.**
Effect of Pluronics on basal P-gp ATPase activity. Pluronic P85, but not Pluronic F127, significantly inhibited basal P-gp ATPase activity in human P-gp expressing membranes (*$p < 0.05$); $n = 9$.
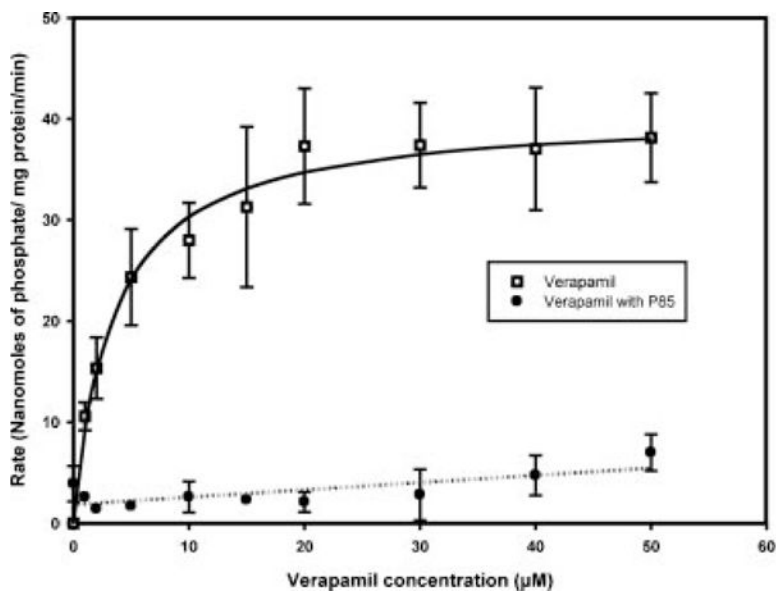
**Figure 3.**
Plot of the P-gp ATPase activity with increasing concentrations of verapamil and the effect of Pluronic P85 on this interaction. The interaction of P-gp ATPase with verapamil was saturable (open squares) and was modeled using the Michealis–Menten enzyme kinetics model (solid line) to obtain estimates of $V_{max}$ and $K_m$. Pluronic P85 inhibited the verapamil-stimulated P-gp ATPase activity (filled circles) and the equation of a straight line was fit to the data (dotted line) to obtain the estimate of $V_{max}/K_m$. Results are expressed as mean ± SD with three replicates. SD not shown for data points measured in duplicate.
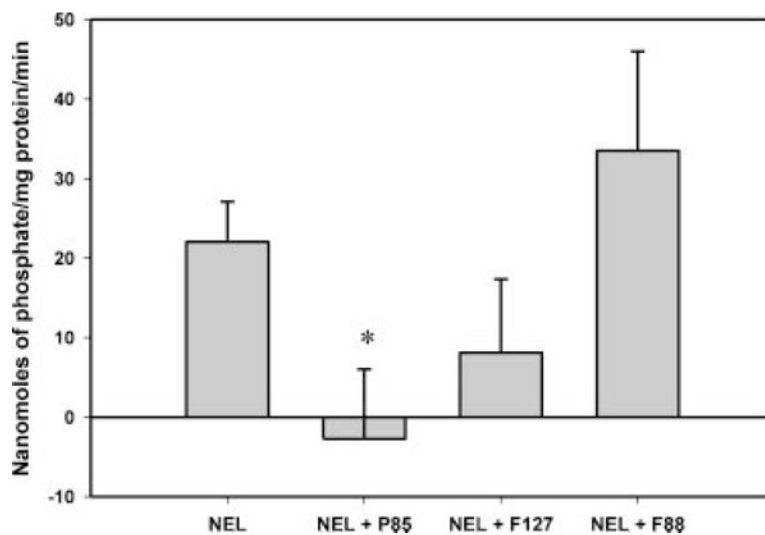
**Figure 4.**
Effect of different Pluronics on nelfinavir-stimulated P-gp ATPase activity. Pluronic P85 significantly inhibited nelfinavir-stimulated P-gp ATPase activity ($*p < 0.05$) while Pluronic F127 and F88 did not show inhibition; $n = 9$.
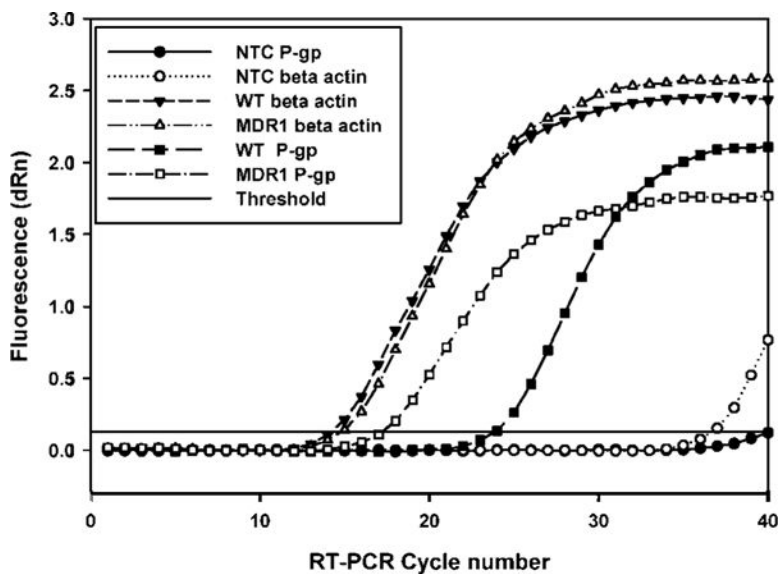
**Figure 5.**
Amplification plots for MDR1 and GAPDH genes using quantitative Real-time Polymerase Chain Reaction (qRT-PCR). The threshold cycle number ($C_t$) for the MDR1 gene was lower for the MDR1-transfected MDCKII cells (open squares) when compared to MDCK Wild-type cells (filled squares). The $C_t$ values for the housekeeping gene, GAPDH were similar between the WT (filled inverted triangles) and the MDR1-transfected cell lines (open triangles). The non-template controls for MDR1 (filled circles) and GAPDH (open circles) did not contain primers in the amplification PCR mix and were used as negative controls; $n$ = 3.
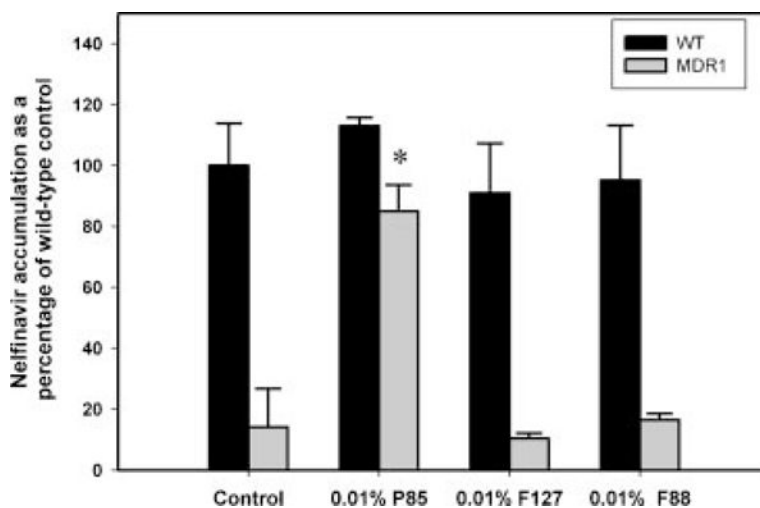
**Figure 6.**
Effect of different Pluronics on the cellular accumulation of nelfinavir in MDCKII WT and MDR1-transfected cells. Nelfinavir accumulation in the MDR1 cells (gray bars) was significantly lower than in the WT cells (black bars). Pluronic P85 significantly increased the accumulation of nelfinavir in the MDR1 cells (*$p < 0.05$). Pluronic F127 and F88 did not have any significant effect on nelfinavir accumulation in the MDR1 cells. Nelfinavir accumulation in the WT cells was not affected by treatment with Pluronics. Results are expressed as mean $\pm$ SD (as percentage of wild-type control); $n = 9$.
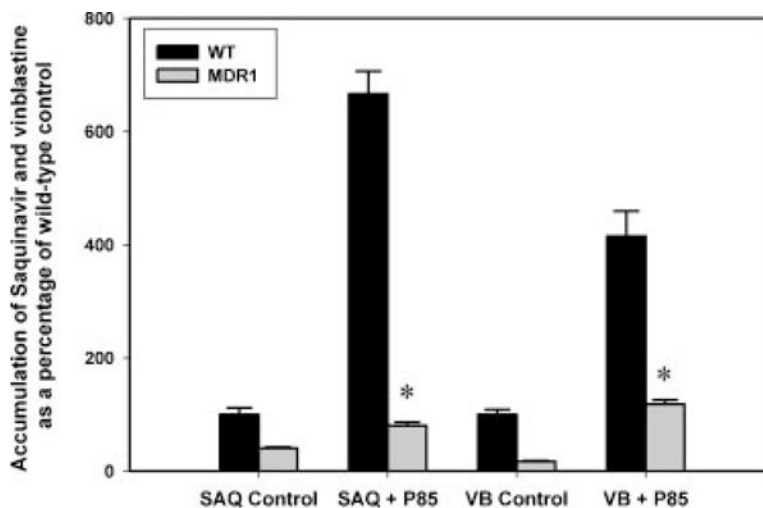
**Figure 7.**
Effect of Pluronic P85 on the cellular accumulation of saquinavir in MDCKII wild-type and
MDR1-transfected cells. Saquinavir accumulation in the MDR1 cells (gray bars) was
significantly lower than in the WT cells (black bars). Pluronic P85 significantly increased
the accumulation of saquinavir in both WT and MDR1 cells (*$p < 0.05$). Vinblastine was
used as the positive control and Pluronic P85 significantly increased vinblastine
accumulation in both WT and MDR1 cells. Results are expressed as mean ± SD (as
percentage of wild-type control); $n = 9$.

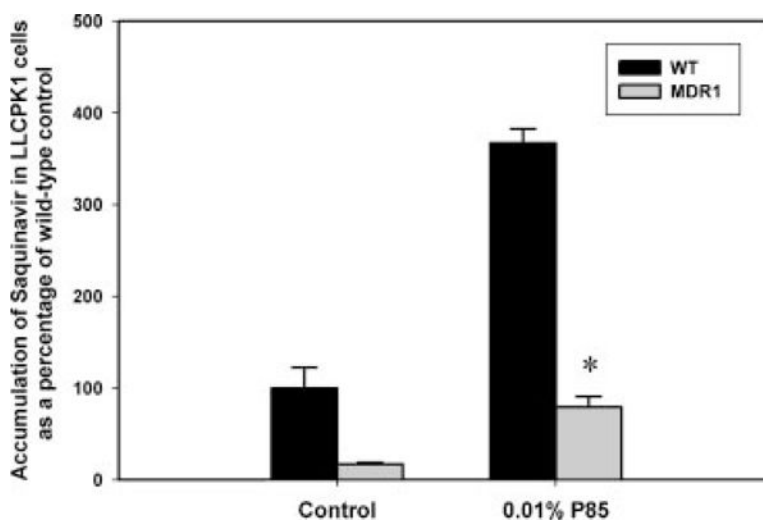Author Manuscript Author Manuscript Author Manuscript Author Manuscript

**Figure 8.**

Saquinavir accumulation in the LLC-PK1 cell model. Saquinavir accumulation in the LLC-PK1 MDR1 cells (gray bars) that over-express human P-gp was significantly lower than in the LLC-PK1 WT cells (black bars). Pluronic P85 significantly enhanced the cellular accumulation of saquinavir in the human P-gp over-expressing LLC-PK1 MDR1 cells (*$p <$ 0.05). Results are expressed as mean $\pm$ SD (as percentage of wild-type control); $n = 9$.