Transport of HIV-Protease Inhibitors across 1α,25di-Hydroxy Vitamin D_3-Treated Calu-3 Cell Monolayers: Modulation of P-Glycoprotein Activity

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Purpose. The presence of P-glycoprotein (P-gp) within the lipid bilayers of the absorptive cells greatly influences drug entry into the HIV-infected sanctuary sites. The objective of this study was to access the potential role of pulmonary cells expressing high levels of P-gp in the efflux of potent anti-HIV drugs such as protease inhibitors.

Methods. Human airway epithelium-derived Calu-3 cells grown in the presence of 0.025 mM 1α,25di-hydroxy Vitamin D_3 (di-OH vit D_3) were used as a model to evaluate the effects of p-glycoprotein efflux of HIV protease inhibitors. Cells used as controls were not treated with di-OH vit D_3. The anti-HIV agents ^{3}H Ritonavir and Saquinavir (50 μM) were used as model compounds for influx and efflux studies.

Results. Di-OH vit D_3 treatment enhanced the differentiation of Calu-3 cells indicated by more cilia and mucus secretion. It also caused elevated P-gp expression as demonstrated by Western Blot analysis and enhanced basal to apical transport of cyclosporine as compared with untreated cells. The amount of Saquinavir transported, after 3 h, across untreated Calu-3 cells (A-B) was 3-fold higher (1.62 μg; Papp = 2.4 (± 0.79) × 10^{-6} cm/s) than di-OH vit D_3-treated cells (0.57 μg with the Papp = 5.02 (± 0.62) × 10^{-7} cm/s). Similar transport profiles were obtained for ^{3}H ritonavir and a significant increase ($p < 0.05$) in the A-B transport (2.5-fold) of ^{3}H ritonavir was observed when the cell monolayers were preincubated with testosterone prior to transport studies. However, transport of AZT remained unaltered in di-OH vit D_3 treated monolayers.

Conclusion. Modulation of P-gp activity may be necessary to increase the therapeutic efficacy of protease inhibitors against HIV-1 reservoirs across alveolar lining cells and fluids.

KEY WORDS: P-glycoprotein; Calu-3; efflux; permeability; induction; anti-HIV agents.

INTRODUCTION

Highly active antiretroviral therapy (HAART) has dramatically improved the clinical management of HIV-1 infection. Despite nondetectable viral RNA in plasma, low levels of replicating viral RNA still exist at the sanctuary sites. HIV reservoirs are primarily formed because effective virucidal concentrations of anti-HIV drugs are not achieved at these sites with current dosage regimens. Subtherapeutic concentrations may either be the result of low permeability associated with efflux of drug molecules into the lumen and/or metabolism in various organs (1,2). The existence of sanctuary sites for HIV-1 may potentially endanger the efficacy of long-term antiretroviral therapy. In addition, complete eradication of HIV-1 may not be possible because of latently infected cells. During HAART, HIV-1 can persist in circulating/resting CD4+ T lymphocytes, lymph node mononuclear cells, pulmonary tissues, placenta and, to a greater extent, in the brain tissues despite sustained suppression of plasma viremia to undetectable levels (3–5). HIV reservoirs, where antiretroviral drug penetration is not optimal, may allow local HIV-1 infection ultimately propagating to other tissues. Possible HIV sanctuary sites include the lungs, testes, lymph nodes, placenta, and the central nervous system (6–9). Lungs act as a separate pharmacokinetic and viriologic compartment. Penetration of xenobiotics into pulmonary epithelial lining and alveolar cells is highly variable. Viral loads in lungs may not necessarily reflect those in plasma and the presence of P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4) in the alveolar cells greatly influence the drug entry into the HIV-1 infected sanctuary sites (10–12).

Lungs are one of the major targets during HIV infection and advanced complications of AIDS. With the onset of AIDS, an opportunistic lung infection is observed along with severe pulmonary complications in more than 50% of the patients (13). Proviral DNA has been detected in lung cells and tissues of HIV infected patients. Hence, lung seems to act as an important reservoir for active HIV-1 replication and subsequent proliferation. Patients with HIV detected in their lungs have extremely poor clinical prognosis with rapid onset of death compared with patients having no detectable HIV in lungs. In addition, patients with pulmonary complications have detectable levels of HIV-infected macrophages along with $CD4^+$, $CD8^+$ T-lymphocytes (14,15).

One major limitation to the penetration of clinically important anti-HIV drugs to HIV sanctuary sites is the active efflux by P-glycoprotein (P-gp), the product of multidrug resistance protein (MDR; 1,2). P-gp, considered as a versatile xenobiotic pump, was initially discovered in cancer cells. This efflux pump is a member of a highly conserved group of the energy-dependent ATP-binding-cassette transporters found in cells from various tissues. This transporter is encoded by a small multigene family, described by *mdr* Class I, II, and III. P-gp is expressed on the lumenal side of the alveolar vessels, where it can actively exclude agents that are substrates of P-gp (16). Recently it has been shown that P-gp limits the permeation of HIV-protease inhibitors across intestinal and central nervous system barriers (17–19). These studies provided the mechanistic basis for the limited cell permeability of the protease inhibitors. The following work was initiated (i) to compare the membrane permeation characteristics of the HIV protease inhibitors, ritonavir, and saquinavir; (ii) to determine the role of P-gp on the permeation of anti-HIV drugs to the HIV-sanctuary site of lungs; and (iii) to examine whether co-administration of other protease inhibitors could elevate the desired protease inhibitor concentration in lungs.

Recently, various cell lines and primary cultures have been used to study the transport of drugs within alveolar linings. A549 cells exhibit many features of alveolar type II cells and have been used to study alveolar drug absorption and metabolism. However, many questions remain over the ability of A549 cells to form functional tight junctions and to

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detect chemically induced alveolar toxicity. Despite the abundance of type II cells in the alveoli, approximately 95% of the alveolar epithelial surface area is composed of type I cells. Hence, suitability of A549 cells as a drug absorption model can also be debated (19,20). Currently, no satisfactory human cell line exists to model the alveolar epithelium. Hence, we are using Calu-3 cells derived from lower airway, anatomically located in vicinity to alveolar tissue, to delineate factors responsible for lower drug concentrations in sanctuary sites during antiretroviral therapy.

The human airway epithelial cell line, Calu-3, has been used extensively as a model to study the drug transport across lower bronchial epithelium (21–24). Because no other suitable cell culture model is currently available to study drug transport across alveolar barrier, we have used Calu-3 cells to study transport of anti-HIV compounds. However, preliminary studies in our laboratory have demonstrated that under conventional growth conditions, Calu-3 cells do not express significant levels of P-gp. Recently, it has been reported that Caco-2 cells grown in presence of 1α,25-di-hydroxy Vitamin D_3 (di-OH vit D_3) express high levels of P-gp and CYP3A4 (25). In this study, we adopted a similar culture technique to induce levels of P-gp in Calu-3 cells and to study its influence on the transport of anti-HIV agents like saquinavir and ritonavir. Results from these studies may demonstrate the utility of this culture system for studying P-gp-mediated drug interactions.

MATERIALS

Zinc sulfate, ferrous sulfate, sodium selenite, trypsin-EDTA, rat-tail collagen type I, AZT, quinidine, cyclosporin A (CsA), and rhodamine 123 (Rh-123) were purchased from Sigma Chemical Company (St. Louis, MO, USA). 1α,25-di-OH vitamin D_3 (di-OH vit D_3) was purchased from Calbiochem (Los Angeles, CA, USA). ^{3}H-Ritonavir and ^{14}C-AZT were obtained from Moravek Chemicals (Brea, CA, USA) ^{14}C-mannitol (50 mCi/mmol) was supplied by Amersham (Piscataway, NJ, USA). Ritonavir and Saquinavir were obtained from National Institute of Health (Rockville, MD, USA) under the Reagents Assistance Program of NIAID.

Cell Culture

Human lung carcinoma-derived Calu-3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The growth medium, Dulbecco's modified Eagle Medium F-12 was obtained from Life Technologies (Grand Island, NY, USA). MEM non-essential amino acids (NEAA), penicillin, streptomycin, sodium bicarbonate, and HEPES were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS, USA). The buffer used in transport studies was Dulbecco's modified phosphate buffer saline (DPBS), containing 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM $MgSO_4$, and 5.3 mM glucose at pH 7.4. Culture flasks (75 cm^2 growth area), polyester Transwells® (pore size 0.4 μm with diameter 6.5mm) were procured from Costar (Bedford, MA, USA).

METHODS

Cell Culture

Human lung carcinoma-derived Calu-3 cells were used at passages 20 to 40.

Conventional Calu-3 Cell Culture

Calu-3 cells were grown at 37°C in an atmosphere of 5% CO_2 in DMEM-F12 containing L-glutamine, supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acids, penicillin (100 U/mL), and streptomycin (100 μg/mL). Semi-confluent cell monolayers were subcultured every 4 days using 0.25% trypsin containing 0.537 mM EDTA. Cells were seeded at a density of 50,000 cells/well in 24-well plates on Transwell® polyester clear inserts (Coaster; diameter: 6.5 mm, pore size: 0.4 μm) coated with rat tail collagen type I followed by laminin and allowed to differentiate completely for 10 days before performing transport experiments. Cells of passage number 20–40 were used through the entire duration of study.

Calu-3 Culture for Enhanced Expression of P-gp

Cellular growth was routinely monitored by optical microscopy (Carl Zeiss Telaval 31). After achieving confluence with conventionally grown Calu-3 cells, the growth medium was replaced with a complete differentiation medium containing 0.1 μM sodium selenite, 3.0 μM zinc sulfate, 5.0 μM ferrous sulfate, and 0.25 μM di-OH vit D_3 supplemented with 5% FBS. The cells were then allowed to differentiate for 5 days before using them for experiments. Upon reaching confluency, the cells were treated with di-OH vit D_3 at least for 6 days before each experiment. Monolayer integrity was monitored by paracellular marker ^{14}C mannitol transport and by measuring the transepithelial electrical resistance (TEER) with an EVOM Epithelial Volt-ohmmeter (World Precision Instruments. Inc., Sarasota, FL, USA).

Microscopy

Transmission electron microscopy (TEM) and scanning electronic microscopy (SEM) were performed to characterize the morphology of Calu-3 cells grown under liquid-covered culture conditions. Cells grown on clear polyester membrane with a 0.4-μm pore size at confluency after 9 days were washed twice with cold DPBS. The cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) and 2% glutaraldehyde in cacodylate buffer for 30 min at 4°C, washed 3 times, and finally dehydrated with graded ethanol. At the end, the membrane was removed from plate, placed on the stub, coated with gold/Pd, and observed using SEM (Philips Electron Microscope, Model XL-30 ESEM-FEG) and pictures were taken.

For TEM, cells were fixed in paraformaldehyde and glutaraldehyde as described previously and postfixed with OsO_4 for 30 min. Then the cell surface was coated with 10% gelatin to protect the surface structures. Cells were processed for thin sectioning using routine procedures (section thickness 90 nm for TEM). Jeol electron microscope (Model JEM 1200 EX II) at 100 kV was used to take pictures.

Immunoprecipitation Western Blot

Confluent Calu-3 cells (both di-OH vit D_3 treated and untreated) grown on T 75 flasks were washed twice with PBS and harvested using a cell scraper. The cells were collected by centrifugation and lysed with immunoprecipitation (IPP) buffer (10 mM Tris, pH 7.4; 150 mM NaCl; 2 mM Na_3VO_4; 5 mM NaF; 1 mM EGTA; 1% Triton X-100; 0.5% NP-40; 0.1 mM phenylmethylsulfonyl fluoride; and 10 μg/mL leupectin) for 10 min on ice. The cells were ultrasonicated (Branson Cleaning Equipment Co., Shelton, CT, USA) for 30 s and then centrifuged at 15,500 g for 10 min at 4°C. The membrane fraction and supernatant were collected, stored at –20°C, and subsequently used for immunoprecipitation. This Calu-3 extract (150 μL) containing 20 μg of protein along with 150 μL of IPP buffer was immunoprecipitated with monoclonal antibody to human anti-P-glycoprotein (#RDI-PRO57043, Research Diagnostics, Inc.) overnight at 4°C. This monoclonal antibody (clone determination: JSB-1) reacts with a conserved cytoplasmic epitope of the plasma membrane-associated P-gp, a 170-kD multidrug resistance-related protein. The tubes were then incubated with protein A-Sepharose for 2 h at 4°C. Sepharose beads containing antigen-antibody complexes were collected by centrifugation, washed three times with IPP buffer, resuspended in 30 μL of SDS sample buffer (62 mM Tris, pH 6.6, bromophenol blue), and then boiled for 5 min. All of these immunoprecipitated samples and molecular weight protein markers were then separated by SDS polyacrylamide gel electrophoresis. The protein markers and the samples were electrophoretically transferred to a nitrocellulose membrane. The portion of the blot containing P-glycoprotein samples was incubated in a blocking buffer containing 5% nonfat dry milk and 3% bovine serum albumin in Tris-buffered saline for 1 h at room temperature. The blot containing immunoprecipitated P-glycoprotein was then incubated with anti-P-glycoprotein (Research Diagnostics, Inc.) antibody at 4°C. Western Blot Amplification Kit from Bio-Rad™ was used to develop the blots according to the manufacturer's protocol.

Diffusion Studies

The permeabilities of various radiolabeled as well as nonradiolabeled molecules and anti-HIV agents across di-OH vit D_3 treated and untreated Calu-3 cells were determined using Transwell® inserts. Before each experiment, cell monolayers were washed three times with DPBS (pH 7.4) at 37°C. Drug solution (100 μL) prepared in DPBS was placed on the donor chamber (upper chamber in case of A-B transport and bottom chamber for B-A). The receiver chamber was filled with 500 μL of DPBS (in case of A-B transport studies). Sampling from the receiver chamber was performed at predetermined time intervals. Entire volume was withdrawn from the receiver chamber and replaced with fresh DPBS solution to maintain sink conditions in receiver chamber. The samples were then analyzed by an appropriate analytical technique such as radiolabelled scintillation counting or HPLC. All experiments were performed at 37°C.

Stock solutions (10 mg/mL) of saquinavir and ritonavir were prepared in DMSO. This stock solution was used to prepare the final concentration of 50 μM with DPBS. Stock solution of Rh123 (200 μM) was prepared in DPBS. Testosterone was first dissolved in ethanol to prepare stock solution of 10 mM, which was then diluted to DPBS to prepare the final working concentration of 100 μM.

To examine the inhibitory effects on the P-gp-mediated efflux of CsA, saquinavir and ritonavir, cells were preincubated on both sides with other P-gp substrates/inhibitors (quinidine, cortisol, testosterone, and Rh-123) 30 min before the experiment. Incubation medium was then removed from donor chamber before replacing it with drug solution.

Radioactivity in the experimental samples was determined by a scintillation counter (LS 6500, Beckman, Fullerton, CA, USA). Nonradioactive samples were analyzed by HPLC. The effective permeability (cm/s) was determined using the following equation:

$$P_{app} = (60 \cdot V/A \cdot Co)(dC/dt)$$

P denotes the permeability in cm/s, dC/dt is the slope of plot of concentration (mM) vs. time (min); Co is the initial donor concentration of drug. A represents the surface area of diffusion, and V is the volume of receiver chamber.

Analytical Method

The amount of ritonavir and saquinavir transported was determined by using reversed-phase HPLC with UV detection. The HPLC system composed of Beckman Gold gradient HPLC pump (128 solvent module), Beckman Gold UV detector (Model 166), and Phenomenix RP-C8 column (5 μm, 4.6 mm i.d. × 15 cm) was used. Detection of both ritonavir and saquinavir was performed at a wavelength of 230 nm. Eluent composition was 67 mM potassium dihydrogenphosphate: acetonitrile (65:35) set at a flow rate of 1.0 mL/min. Elution of ritonavir and saquinavir occurred at 12.8 min and 16.6 min respectively.

Statistical Analysis

All studies were performed at least in triplicates. Data are presented as mean ± standard deviation. Statistical significance was inferred through unpaired student's t test (INSTAT, version 1.2). Statistical significance was expressed at the 95% confidence level.

RESULTS

Morphology of Calu-3 Cells

We have described in a previous report that Calu-3 cells form tight monolayers along with cilia and active mucus secretion in conventionally grown liquid immersed surface (26). To elucidate whether change in media conditions result in accelerated morphologic differentiation, electron micrographs of Calu-3 cells grown in both conventional medium and differentiation medium were obtained. Cells grown under liquid-immersed conditions on collagen and laminin coated Transwell filters formed a single layer of cells at confluency, as shown by light microscopy, which also revealed that no observable difference in the monolayer characteristic existed between untreated and di-OH vit D_3 treated culture monolayers. Presence of abundant cilia was evident from the SEM of di-OH vit D_3 treated Calu-3 cells (Fig. 1 A and B). The main difference was in the shape of cilia, i.e., thick and short in conventionally cultured cells compared with long and thin

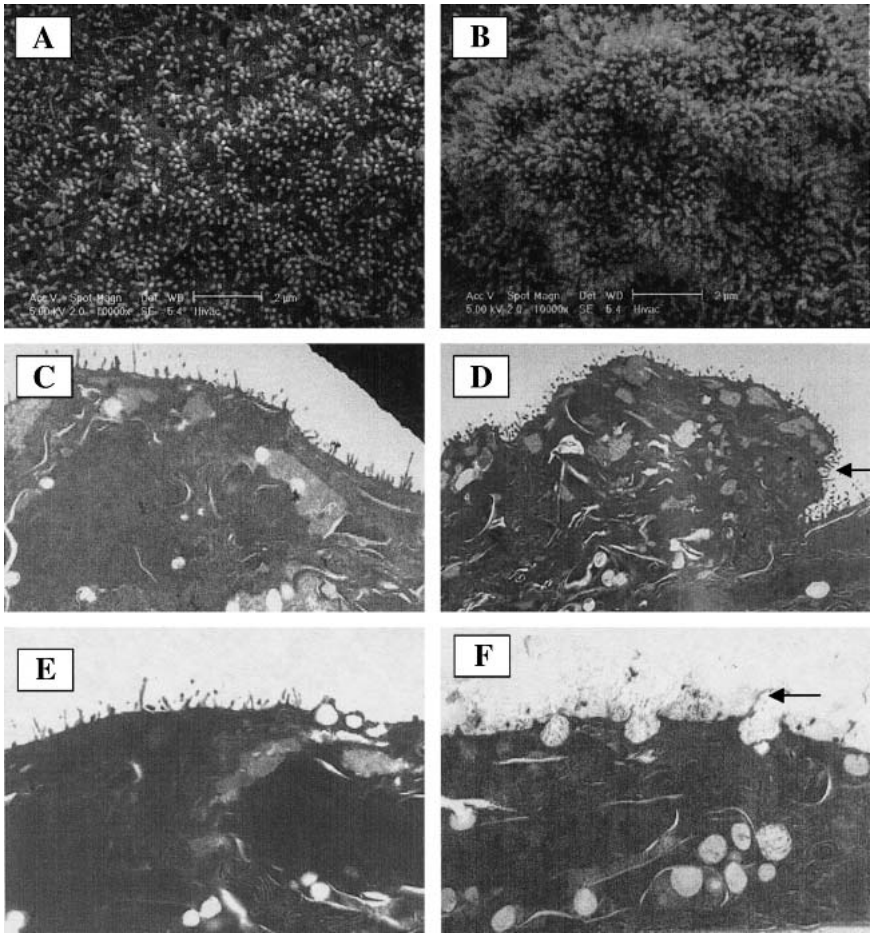

Fig. 1. Scanning electron microscopy pictures of Calu-3 cells showing abundant cilia and mucus secretion in di-OH vit D_3-treated cells (B, ×20,000), when compared to untreated cells (A, ×20, 000). Transmission electron microscopy pictures of Calu-3 cells grown under liquid immersed conditions on collagen and laminin coated filters. Figures illustrate enhanced growth of cilia (D) and mucus secretion (F) in di-OH vit D_3-treated Calu-3 cells compared with untreated cells (C and E, respectively; scale 1 μm). Increase in the number of cilia and mucus secretion is indicated by bold arrow (←).

in di-OH vit D_3-treated cultures. TEM analysis revealed that di-OH vit D_3 cells express well-defined apical cilia (Fig. 1C) in comparison with untreated cells (Fig. 1D). Also, an enhanced secretion of mucus was evident in di-OH vit D_3-treated cells (Fig. 1 E and F) Hence, Calu-3 cells grown with differentiation medium containing di-OH vit D_3 seems to be a suitable model to study *in vitro* drug delivery across airway epithelium.

In addition, Western Blot analysis clearly demonstrated higher levels of P-gp expression in di-OH vit D_3 treated cells than conventionally grown untreated cells (Fig. 2).

Calu-3 Cell Monolayer Integrity

Cellular integrity of Calu-3 cells was regularly monitored by TEER values associated with the monolayer. The transepithelial resistance of the monolayers reached a stable value at 1200 ± 220 $\Omega \cdot cm^2$ in di-OH vit D_3-treated monolayers as compared with 900 ± 150 $\Omega \cdot cm^2$ in untreated cells. The integrity of the cell monolayers during a transport experiment was further investigated using both paracellular and transcellular markers, ^{14}C mannitol and ^{14}C diazepam, respectively. Appearance of these markers in the receiver chamber was linear with time in both A-B and B-A directions. The apparent permeabilities of mannitol and diazepam across di-OH vit D_3-treated Calu-3 cells were 5.2 ± 0.44 × 10^{-7} cm/s and 1.7 ± 0.38 × 10^{-5} cm/s, respectively compared with 3.9 ± 0.96 × 10^{-7}

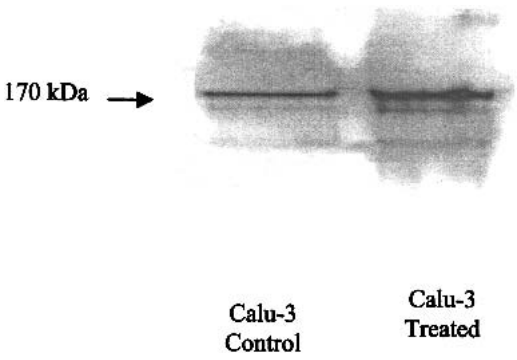

Fig. 2. Comparison of P-gp expression in untreated Calu-3 cells and di-OH vit D_3-treated Calu-3 cells. Presence of 1α,25di-hydroxy vitamin D_3 is responsible for enhanced expression of P-gp in Calu-3 cells.

cm/s ($p < 0.05$) and $1.7 \pm 0.38 \times 10^{-5}$ cm/s across untreated cells. These results suggests that treating the cells with di-OH vit D_3 does cause differential changes in the properties of tight junction, limiting the paracellular transport of mannitol ($p < 0.05$), but this treatment does not appear to alter transcellular transport characteristics.

Effect of Quinidine: A Specific P-gp/MRP1 Inhibitor on CsA Transport

To confirm the existence of P-gp-mediated efflux in di-OH vit D_3-treated Calu-3 cells, transport of CsA, a well-established P-gp substrate was examined. Quinidine, a known P-gp/MRP1 inhibitor, was used at various concentrations to determine the effect of P-gp on cellular transport of CsA in both untreated and di-OH vit D_3-treated Calu-3 cells.

Transport of ^{3}H CsA (0.5 μCi/ml, 7.0 Ci/mmol) in presence of various concentrations of quinidine across both untreated and di-OH vit D_3-treated Calu-3 cells is depicted in Fig. 3, A and B, respectively. A 2-fold increase in the B-A transport of CsA (5.54% of donor concentration) compared with A-B transport (3.37% of donor concentration) was noticed across the untreated cells ($p < 0.05$). However, in case of di-OH vit D_3-treated cell monolayers, a 4-fold increase in the B-A transport of CsA (19.53% of donor concentration) compared with A-B transport (4.8% of donor concentration) was observed ($p < 0.05$). Quinidine inhibited the efflux of ^{3}H CsA mediated by P-gp in both the culture systems, in a concentration dependent manner. The maximum inhibitory concentration used in both cell monolayers was 75 μM. However, the inhibitory effect of quinidine on CsA transport was more pronounced in the di-OH vit D_3-treated Calu-3 cells (Fig. 3 A and B).

An involvement of P-gp in the efflux of CsA and its inhibition by quinidine was further evaluated by studying the bidirectional transport of ^{14}C AZT (0.5 Ci/ml, 54 mCi/mmol). The major pathway of AZT transport is considered to be by transcellular passive diffusion and is not a known substrate for P-gp. P_{app} values (A-B and B-A) of AZT were 3.99 ($\pm$ 1.07) $\times 10^{-5}$ cm/s and 4.06 ($\pm$ 0.83) $\times 10^{-5}$ cm/s for untreated and 2.7 ($\pm$ 1.3) $\times 10^{-5}$ cm/s and 3.9 ($\pm$ 0.77) $\times 10^{-5}$ cm/s for di-OH vit D_3-treated Calu-3 cells, respectively. These values were not significantly different from each other ($p > 0.05$), clearly suggesting that P-gp induction does not play a role in the transport of AZT.

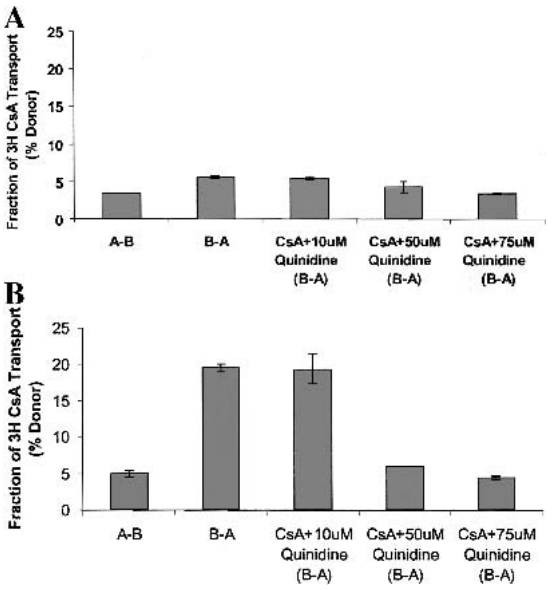

Fig. 3. Concentration dependent inhibitory effect of quinidine on P-gp mediated efflux of ^{14}C CsA across (A) untreated and (B) di-OH vit D_3-treated Calu-3 cells. A 4-fold increase in B-A transport is pronounced in treated cells compared with untreated cells. Quinidine shows maximum inhibitory at 75 μM ($p < 0.05$) compared with a nonsignificant effect at 10 μM ($p > 0.05$) (Mean ± SD, n = 3–6).

Interaction of P-gp Substrate/Inhibitor with Protease Inhibitor

Directional studies were conducted to evaluate the influence of P-gp efflux on ^{3}H ritonavir (0.5 μCi/mL, 1.0 Ci/mmol) transport. P-gp substrate, such as Rh-123 (0.2 mM) and P-gp inhibitor, like testosterone (0.1 mM) were used to study the inhibitory effect of these compounds on ritonavir efflux. Figure 4 depicts the effect of Rh-123 and testosterone on the transport of ^{3}H ritonavir across di-OH vit D_3 treated Calu-3 cell monolayers. A significant increase ($p < 0.05$) in the A-B transport (2.5-fold) of ^{3}H ritonavir was observed when the cell monolayers were either pre or co-incubated with testosterone. The permeability values have been summarized in Table I. These results indicate that testosterone, like other p-gp inhibitors, interferes with the efflux of protease inhibitor, ritonavir. In addition, Rh-123 did not exhibit remarkable inhibition on ritonavir efflux since it is not an inhibitor for P-gp.

Transport of Saquinavir and Ritonavir across Calu-3 Cells

This study was designed to investigate the combined effect of P-gp and CYP3A4 induction on the transport of saquinavir, an anti-HIV compound, which is known to be a substrate for both P-gp and CYP3A4. Figure 5 shows the effect of P-gp induction on transcellular transport of saquinavir. The cumulative amount of saquinavir transported, after 3 h, across untreated Calu-3 cells (A-B) was 1.62 μg with P_{app} = 2.4 ($\pm$ 0.79) $\times 10^{-6}$ cm/s, which was 3-fold higher than di-OH vit D_3-treated cells. Same value for treated cells was 0.57 μg with the P_{app} = 5.02 ($\pm$ 0.62) $\times 10^{-7}$ cm/s.

Once the induction of P-gp in the di-OH vit D_3-treated Calu-3 cells was functionally determined, further studies were performed to understand the extent of P-gp activity in the treated cells by studying the bi-directional transport of sa-

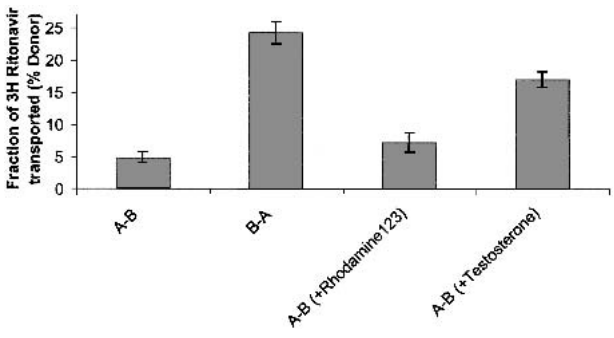

Fig. 4. Bidirectional transport of ^{3}H ritonavir in di-OH vit D_3-treated Calu-3 cells and the inhibitory effect of P-gp-mediated efflux by Rh123 and testosterone (*$p < 0.05$) (Mean ± SD, n = 3–6).

Table I. Comparative P_{app}s of Ritonavir Transport across di-OH vit D_3-Treated Calu-3 Cells

Compound	P_{app} (A-B) cm/s	P_{app} (B-A) cm/s
Ritonavir	3.16×10^{-7} (± 0.22)	5.83×10^{-6} (± 0.29)
Ritonavir + Rh123	3.27×10^{-7} (± 0.37)	–
Ritonavir + testosterone	7.29×10^{-6} (± 0.28)	–

quinavir across Calu-3 monolayers. Figure 5B illustrates the effect of P-gp on the bi-directional transport of saquinavir across di-OH vit D_3-treated Calu-3 cells. The cumulative amounts of saquinavir transported after 3 h were 0.57 μg with the P_{app} of 5.02 (± 0.62) $\times 10^{-7}$ cm/s in A-B direction and 5.73 μg with the P_{app} of 9.07 (± 1.17) $\times 10^{-6}$ cm/s in B-A direction.

To further delineate the effects of P-gp and CYP3A4 on saquinavir transport, a known P-gp and CYP3A4 inhibitor, cortisol, was included to inactivate the enzymes before the transport experiments. Figure 6 demonstrates the inhibitory effect of cortisol on the P-gp mediated efflux of saquinavir, across P-gp induced Calu-3 cell monolayers at two different concentrations. At the end of the 3-h experimental period, the cumulative amount of saquinavir transported (A-B) was 176 ng. On pretreating the cells with 0.05 mM cortisol, no significant increase in A-B transport of saquinavir was observed with the value of 174 ng in 3 h. However, the transport of saquinavir was significantly increased to 340 ng with pretreatment of a higher concentration of cortisol (0.2 mM).

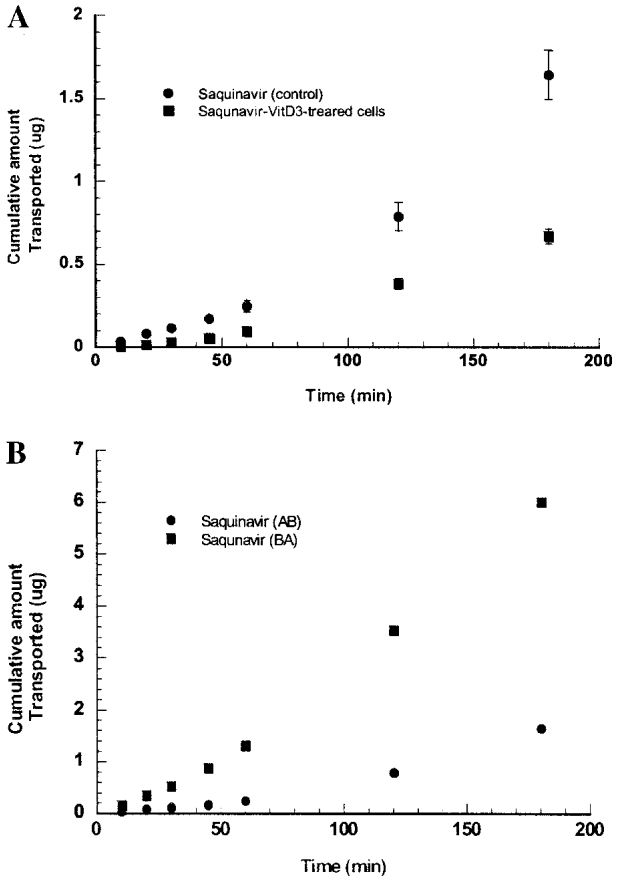

Fig. 5. (A) Transport of saquinavir across untreated and di-OH vit D_3-treated Calu-3 cells. About a 3-fold decrease in transport (A-B) of saquinavir is observed in treated cells because of P-gp induction (*p < 0.05). (B) Bidirectional transport of saquinavir across di-OH vit D_3-treated Calu-3 cells. Approximately a 12-fold increase in (■) B-A transport of saquinavir (cumulative amount of 5.71 μg) is found compared with (●) A-B transport (cumulative amount of 0.49 μg) (Mean ± SD, n = 3–6).

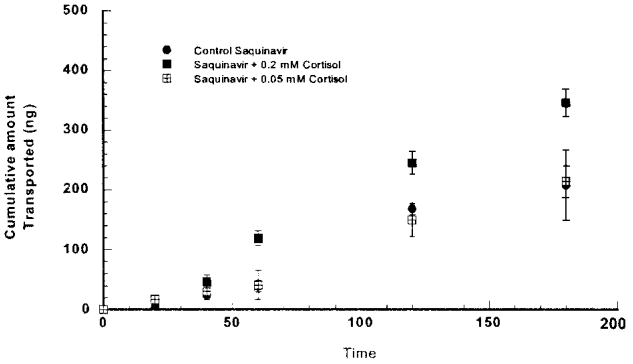

Fig. 6. Inhibitory effect of (■) 0.05 mM and (□) 0.2 mM cortisol on (●) A-B transport of Saquinavir in di-OH vit D_3 Calu-3 cells (Mean ± SD, n = 3–6).

DISCUSSION

Previous studies on changes in epithelial cell growth have demonstrated that filters and precoatings can significantly influence the morphology and protein expression in various cultures (27). The aim of this study was to modify an established cell line to increase its utility in screening the transport of drug molecules against P-gp-mediated efflux. This study used the human airway epithelial cell-line Calu-3 as a model to study the efflux of protease inhibitors. In this article, we report that submicromolar concentrations of di-OH vit D_3 stimulate P-gp expression in human airway epithelial cell line. This is the first demonstration of the induction of P-gp efflux pump in Calu-3 cell monolayer stimulated with di-OH vit D_3. This observation is consistent with a previous report of induction in P-gp expression by di-OH vit D_3 in Caco-2 cells (25,27). In the induced Calu-3 cells, a significant expression of P-gp was functionally determined. Such enhancement in the P-gp expression by di-OH vit D_3 was confirmed by immunoblotting using P-gp antibody (which does not cross react with other MRPs), as well as inhibition of P-gp with specific inhibitors like quinidine and testosterone, thus establishing that the efflux of the protease inhibitors was primarily P-gp mediated.

Previous studies have demonstrated a time-dependent increase in induction of enzyme activity of cells on exposure to di-OH vit D_3 (25). Therefore, all the efflux studies using Calu-3 cells were conducted after a specified period of exposure to the differentiation medium. Generally, Calu-3 cells have been used for transport experiments at 10–12 days post-seeding, which measures up to 5–6 days post-confluence (26). Hence, we established a 6-day exposure protocol with di-OH vit D_3 medium. Six days of exposure to this differentiation medium was kept constant with an estimation of equal protein expression during all experimental conditions. Similarly, for untreated cultures, the cells were exposed to conventional growth medium for the same duration. However, studies showing time dependent induction of P-gp and its mRNA expression in response to di-OH vit D_3 are in progress.

Various cell lines and primary cultures have been used to study the transport of drugs within alveolar linings. Currently no satisfactory human cell line exists to model the alveolar epithelium. Hence, we are using Calu-3 cell line derived from lower airway, anatomically located in vicinity to alveolar tissue. Our liquid-immersed culture technique has demonstrated a morphologic similarity of cilia and mucus secretions comparable to air-interface culture system (24,26). We have reported that the cells grown under di-OH vit D_3 medium-immersed conditions expresses elongated cilia and considerable amount of mucus secretion, similar to the cells grown under air-interface. In addition, the functional tight junctions are more pronounced compared to conventionally grown cells, which is demonstrated by lower ^{14}C mannitol flux. However, no difference is observed in the characteristic of the lipid bilayer's property, as demonstrated by no statistically significant differences in the flux of ^{14}C diazepam across both untreated and di-OH vit D_3 treated Calu-3 cells.

A number of clinical data revealed that multi-drug resistance in epithelial cells is associated with the over expression of certain MDR proteins. Numerous drug efflux pumps (P-gp, MDR-1, MRP-1 etc) present on the apical surface of the epithelial cells, are known to limit absorption of several therapeutic agents. Modest modulation in the P-gp activity was achieved by pre-incubation with potent P-gp inhibitors. Thus, the efflux of ^{3}H CsA across Calu-3 was completely inhibited by quinidine at a concentration of 75μM (Fig. 3 A and B). It was also observed that there was no significant inhibition of B-A transport of CsA across untreated cells as compared with di-OH vit D_3-treated cells with 50 μM quinidine. This may be explained by the fact that only 2-fold increase in B-A transport was observed across untreated cells as compared with A-B. Hence, the inhibition in presence of 50 μM quinidine is not significant when compared with 75 μM quinidine. The inhibition of ^{3}H CsA efflux in the B-A direction by quinidine suggests that the efflux of CsA be mainly driven by P-gp. Previous report suggested that P-gp mediated efflux could be completely inhibited by specific P-gp inhibitors like CsA (22). In present case, the CsA is used as a specific substrate for P-gp and inhibition of its efflux demonstrates the inactivation of P-gp pump. Results from P-gp induction were further validated by performing the bi-directional transport of ritonavir (Fig. 4), saquinavir (Fig. 5B), and AZT. Because both ritonavir and saquinavir are substrates for P-gp, whereas AZT is not, their transport characteristics are indicative of P-gp induction. Our studies demonstrate that there is no significant difference in AZT flux across both untreated and di-OH vit D_3-treated Calu-3 cells. Hence, induction of P-gp does not alter transport characteristics of molecules that are not substrate for P-gp.

The present study also analyzed A-B transport of ^{3}H ritonavir in presence of various P-gp substrates such as Rh-123 and testosterone. These substrates interact with ritonavir efflux by inhibiting P-gp efflux pump. Based on our results (Fig. 4), we hypothesize that since Rh-123 is a substrate for P-gp and does not inhibit the transport of other substrate (ritonavir in this case), thus was not able to significantly increase its flux (28). However, testosterone is a known inhibitor for P-gp, and hence demonstrated a significant increase in A-B transport of ritonavir (29,30). Because the HIV protease inhibitors are substrates for both CYP3A4 and P-gp, their transport can be highly influenced by the molecules capable of inducing or inhibiting CYP3A4 P-gp. Because di-OH vit D_3 induces both P-gp and CYP3A4, treated cells could be used as an *in vitro* model for both P-gp and CYP3A4 effects (27). Transcellular transport of saquinavir (A-B) was significantly increased in the presence of 0.2 mM cortisol, which is possibly caused by combined inhibition of both P-gp and CYP3A4. The contribution of P-gp inhibition by cortisol can be established easily based on the other supportive data from this report. However, the role of metabolic inactivation in increasing the saquinavir transport needs further investigation.

In conclusion, P-gp and CYP3A4 limit the transport of anti-HIV protease inhibitors across absorptive cells. This report demonstrates that induction of P-gp in Calu-3 cells by 0.25 μM di-OH vit D_3 provides a practical model to perform *in vitro* transport studies to assess drug availability to the HIV sanctuary site of lower pulmonary region. The induced cells express increased levels of P-gp whereas retaining the monolayer characteristic of the cell line. Although Calu-3 cells express different types of cytochrome enzymes (25), induction of CYP3A4 in response to di-OH vit D_3 needs further exploration. The induced cell culture model may predict the alveolar permeation rates and metabolism of anti-HIV agents thus providing a more realistic model to study the drug-drug interactions during the HAART.

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