Rapid Assessment of P-Glycoprotein Inhibition and Induction *in Vitro*

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Purpose. Using rhodamine123 (RH123) cell exclusion, 17 clinically used compounds were screened for their inhibitory effect on P-glycoprotein (P-gp), which was compared with the drugs' inhibitory activity against CYP3A4. The same assay was used to study induction of P-gp activity.

Methods. P-gp inhibition was assessed using RH123 accumulation into LS180V cells as well as Rh123 transport across Caco-2 monolayers. Inhibition of CYP3A4 was determined in human liver microsomes using triazolam-4-hydroxylation. Induction of P-gp expression and activity was measured using western blot analysis and RH123 accumulation into LS180V cells, respectively.

Results. The observed inhibition of RH123 cell exclusion ranged from little or no effect (digoxin, indinavir, fexofenadine) up to a nearly 10-fold increase in RH123 accumulation (ivermectin, terfenadine). No correlation between P-gp and CYP3A4 inhibition was observed. The rank order in P-gp inhibitory potency for terfenadine, verapamil, ritonavir, and indomethacin was identical in both LS180V and Caco-2 models. Ritonavir and St. John's wort extract showed a concentration-dependent P-gp induction, with good correlation between western blot analysis and RH123 accumulation.

Conclusions. The RH123 accumulation assay in LS180V cells can be used as a valuable screening tool to study both inhibition and induction of P-gp activity and expression. This assay has the potential to predict P-gp-mediated alterations in intestinal absorption of drugs.

KEY WORDS: P-glycoprotein; inhibition; induction; *in vitro*; LS180; screening.

INTRODUCTION

In addition to the effects of drugs on metabolizing enzymes, drug interactions with transport proteins are receiving increasing attention. The 170-kDa membrane protein P-glycoprotein (P-gp) is an ATP-dependent drug efflux pump that is constitutively expressed in several human tissues (intestinal epithelia, blood–brain barrier endothelia, liver, kidney, testes, lymphocytes; Refs. 1,2) as well as in cancer cells (3). A multitude of drugs has been identified as P-gp substrates, inhibitors, or inducers, and the rapid assessment of those characteristics of a drug or drug candidate is crucial to anticipate potentially serious drug interactions or therapy failures.

The fluorescent dye RH123 has been extensively used as an index of P-gp-mediated transport in rodent and tissue culture models. Although transport of RH123 by MRP1 has been argued, RH123 transport was unaffected by the MRP1 inhibitors probenecid and indomethacin (4) in both Caco-2 monolayers and LS180V cells, indicating a minimal contribution of MRP1 in those models. Using different cell lines, RH123 cell exclusion has also been suggested for the identification of multidrug-resistance reversal agents in chemotherapy (5,6). Additionally, a drug screen by the National Cancer Institute showed a good correlation of RH123 transport with the expression of P-gp, but not MRP, in 58 different cell lines (7).

We used LS180V cells, a P-gp-expressing human colon adenocarcinoma cell line selected with vinblastine for consistently elevated P-gp levels (4,8,9), to develop an assay for the rapid assessment of the effects of drugs on P-gp activity. Using the exclusion of the fluorescent dye rhodamine123 (RH123) from LS180V cells as an index for P-gp activity, 17 compounds were screened for their inhibitory effect on P-gp, which was compared with the drugs' inhibitory activity against CYP3A4. The same LS180V cell assay was used to study the induction of functional P-gp activity caused by chronic (3-day) exposure to the study drugs as well as the combined effect of P-gp inducing and inhibiting drugs on transporter activity.

MATERIALS AND METHODS

Chemicals

St. John's wort (Natrol Inc., Chatsworth, CA, USA) and ritonavir (RIT) were extracted into methanol from commercially available dosage forms. Purity was verified by high-performance liquid chromatography against hypericin (an active moiety within SJW, Carl Roth GmbH & Co., Karlsruhe, Germany) and RIT standard (Abbott Laboratories, N. Chicago, IL, CA). All other drugs and chemicals were purchased from commercial sources, or were kindly provided by their pharmaceutical manufacturers.

Cell Lines and Liver Microsomes

The human colon adenocarcinoma cell line LS180 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were selected for elevated P-gp levels with increasing concentrations of vinblastine (1-2-4-8 ng/mL) and maintained at 4 ng/mL until initiation of induction experiments. The selected cell line was denoted LS180V and was grown in MEM (Minimal Essential Media, GibcoBRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids (GibcoBRL, Rockville, MD, USA), 1 mM pyruvic acid, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 4 ng/mL vinblastine.

The human colon adenocarcinoma cell line Caco-2 was kindly provided by Dr. William Jefferson (Tufts-New England Medical Center and Tufts University School of Medicine, Boston, MA, USA) and used at passages 30-40. Caco-2 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium, GibcoBRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids (GibcoBRL, Rockville, MD, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

Liver samples from human donors with no known liver

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disease were provided by the International Institute for the Advancement of Medicine (Exton, PA, USA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN, USA), or the National Disease Research Interchange (Philadelphia, PA, USA). Four human livers samples, from a panel of livers, were pooled and used for all studies.

P-gp Induction

LS180V cells were seeded at $2 \times 10^4/cm^2$ in tissue-culture dishes (Corning Costar Corp., Cambridge, MA, USA). Cells were grown at 37°C, 5% CO_2 to 50% confluence (3 to 4 days), when media was replaced by vinblastine-free media containing the study drug and 0.5% DMSO. Experiments were performed in triplicate and vehicle (media with 0.5% DMSO) served as a negative control. After 72 h, cells were prepared for western blot analysis, subjected to RH123 cell exclusion experiments, or used for fluorescent microscopy as described below.

Western Blotting

Cells were lysed with 1% Triton-X and 0.5% deoxycholate, (in phosphate buffer, pH 7.4, 1 mM phenylmethylsulfonyl fluoride and centrifuged (1000 g). The supernatant was collected, and the total protein concentration was determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples were separated at 4°C by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 4–15% gradient polyacrylamide gel and immunoblotted using monoclonal mouse anti-human MDR1 (C219) with sheep anti-mouse Ig as the secondary antibody. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to activate the horseradish peroxidase signal. Blots were exposed to radiographic film and quantified via computer image analysis (Scion Image, Scion Corp., Frederick, MD, USA). Because a pure P-gp standard that comigrates at the established of molecular weight of 170-180 kDa is currently not available, highly induced LS180V cell preparations were used to generate a standard curve (Fig. 4A), which allowed comparison of relative P-gp contents between samples. Intrablot variability was 7.1%, and interday variability was 17.4%. Ritonavir (10 μM), previously established as a P-gp inducer (4,9), was used as a positive control inducer in all studies.

Drug Accumulation Studies

LS180V cells were preincubated for 30 min with media containing the study drug or media (0.5% DMSO) alone. RH123 solution was then added to each well such that they contained 10 μM RH123 and 1% methanol. After incubation for 60 min, the cells were thoroughly washed five times with warm media and solubilized with 0.5% deoxycholate and 1% Triton X. The supernatant from each well was analyzed for RH123 fluorescence and total protein, which served as an additional measure (aside from microscopic cell evaluation) of comparable cell density and viability. The interday variability of RH123 accumulation was 14.2%. While there was interday variability in absolute RH123 accumulation values in inhibition experiments, the relative rank orders were unchanged. In all studies, ritonavir (10 μM) was used as a positive control inducer. Verapamil (10 μM) was used as a positive control inhibitor.

Fluorescence Microscopy

After the drug accumulation experiment described above, the cells were not lysed but subjected to epifluorescence microscopy using a Nikon Optishot microscope. RH123 was visualized using an FITC filter set (ex: 450-490, em: 510-530). Images were captured using a Photometric Quantix Digital Camera and V++ software (Digital Optics, Auckland, New Zealand). Incandescent images of the same fields were taken to demonstrate similar cell density.

Inhibition of CYP3A4-Mediated Metabolism of TRZ in Human Liver Microsomes

All incubation, inhibition procedures and TRZ analysis were performed as previously described (10). The inhibition profile of each drug was tested at 10 μM with 250 μM TRZ substrate. The interday variability for inhibition of TRZ hydroxylation (using 1 μM RIT as a control) was 4.9%.

Inhibition of P-gp-Mediated Transport in Caco-2 Cell Monolayers

Caco-2 cells were seeded at $2 \times 10^4/cm^2$ in polycarbonate membrane transwell plates (2.5 cm diameter, 3-μm pore size; Corning Costar Corp., Cambridge, MA, USA). Cells were grown at 37°C, 5% CO_2 with media changes every 3–5 days. Transport experiments were conducted on day 18–24 post-seeding. Transepithelial electrical resistance was >16000 Ω/cm^2, confirming high monolayer confluence. Drug solutions were prepared in Opti-MEM (GibcoBRL) serum free media containing 0.5% DMSO and 0.5% methanol. Inhibition of RH123 transport was tested with the inhibitor (10 μM) present in both chambers and RH123 (5 μM) present in either the apical or basolateral chamber. After 120 min incubation, aliquots were removed from the chamber initially not containing RH123 and were subjected to fluorescence analysis at 500/550 nm (excitation/emission). Caco-2 cells were viable and the monolayer stayed functional during the course of the experiment as demonstrated by the linearity of RH123 B to A transport between 60 min and 240 min ($\mathbf{r}^2 = 0.988$).

RESULTS

Fluorescence Microscopy

The measure of P-gp activity in this cell accumulation assay is the intracellular concentration of the P-gp substrate RH123. Accordingly, inhibition of P-gp activity results in increased intracellular RH123 concentrations reflected by higher fluorescent signals (Fig. 1AI and 1BI) compared to media treated control cells (Fig. 1AII and 1BII). In contrast, induction of P-gp activity causes enhanced efflux of RH123 leaving intracellular RH123 concentrations below control values (Fig. 1AIII and 1BIII).

P-gp Inhibition

Using the RH123 cell exclusion assay, 17 clinically used drugs were screened for their inhibitory effect on P-gp activity. The observed inhibition of RH123 cell exclusion (Fig. 2A)

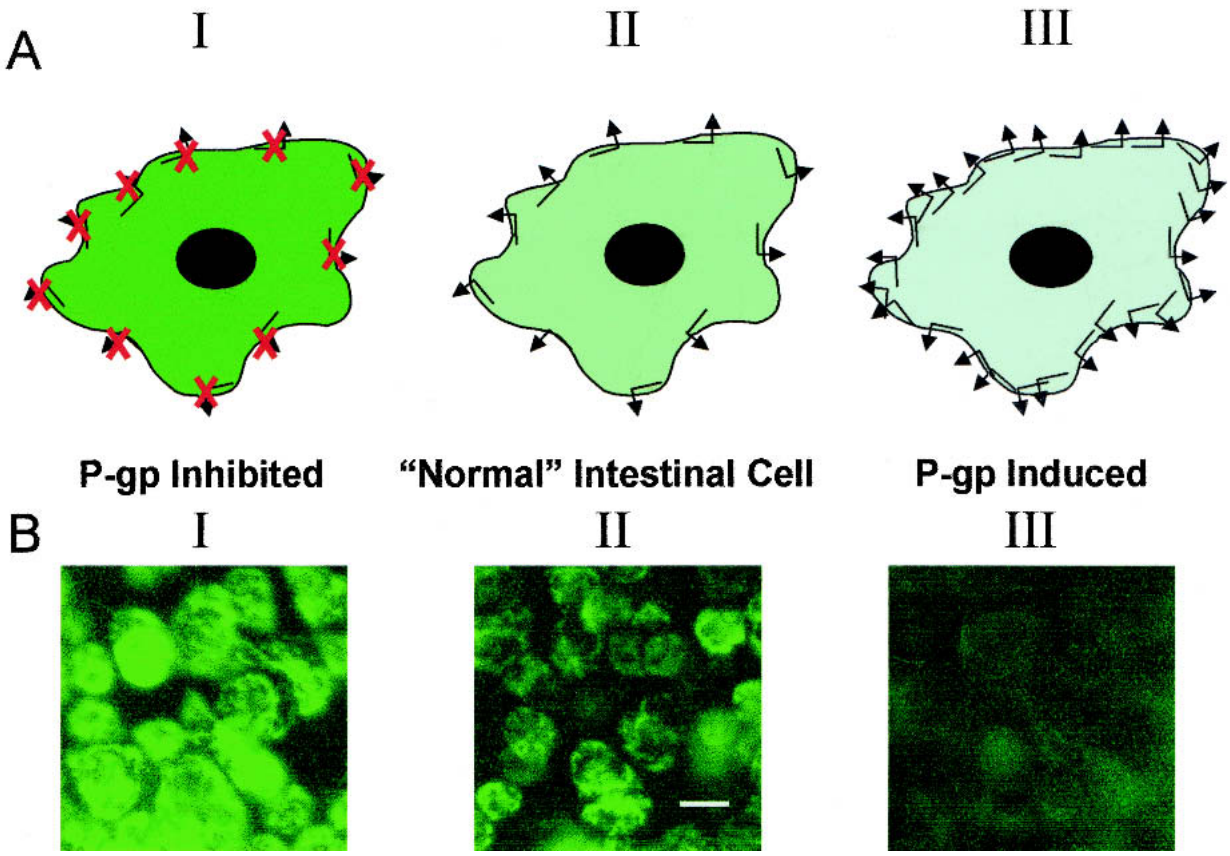

Fig. 1. RH123 cell accumulation schematic and microscopy. A, Intestinal cells with inhibited P-gp accumulated more RH123 (A.I) than vehicle controls (A.II). Conversely, intestinal cells with induced P-gp expression showed higher transporter-mediated cell exclusion of RH123 (A.III). B, RH123 cellular accumulation in LS180V cells was visualized by epi-fluorescence microscopy using an FITC filter set. Increased RH123 accumulation was evident after 1 h of acute exposure to VER (100 μM) treatment (B.I) compared with control cells exposed to vehicle alone (B.II). In contrast, 3 days of exposure to RIT (10 μM) resulted in decreased RH123 accumulation (B.III). Each image was acquired under identical conditions at 200× magnification. Incandescent light images of each field verified comparable cell density. Representative fields are shown. Scale bar = 25 μ.

ranged from little or no effect (digoxin, midazolam, indinavir, fexofenadine) over moderate inhibition, leading to a 2- to 5-fold increase in intracellular RH123 (ritonavir, quinidine, verapamil), up to very potent inhibition associated with an almost 10-fold increase in RH123 accumulation (ivermectin, terfenadine).

Four compounds representing a range of P-gp inhibitory potency in LS180V cells showed the same rank order in the inhibition of RH123 transport across Caco-2 monolayers with terfenadine (most potent inhibitor) > verapamil > ritonavir > indomethacin (Fig. 3A and B).

CYP3A4 Inhibition

The inhibitory activity of the same 17 study compounds on triazolam-4-hydroxylation in human liver microsomes ranged from <10% (digoxin, fexofenadine, ivermectine) up to almost 100% inhibition (ritonavir, indinavir; Fig. 2A). Although some overlap between P-gp and CYP3A inhibitory activity exists, a general correlation was not apparent (Fig. 2B).

P-gp Induction

Ritonavir and St. John's wort extract caused a significant increase in both P-gp expression (Fig. 4A) and P-gp activity (Fig. 4B) in LS180V cells after 3-day exposure. The effect was concentration dependent and showed an excellent correlation between western blot analysis and cell exclusion assay (Fig. 4C). The positive control (ritonavir, 10 μM) consistently produced an increase in P-gp expression of 4-fold, with an interday variability of 15%. Ritonavir likewise increased RH123 cell exclusion by 50% with an interday variability of 6.6%.

DISCUSSION

RH123 accumulation into LS180V cells was used as an assay for P-gp inhibition and induction in the screening of 17 clinically used drugs. The most potent P-gp inhibitors were ivermectin and terfenadine, causing a 9-fold increase in RH123 accumulation compared to control cells (Fig. 2A). The screening concentration of 10 μM was chosen based on concentration-response experiments (data not shown) as a concentration where potent inhibitors begin to plateau, while modest inhibitors show an effect. Since Caco-2 cell monolayers are a frequently used model for drug transporter studies (11), we compared the rank order of P-gp inhibition between LS180V and Caco-2 cells. Four compounds representing a range of P-gp inhibitory potency in LS180V cells showed the same rank order in both models with terfenadine (most potent inhibitor) > verapamil > ritonavir > indomethacin (Fig.

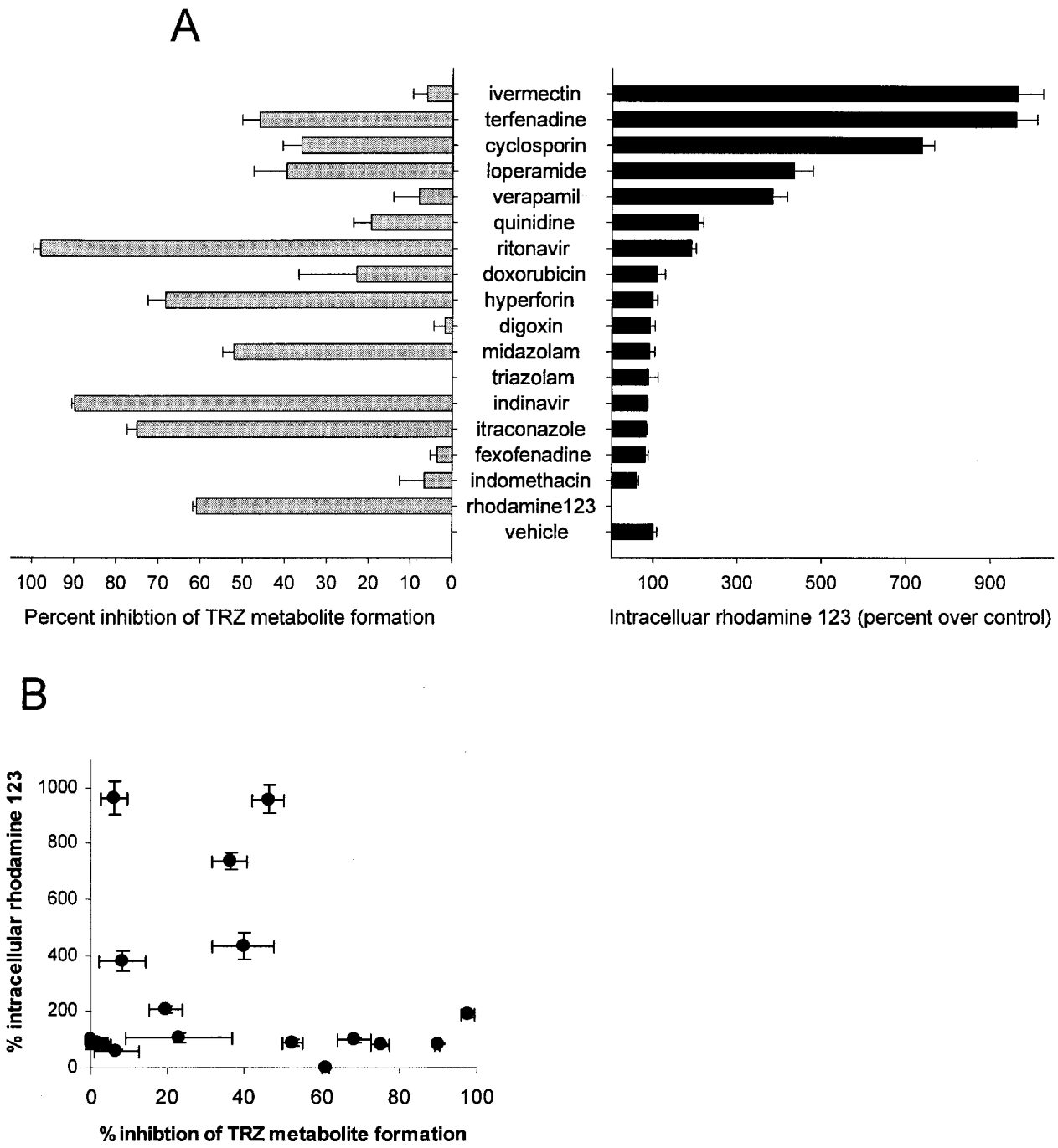

Fig. 2. P-gp and CYP3A inhibition by 17 drugs. A, Comparison of P-gp and CYP3A inhibition by 17 drugs at 10 μM. Inhibition of P-gp activity was assessed using RH123 drug accumulation into LS180V cells (right panel). CYP3A inhibition was determined in human liver microsomes using triazolam-4-hydroxylation as an index reaction (left panel). B, Scatter plot comparison of P-gp and CYP3A inhibition (the data presented in panel A) by 17 drugs at 10 μM. Bars (A) and data points (B) represent the mean ± SD (n = 3).

3A and B). Using the relative ratio to verapamil inhibition as a means to compare different studies, this rank order also matches the literature (12–14).

For a number of compounds, the effects on P-gp mediated RH123 transport parallel their effects on CYP3A (9,15,16). In an approach similar to that of Wandel *et al.* (17), we tested the inhibitory activity of the 17 study compounds on triazolam-4-hydroxylation in human liver microsomes as an index of CYP3A activity (Fig. 2A). Although several P-gp inhibitors were potent inhibitors of CYP3A (e.g., terfenadine, cyclosporin. loperamide), some did not substantially affect CYP3A (e.g., ivermectin), and others potently inhibited CYP3A without affecting P-gp activity (e.g., indinavir, itraconazole). This finding confirms results in other cell lines showing that variability of inhibition potency and dissociation of inhibitory activity for a given drug is common (14) and emphasizes the need for a rapid and simple screening technique for the assessment of P-gp inhibition.

In addition to its properties as a P-gp substrate and its potential to inhibit P-gp activity, another factor contributing

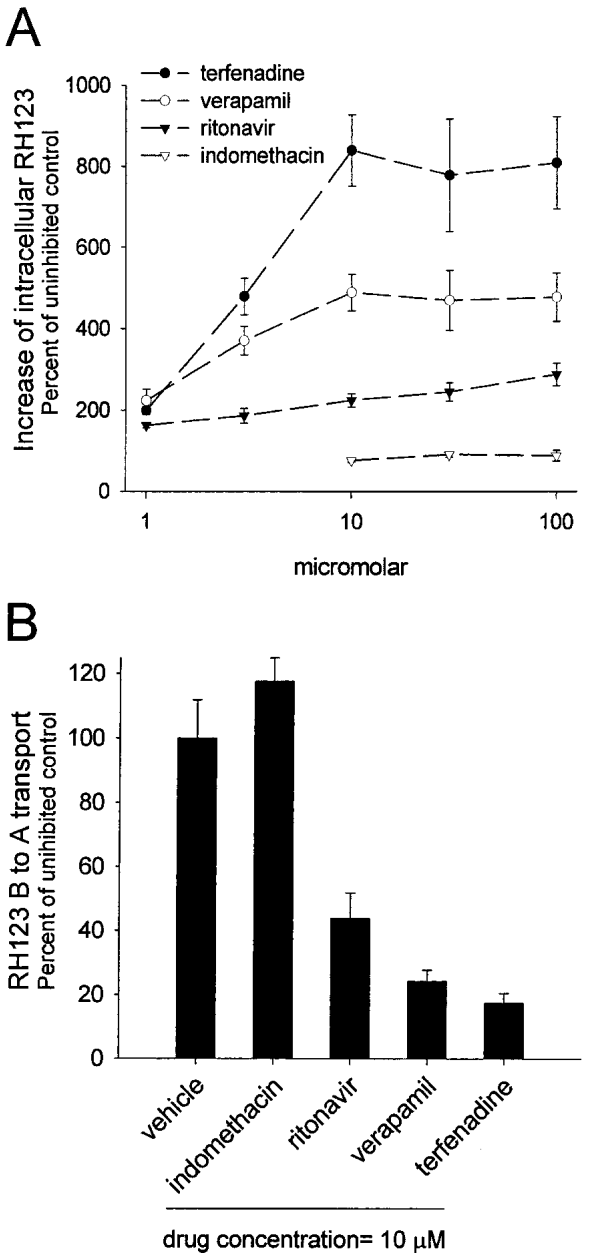

Fig. 3. Comparison between LS-180V cells and Caco-2 monolayers as a model for P-gp inhibition. A, Inhibition of RH123 LS180V cell exclusion in relation to concentration of the index drugs terfenadine, verapamil, and ritonavir (1, 3, 10, 30, and 100 μM) and negative control indomethacin (10, 30, and 100 μM) was assessed by comparison to vehicle control (0.5% DMSO). Data represent intracellular drug concentration as a percent of the uninhibited vehicle control (0.5% DMSO). B, Inhibition of RH123 transport across Caco-2 monolayers by the same four index drugs (10 μM) was assessed by comparison to vehicle control. Data represent drug transport from the basolateral to the apical chamber as a percent of the uninhibited control. Data points (A) and bars (B) represent the mean ± SD (n = 3).

to P-gp interactions of a drug is its ability to cause induction of P-gp expression after chronic exposure, an effect previously studied in LS180V cells (4,9,18–20). In the clinical setting, this effect has been verified in the intestine, where induction of P-gp activity can reduce the bioavailability of

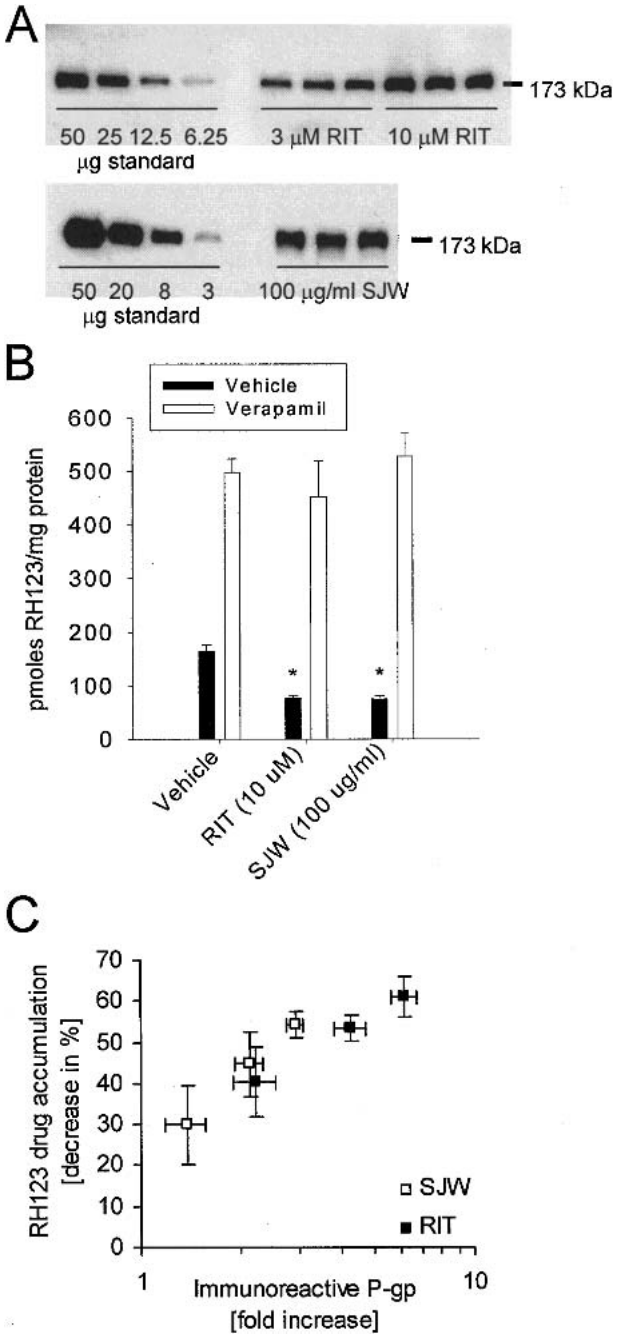

Fig. 4. RH123 cell accumulation and quantitative western blotting as an index of P-gp induction LS180V cells were incubated with vehicle alone (0.5% DMSO), or with St. John's Wort (SJW; 10, 30, 100 μg/ml) or ritonavir (RIT; 3, 10, 30 μM). A, After a 72-h incubation, immunoreactive P-gp was quantified by western blotting and computer image analysis. B, Alternatively, cells were incubated with 10 μM RH123 and analyzed for intracellular drug concentrations. Control experiments under the same conditions were performed using acute exposure to 100 μM VER. Bars represent the mean ± SD (n = 3). Asterisks indicate a significant difference compared to the vehicle control ($p < 0.05$, analysis of variance with Dunnett's *t* test). C, Immunoquantified P-gp and RH123 cell accumulation for RIT and SJW was concentration dependent and showed a log-linear correlation. Mean immunoreactive protein (± SD; n=3) is expressed as a relative increase over vehicle control.

orally administered drugs that are P-gp substrates (21–23). In the intestine, concentrations achieved after oral administration of most drugs will considerably exceed plasma concentrations and may reach high micromolar or even millimolar levels. While induction studies have been less successful with Caco-2 cells because of the late onset of P-gp expression in this cell line (24), LS180V cells are suitable for studies of both inhibition and induction of P-gp. Accordingly, we exposed LS180V cells to increasing concentrations of two P-gp inducers, ritonavir (4) and St. John's wort extract (18), for 3 days. The observed effect was concentration dependent, and there was a high correlation between results of western blot analysis and cell exclusion assay (Fig. 4C).

Considering that the treatment of multiple conditions (e.g., antiretroviral therapy, immunosuppressant treatment, cancer chemotherapy) currently requires a complex regimen of combination drug therapy, the need for an *in vitro* assay modeling the effects of combined exposure to both P-gp-inducing and P-gp-inhibiting drugs is evident. Using non-nucleoside HIV-1 reverse transcriptase inhibitors, we have recently demonstrated that this RH123 cell accumulation assay in LS180V cells can be used to elucidate such combined effects that result from chronic exposure to P-gp inducing drugs followed by acute exposure to P-gp inhibitors (9).

In summary, the present *in vitro* assay uses the human intestinal cell line LS180V to assess the effects of drugs on functional P-gp activity in order to anticipate P-gp mediated alterations. Data generated could be used to predict changes in intestinal drug absorption and potentially other effects on drug bioavailability (e.g. CNS uptake and renal excretion). The assay has two distinct advantages over previously used approaches: the use of the same cell line for both inhibition and induction studies, and the incorporation of an activity assay for P-gp induction. Unlike the frequently used cell monolayers, the LS180V model is suitable for studies of acute inhibition, chronic induction, or combinations of both and therefore allows for an *in vitro* modeling of combination drug treatment. While previous studies have focused on western blot analysis of induced P-gp expression (8,19), the LS180V cell accumulation assay provides a direct assessment of functional P-gp activity while the option for additional western blot analysis still exists. Additionally, LS180V cells require only 3–5 days between seeding and initiation of experiments, while Caco-2 monolayers take about 3 weeks to reach confluence. If scaled up to 96-well plates, combined with automated sample handling equipment and a plate reading fluorimeter, the assay could generate an extensive database of valuable information on potential P-gp related interactions of drugs or drug candidates.

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