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Binary and ternary combinations of anti-HIV protease inhibitors: effect on gene expression and functional activity of CYP3A4 and efflux transporters

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Abstract

Background—The purpose of this study is to identify the effect of binary and ternary combinations of anti-HIV protease inhibitors (PIs) on the expression of metabolizing enzyme (CYP3A4) and efflux transporters [multidrug resistance-associated protein 2 (MRP2), P-glycoprotein (P-gp) and breast cancer resistant protein (BCRP)] in a model intestinal cell line (LS-180).

Methods—LS-180 cells were treated with various combinations of PIs (amprenavir, indinavir, saquinavir and lopinavir), and the mRNA expression levels of metabolizing enzyme and efflux

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transporters were measured using quantitative reverse transcription polymerase chain reaction. The alteration of gene expression was further correlated to the expression of nuclear hormone receptor PXR. Uptake of fluorescent and radioactive substrates was carried out to study the functional activity of these proteins. Cytotoxicity and adenosine triphosphate (ATP) assays were carried out to measure stress responses.

Results—Binary and ternary combinations of PIs appeared to modulate the expression of CYP3A4, MRP2, P-gp and BCRP in a considerable manner. Unlike the individual PIs, their binary combinations showed much greater induction of metabolizing enzyme and efflux proteins. However, such pronounced induction was not observed in the presence of ternary combinations. The observed trend of altered mRNA expression was found to correlate well with the change in expression levels of PXR. The gene expression was found to correlate with activity assays. Lack of cytotoxicity and ATP activity was observed in the treatment samples, suggesting that these alterations in expression levels were probably not stress responses.

Conclusions—In the present study, we demonstrated that combinations of drugs can have serious consequences toward the treatment of HIV infection by altering their bioavailability and disposition.

Keywords

binary combinations; drug interactions; drug metabolism; efflux transporters; protease inhibitors; ternary combinations

Introduction

The introduction of highly active antiretroviral therapy (HAART) diminished the mortality rate of human immunodeficiency virus (HIV)-infected individuals besides a greater improvement in the quality of life. HIV protease inhibitors (PIs), which are potent antiretroviral agents, still remain a vital constituent of HAART. PIs prevent viral replication by inhibiting the activity of HIV-1 protease, an enzyme used by viruses to cleave nascent proteins for final assembly of new virions. Several PIs and their combinations are widely indicated in the treatment of AIDS, comprising a multidrug therapy regimen owing to the risk of developing drug-resistant mutated viruses. However, treatment options vary with individuals and remain very complex. Multidrug therapy is often associated with severe drug-drug interactions leading to potential adverse reactions resulting in treatment failure [1, 2]. These effects are mainly attributed to the development of viral [3] and cellular resistance [4, 5]. Medications alone or in combinations indicated in the treatment of AIDS are usually administered orally and are absorbed through the gastrointestinal tract. Oral administration achieves relatively high concentrations of drugs exposed to the intestinal lumen, causing potential drug-drug interactions.

The bioavailability of PI combinations following oral administration is severely affected by their ability to either inhibit or induce the activity/expression of efflux pumps and metabolizing enzymes. The intestine is a site of expression of CYP450s and multiple efflux transporters. Efflux pumps, i.e., P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistant protein (BCRP), along with metabolizing

enzyme (CYP3A4) have been reported to be responsible for alteration in drug bioavailability due to altered pharmacokinetics [6–8]. This can cause suboptimal drug concentrations in plasma and poor penetration into the intracellular compartment, resulting in reduced antiviral activity [9].

In general, drug-drug interactions arise when the disposition of one drug is altered by the other [1, 2]. Such interactions may generate additive, synergistic, or antagonistic effects depending on the combinations [1]. Clinicians are often required to adjust dosage owing to PIs being substrates, inducers or inhibitors of metabolizing enzymes and efflux transporters [10]. Appropriate dose adjustment or choice of a relatively safer combination can produce better therapeutic efficacy without causing toxicity. The drug combination may have different pharmacokinetic interaction when coadministered in short-term. However, different combinations may generate induction of metabolizing enzyme and efflux transporter to different extents when coadministered for extended periods of time than what is observed when the drugs are administered individually [1]. Thus, many of the existing interactions when conducted clinically turn out to be often inconclusive with results that are difficult to predict. Additionally carrying out clinical studies for drug interactions end up being very expensive as the number of possible combinations is very large. Delineation of drug-drug interactions through clinical pharmacokinetic studies involves substantial cost, time and exposing human volunteers to a high degree of risk. Therefore, there is a severe need to delineate drug-drug interactions among various PIs *in vitro* and predict *a priori* clinical drug interactions.

However, lack of a well-characterized cell line model to examine the expression of efflux transporters and metabolizing enzymes still poses a challenge in predicting *in vivo* drug-drug interactions. LS-180 cells have been employed to study the induction of these transporters because of the higher, natural abundance of PXR in LS-180 compared to Caco2 cells [11,12]. Because the efflux proteins and metabolizing enzymes work in tandem to keep xenobiotics out of cells, the effect of the binary and ternary combinations of PIs on these proteins is crucial in preventing potential drug interactions and therapeutic failures [1, 13]. A large number of binary and ternary combinations of drugs can be studied together for their induction potential and a theoretical list of possibly safe clinical combinations of PIs can be generated. Therefore, the objective of the present study was to observe the effect of binary and ternary combinations of anti-HIV PIs on the expression of efflux transporters (P-gp, MRP2 and BCRP) and metabolizing enzyme (CYP3A4) in a model intestinal cell line (LS-180). This study is anticipated to provide a database for clinicians in selecting drug combinations to allow better outcome with minimal side effects.

Materials and methods

PIs amprenavir, saquinavir and lopinavir were generous gifts from (GlaxoSmithKline, Brentford, Middlesex, UK), (Roche, Basel, Switzerland) and (Abbott Laboratories, Chicago, IL, USA), respectively. Indinavir was purchased from Fisher Scientific (Fair Lawn, NJ, USA). LS-180 cells were obtained from American Type Culture Collection (Manassas, VA, USA). [¹⁴C]Erythromycin (specific activity: 51.3 mCi/ mmol) was procured from Moravek Biochemicals (Brea, CA, USA). TrypLE™ Express, Dulbecco's modified Eagle's medium

(DMEM), Vivid® CYP450 Screening Kit and ATP determination kit were obtained from Invitrogen (Carlsbad, CA, USA). OligodT, dNTP, MgCl₂, M-MLV Reverse Transcriptase and CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) were purchased from Promega Corporation (Madison, WI, USA). LightCycler 480® SYBR I Green Master Mix was obtained from Roche Applied Science (Indianapolis, IN, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Culture flasks (75 cm² growth area), 12-well plates (3.8 cm² growth area per well) and 96-well plates (0.32 cm² growth area per well) were purchased from Corning Costar Corp. (Cambridge, MA, USA). All other chemicals were products of commercial grade and were purchased from Sigma Chemicals (St. Louis, MO, USA) and Fisher Scientific.

Cell culture

LS-180 cells were used for all the molecular expression and functional activity studies. Cells were cultured in T-75 flasks at 37°C in a humidified atmosphere using 5% CO₂ and were maintained with DMEM supplemented with 10% FBS (heat inactivated), 1% nonessential amino acids, 20 mM HEPES, 29 mM sodium bicarbonate and 100 µg/mL of penicillin and streptomycin each. The medium was changed every alternate day. Cells were allowed to reach 80%–90% confluence, which was confirmed with a microscope, and then passaged using TrypLE™ Express solution. Cells were seeded at a density of 250,000 cells/well in 12-well tissue culture treated plastic plates and at a density of 10,000 cells/well in 96-well plates. These cells were then allowed to grow for 5–7 days and used for further studies.

Preparation of drug solutions and drug treatment on LS-180 cells

Individual PI stocks were prepared in dimethyl sulfoxide (DMSO) and sterile filtered through 0.22-µm filters. Medium containing drugs either individually or for binary and ternary combinations was then prepared by spiking appropriate amounts from the respective drug stocks just before initiating the treatment. Cells were treated for 5 consecutive days with the following drugs alone and in combination (binary and ternary combinations used are summarized in Figure 1): A (amprenavir), I (indinavir), S (saquinavir), L (lopinavir), AI (amprenavir+indinavir), AS (amprenavir+saquinavir), LI (lopinavir+indinavir), SL (saquinavir+lopinavir), AL (amprenavir+lopinavir), ALI (amprenavir+lopinavir+indinavir), ASI (amprenavir+saquinavir+indinavir), ASL (amprenavir+saquinavir+lopinavir), SLI (saquinavir+lopinavir+indinavir). Medium containing drugs was changed every day in all the studies performed. The final concentration of DMSO in all the drug solutions did not exceed 0.5% (v/v). The drug concentration of the single PI solutions was 10 µM and net concentrations for the combinations were additive, i.e., 20 µM for binary combination and 30 µM for ternary combinations. [¹⁴C]Erythromycin was selected as a model substrate to study MDR1- and MRP2-mediated efflux [14–16].

Quantitative gene expression analysis using real-time reverse transcription polymerase chain reaction

Total RNA was isolated from treated cells using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was quantified by Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). Two micrograms of total RNA was added to 1.25 µL of oligodT primer and denatured at 70°C for 10 min. After denaturation, it was reverse

transcribed using M-MLV Reverse Transcriptase. Quantitative real-time PCR was conducted using LightCycler SYBR Green technology (Roche) using cDNA equivalent to 80 ng of RNA. PCR products were subjected to a melting curve analysis to confirm the PCR specificity. Primers were designed using PRIMER BLAST tool through PubMed. All the primers were designed such that the amplicons generated will be 50–150 bp long to increase the efficiency of simultaneous amplification of target and reference genes. The specificity of the target amplicon was tested by melting curve analysis. The primer sequences are summarized in Table 1. Quantitative values were obtained above the threshold PCR cycle number (CT) at which the increase in signal associated with an exponential growth for PCR products is detected. The relative mRNA levels in each sample were normalized according to the expression levels of glyceral-dehyde 3-phosphate dehydrogenase (GAPDH), which was used as an internal control. Quantitative real-time PCR was carried out to study the alteration in the expression of metabolizing enzyme (CYP3A4) and efflux transporters (P-gp, MRP2 and BCRP) and nuclear hormone receptors (PXR) after treatment with PI combinations in LS-180 cells. Induction levels of each test gene were normalized to GAPDH according to the following formula: $CT_{\text{Test Gene}} - CT_{\text{GAPDH}} = CT$. Thereafter, the relative mRNA induction levels of each gene were calculated using the CT method:

$CT_{\text{Test Gene}} - CT_{\text{Test Gene in DMSO control}} = CT_{\text{Test Gene}}$. The Test Gene Test Gene in DMSO control Test Gene fold changes of mRNA levels were expressed as the relative expression 2^{-CT} [12].

VIVID™ assay

The functional activity of metabolizing enzyme (CYP3A4) in LS-180-treated cells was assayed using VIVID™ CYP3A4 Red Substrate (Invitrogen), which was prepared as a 20 mM stock solution in acetonitrile. Briefly, cells were plated in 96 wells and treated as described earlier. After 5 days of treatment, CYP3A4 activity assay was performed after lysing the cells and reagents were added according to the manufacturer's protocol. Fluorescent readings were monitored kinetically at 37°C over a period of 30 min using a 96-well plate reader (SpectraFluor Plus, Maennedorf, Switzerland) at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. Activity was measured as the rate of fluorescent metabolite production from the metabolism of a Vivid® CYP450 substrate over the course of the reaction for all the drug combinations tested.

Uptake studies

Uptake of [¹⁴C]erythromycin was performed according to a previously published procedure [14] to correlate the gene expression levels to that of functional activity. Briefly, cells were plated in 12-well tissue culture treated plates and treated every day. Upon terminating treatment after 5 days, medium containing drugs was aspirated from wells and cells were washed (3×10 min) with 2 mL of Dulbecco's phosphate-buffered saline (DPBS) containing 130 mM NaCl, 2.5 mM KCl, 7.5 mM Na₂HP₄ 1.5 mM KH₂PO₄, 1 mM CaCl₂ 0.5 mM MgSO₄, 5 mM glucose, and 20 mM HEPES at pH 7.4. Then, 1 mL of DPBS containing [¹⁴C]erythromycin (0.25 µCi/mL) was added to the cells in each well and incubated at 37°C for 30 min. Solutions were removed and uptake was terminated with ice-cold stop solution (200 mM KCl and 2 mM HEPES). Later, cells were lysed overnight at room temperature with 1 mL of 0.1% (v/v) Triton-X in 0.3 N sodium hydroxide solution. Aliquots (500 µL) of

the cell lysate were then added to scintillation vials containing 3 mL of scintillation cocktail (Fisher Scientific) and radioactivity was measured with a scintillation counter (Model LS-6500; BeckmanCounter, Fullerton, CA, USA) with an efficiency of 95% to measure [¹⁴C] compounds. Uptake in each well was normalized to protein content, estimated by the Bio-Rad protein estimation kit using bovine serum albumin (BSA) as the standard.

Cell viability assay

Cell viability assay was performed to examine the toxicity of PIs, and their binary and ternary combinations used in this study. For this assay, CellTiter 96 AQueous Non-radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA) was used, which constitutes a colorimetric method for determining the number of viable cells in culture. Briefly, cells were seeded onto 96-well plates for the assay and the treatment was initiated after 24 h of seeding and continued for 5 days. One hundred microliters of respective drug solution/combinations was added to each well to evaluate the cytotoxic effect of the drug combinations tested. Medium was replaced by freshly prepared drug solutions every day for 5 days and the assay was performed according to the manufacturer's directions. Cell viability was measured as the rate of fluorescent metabolite production (color formation) over the course of the reaction, which is proportional to the number of viable cells present. Color determination (absorbance) was then measured at 485 nm (reference at 590 nm) using a 96-well microtiter plate reader (SpectraFluor Plus).

ATP determination assay

The cells were seeded in 96-well plates at a density of 10,000/well and treatment was initiated with 100 µL of the respective PIs and their binary and ternary combinations. Subsequently, upon termination of the treatment after 5 days, medium containing drug solutions was removed and the cells were washed with DPBS (3×10 min). Lysis solution (100 µL) was then added and the lysate was further utilized for the quantitative detection of ATP using ATP Determination Kit (Molecular Probes, Invitrogen) following the manufacturer's instructions. Ouabain (1 mM), sodium azide (1 mM) and 2,4-dinitrophenol (1 mM) served as ATP inhibitors. Luminescence was then measured using a microtiter plate reader (SpectraFluor Plus) and it was normalized to protein content. It was estimated by the Bio-Rad protein estimation kit using BSA as the standard.

Results

Expression of CYP3A4 and efflux transporters mRNA in LS-180 cells

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was carried out to study the changes in expression levels of CYP3A4 and efflux transporters (P-gp, MRP2 and BCRP) mRNA following different treatments with PI combinations in LS-180 cells. Binary and ternary combinations of PIs appeared to modulate the expression of various efflux transporters and CYP3A4 in a considerable manner. Unlike the individual PIs, binary combinations showed much greater induction of the gene expression (5- to 15-fold) which was not found to be as pronounced in the presence of their ternary combinations (1- to 4-fold). This trend was observed to be similar for all the mRNA tested (Figure 2A–D).

The fold induction for each drug combination and gene was compared to that of cells exposed to DMSO.

Expression of nuclear hormone receptor mRNA in LS-180 cells

qRT-PCR analysis was carried out to study the alteration in the expression of nuclear hormone receptor (PXR) mRNA after treatment with PI combinations in LS-180 cells. This was performed to correlate the induction of nuclear hormone receptors with the levels of gene expression of efflux transporters and metabolizing enzymes. The induction levels of PXR corroborated well with the expression levels of CYP3A4 and efflux transporters as observed in gene expression studies. Binary combinations showed much greater induction of the gene expression of PXR when compared to individual PIs or their ternary combinations (Figure 3). Similar to what has been observed in previously published reports [12], CAR was found to be noninducible in LS-180 cells (data not shown).

VIVID™ metabolism assay for CYP3A4 functional activity

To study whether the change in CYP3A4 mRNA expression translates into functional activity, the activity of CYP3A4 enzyme was measured under similar treatment conditions by VIVID™ CYP3A4 assay kit containing red fluorescent substrate. Fluorescent metabolite formation was found to be significantly higher in cells treated with binary combinations of PIs as compared to individual PIs or their ternary combinations (Figure 4). Highest CYP3A4 activity was observed in the AS combination. Despite a multifold increase in mRNA levels, PI treatment regimens did not translate into similar levels of functional activity.

[¹⁴C]Erythromycin uptake in LS-180 cells

Erythromycin being a known substrate of P-gp and MRP2 [14], [¹⁴C]erythromycin uptake assay was performed in the cells treated with various PI combinations. Significant reduction in erythromycin uptake was observed in cells treated with all binary combinations of PIs except SL combination. There was no significant reduction in uptake upon treatment with either individual or ternary combinations of PIs except in the case of amprenavir alone or the ternary combination of saquinavir, lopinavir and indinavir (Figure 5).

Cell viability (MTT) assay

MTT assay was performed to study the cytotoxicity of individual PIs and their combinations. None of the combinations except binary combination SL and ternary combination SLI produced any significant toxicity at the concentrations utilized (Figure 6). However, the ternary combination of SLI appeared to be lethal in these drug combinations.

ATP activity assay in LS-180 cells

ATP activity assay was performed in LS-180 cells after PI combination treatment. There was no significant difference in ATP activity upon PI treatment regimens with either individual or binary combinations of PIs when compared to vehicle control. However, a significant rise in ATP activity was observed in cells treated with some ternary combinations (ALI, ASI, ASL) (Figure 7). The ATP activity trend did not correlate well with the efflux transporter activity observed in previous studies.

Discussion

LS-180 cells have been shown to be a good model for predicting long-term drug-drug interactions on the basis of inductions of efflux transporter and metabolizing enzymes [17,18]. Our long-term goal is to further validate it as a model cell line for studying potential drug interactions for chronic multidrug treatment regimens. Previous studies have shown that individual PIs are inducers of metabolizing enzymes and efflux transporters [12, 17] and that LS-180 cells can be effectively used to study this induction potential. Due to high occurrence of drug resistance, antiretroviral drugs including PIs are generally given in binary or ternary combinations to overcome potential therapeutic failure. It is well known that individual PIs can induce various efflux transporters and metabolizing enzymes. Our study indicates an altered induction potential when given in various combinations. This is the first time that binary and ternary combinations of PIs are being studied for their effect on the expression and activity of various efflux transporters and metabolizing enzymes.

PIs have been shown to induce multiple enzymes of the cytochrome P450 family [19, 20]. For the present studies, only CYP3A4 expression and activity was tested because LS-180 cells do not induce other CYP enzymes significantly even in the presence of known inducers [12]. A, S, L and I were all previously reported to be inducers of CYP3A4 in LS-180 cells at 10 μ M concentration; however, at present, only A and S showed significant induction. The reason for nonresponse to other PIs may be related to the culture condition and duration of the treatment period. All five binary combinations tested in our studies showed much higher induction of CYP3A4 as compared to individual PIs. Despite LI combination showing more than 5-fold induction, it was still the weakest inducer among all binary combinations tested. AI combination resulted in the most statistically significant induction potential among all the combinations studied. The significant elevation in levels of induction cannot be attributed only to the additive response to increased levels of PIs. Apparently, all ternary combinations displayed 3- to 5-fold induction; surprisingly this induction potential for all ternary combinations was relatively smaller than that of binary combinations (Figure 2A), further confirming that the induction levels do not represent an additive response.

Among all the binary combinations tested AI, AL and LI demonstrated significant induction for all the efflux transporters. However, AI combination was found to be the most potent inducer for *MRP2* and *BCRP* genes (Figure 2B and D). Interestingly, AL combination caused the highest induction potential for CYP3A4 and P-gp (Figure 2A and C). Surprisingly, none of the individual PIs or their ternary combinations demonstrated any significant induction of *MRP2* in LS-180 cells, yet all of the binary combinations (except AS) showed very high induction potential for *MRP2* (Figure 2B). At the concentration tested, A produced very high induction of P-gp along with the three previously described binary combinations (Figure 2C). Conversely, ASI, ALI and SL combinations indicated a noticeable reduction of P-gp expression. In the case of *BCRP*, A, AI, AL, LI and SLI demonstrated significant induction and L showed significant inhibition at the tested concentrations (Figure 2D).

Several nuclear hormone receptors and transcriptionally mediated pathways have been reported to be responsible for chemically induced elevated expression of various proteins in

response to xenobiotic exposure. PXR and CAR have been especially reported to play a significant role in the regulation of expression levels of multiple efflux transporters and metabolizing enzymes [21–23]. Previous reports have shown that the expression levels of PXR correlate well with the expression levels of metabolizing enzymes both *in vitro* and *in vivo*. Recent studies have also shown that flavonoids can induce mRNA and protein levels of PXR and can play an important role in regulation of its downstream proteins [24]. Induction of mRNA levels of PXR upon similar treatments with PI combinations was also tested. PXR induction levels (Figure 3) were found to correlate to some extent with the induction levels of efflux transporters and metabolizing enzymes (Figure 2A–D). The PXR expression levels in response to various PIs along with combinations were strikingly very similar with P-gp expression except amprenavir-mediated induction. These results clearly indicate that PXR controls P-gp expression. Previous reports have suggested that LS-180 cells may not be the best cell line to study CAR-mediated induction of genes [12], and our results corroborated with this hypothesis as none of the drugs or their combinations showed any significant induction of CAR (data not shown) when measured with qRT-PCR.

The mRNA expression levels may be a good indicator of the downstream protein activity, but to confirm the results, various metabolic as well as efflux transporter activity assays were carried out. VIVID™ CYP3A4 assay is a well-known method to quantify the CYP3A4 activity in cells [6, 14]. The functional activity of CYP3A4 (Figure 4) for the treated cells was fairly correlated with the mRNA levels (Figure 2A). The functional activity of CYP3A4 was only 1.5-to 2-fold compared to the mRNA induction (5- to 10-fold). The ternary combinations of ALI and ASI did not show significant increase in activity (Figure 4) even though it was observed at genetic level. There may be multiple reasons behind this discrepancy. The protein translation may not be highly correlative to the genetic levels observed. The RNA stability might also be another reason for the quantitative difference even though the trend is observed to be qualitatively similar. More in-depth studies are required to identify the exact reasons for these inconsistencies. However, these observations further strengthen our hypothesis that the drug combinations should be tested in combination rather than individually because even though a drug may give statistically insignificant interactions with metabolizing enzymes or MDR proteins individually, when treated in combination the interaction may become statistically significant and thus clinically relevant.

Erythromycin is a known substrate of both MRP2 and P-gp and hence uptake of [¹⁴C]erythromycin was investigated to correlate the functional activity of P-gp and MRP2 after PI treatments. Amprenavir (A) caused very high induction of P-gp mRNA with no effect on MRP2 mRNA. As a result, very little decrease in uptake of [¹⁴C]erythromycin was observed (Figure 5). This is possibly because erythromycin is a better substrate for MRP2 than P-gp. Our earlier report indicated that [¹⁴C]erythromycin uptake was much higher in the presence of MK-571 compared to GF120918, the specific inhibitors of MRP and P-gp, respectively [15]. All binary combinations other than SL showed significant decrease of [¹⁴C]erythromycin uptake, showing a similar trend to that observed in gene expression studies. Only SLI showed a significant decrease in erythromycin uptake among all the ternary combinations tested.

The reason for the increase in the expression and activity levels of efflux transporters and metabolizing enzymes from single PI treatment to binary combinations can be a direct additive effect of increase in molar content of drug being exposed to the cells, but a similar additive effect should be observed in the case of ternary combination if it is only a result of stoichiometry. The reason for this discrepancy in the expression levels observed with ternary combinations can be a stress response due to cytotoxic effects of ternary combinations [25, 26]. To test this hypothesis, an MTT assay was performed to study the cytotoxic effects of all the combinations (Figure 6). All combinations tested were found to be noncytotoxic to the cells except SL and SLI. The induction of various genes observed with SLI may be attributed as a cellular stress response. Yet, the general lack of cytotoxicity observed with other ternary combinations excludes the possibility of the decrease in induction levels observed with ternary combinations being a stress response.

Previous studies have shown that when ATP levels were altered upon any treatment, the uptake of efflux transporter substrates was affected [27]. To further confirm this relationship, an ATP assay was performed. None of the individual PIs or their binary combinations showed any significant increase in ATP activity after treatment (Figure 7). Hence, the increase in efflux activity observed was probably a result of enhanced expression and not an alteration of a cofactor activity. A slight change in ATP activity was observed in three of the ternary combinations (ALI, ASI, ASL), none of which had shown any increased efflux after treatment.

The induction of gene expression might have been a simple additive effect due to increased molar quantities of PIs. But if this was the case, then the ternary combinations should show higher induction than the binary combinations. Docking studies with different PXR agonists and antagonists using novel computational models have shown that there might be different binding sites present in PXR [28–30]. The increased induction in binary combinations may be due to occupation of allosteric activators. The subsequent decrease of induction in ternary combinations may be then due to eventual binding with exposed inhibitory sites. Further computational and activity-based studies are needed to further elucidate this phenomenon. The current concentrations were selected based on previously published results for LS-180 induction and cytotoxicity. The concentration ranges used were close to clinically observed $C_{\max}$ values but may not exactly mimic tissue concentration in the clinic. These studies act as a proof of concept, but further studies may be needed to make the data more clinically relevant.

Conclusions

This is the first time a comprehensive study has been undertaken in which variations in stimulation potential of binary and ternary combinations of PIs for the induction of CYP3A4 and efflux transporter transcripts in LS-180 cells has been illustrated. For the first time, it has been demonstrated that binary combinations in general caused greater modulation of gene expression and functional activity of CYP3A4 and efflux transporter as compared to the individual PIs or their ternary combinations. This induction was found to be mostly PXR mediated. As there is not a simple additive effect responsible for this induction, further studies need to be performed to identify the possibility of allosteric inhibition of PXR

activity in case of certain combinations. The cell lines and techniques used in this study can be used for rapid screening of different drug combinations for their potential inducibility of efflux transporters and metabolizing enzymes for predicting potential drug-drug interactions.

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Figure 1.
Scheme of PIs and their combinations used for treatment of LS-180 cells.

Figure 2. Relative fold mRNA induction of (A) CYP3A4, (B) MRP2, (C) P-gp, (D) BCRP after treatment with PIs in LS-180 cells. n=4, *p<0.05, **p<0.01.

Figure 3.
Relative fold mRNA induction of PXR after treatment with PIs in LS-180 cells. n=4,
*p<0.05, **p<0.01.

Figure 4. Vivid assay for CYP3A4 activity in LS-180 cells after PI treatment. n=8, *p<0.05, **p<0.01.

Figure 5.
Percentage uptake of [¹⁴C]erythromycin in LS-180 cells after PI treatment. n=8,
*p<0.05, **p<0.01.

Figure 6.

Cell viability (MTT) assay carried out on LS-180 cells after PI treatment. n=8,

*p<0.05,**p<0.01.

Figure 7.
ATP activity assay in LS-180 cells after PI treatment. n=8, *p<0.05.

Table 1

qRT-PCR primer sequences.

Gene	Accession number	Sequence (5'→3')	Template	T _m	GC, %	Product length
<i>GAPDH</i>	NM_002046.3	Forward	ATCCCTCCAAAAATCAAGTGG	49.41	45.00	260
		Reverse	GTTGTCATGGATGACCTTGG	50.65	50.00	
<i>ABCB1</i> (MDR1)	NM_000927.3	Forward	CTTATGCTCTGGCCCTTCTGG	52.25	55.00	137
		Reverse	TG CTTCAATG CTTGG AG ATG	50.64	45.00	
<i>ABCC2</i> (MRP2)	NM_000392.3	Forward	AAATATTTTGGCTGGGAACC	48.24	40.00	147
		Reverse	TGTGACACACAGATACCAGGA	51.74	50.00	
<i>ABCG2</i> (BCRP)	NM_004827.2	Forward	AGTGCAAGGAAAAGATCCAA	51.51	45.00	119
		Reverse	TG CCCATCACAAACATCATCT	51.29	45.00	
<i>CYP3A4</i>	NM_017460.3	Forward	ACCGTGACCCAAAAGTACTGG	53.76	55.00	119
		Reverse	GTTTCTGGGTCCACTTCCAA	51.78	50.00	
<i>NR1I2</i> (PXR)	NM_022002.2	Forward	GCAGGTGGCTTCCAGCAACT	63.32	60.00	128
		Reverse	GGGCGGTCTGGGGAGAAAGAG	64.12	70.00	