Reduced ABCB1 Expression and Activity in the Presence of Acrylic Copolymers

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ABSTRACT

Purpose: P-glycoprotein (P-gp; ABCB1), an integral membrane protein in the apical surface of human intestinal epithelial cells, plays a crucial role in the intestinal transport and efflux leading to changes in the bioavailability of oral pharmaceutical compounds. This study was set to examine the potential effects of three Eudragits RL100, S100 and L100 on the intestinal epithelial membrane transport of rhodamine-123 (Rho-123), a substrate of P-gp using a monolayer of human colon cancer cell line (Caco-2).

Methods: The least non-cytotoxic concentrations of the excipients were assessed in Caco-2 cells by the MTT assay. Then the transepithelial transport of Rho-123 across Caco-2 monolayers was determined with a fluorescence spectrophotometer. Besides, the expression of the P-gp in cells exposed to the polymers was demonstrated using Western-blotting analysis.

Results: Treatment of cells with Eudragit RL100 and L100 led to a very slight change while Eudragit S100 showed 61% increase in Rho-123 accumulation (P<0.001) and also reduced transporter expression.

Conclusion: Our studies suggest that using proper concentrations of the Eudragit S100 in drug formulation would improve intestinal permeability and absorption of p-gp substrate drugs.

Introduction

Although oral route for drug administration is the most convenient and favored choice for patients, most hydrophilic drugs and some high molecular weight hydrophobic drugs show poor intestinal permeability and absorption which is a key factor that determines the pharmacokinetics of oral drug compounds and alters their both bioavailability and pharmaceutical effects.1-4 P-gp, also known as ABCB1, is a plasma-membrane associated efflux pump in humans with an energy dependent function which plays a crucial role in the intestinal transport and is known to be responsible for the occurrence of drug resistances.5-7 This effect of P-gp, which is encoded by MDR1 gene, is considered to be one defense against toxic agents where it can reduce the bioavailability of a wide range of pharmaceutical compounds as well.8-10 Caco-2 cells which are human colonic adenocarcinoma cell line and expressing P-gp appear to be used widely for in vitro permeability studies.11 Previous studies have revealed that some drugs and commonly used substances in drug formulations can alter the ability of P-gp in pumping its substrates.12,13 That means excipients, substances other than the pharmacologically active drugs, are not considered to be inert components and may have an important effect on drug metabolism and efflux.11,14 Polyaacrylate polymers have been used widely to achieve the desired drug release profile with the drug being released at the right place and time or, if necessary, over a desired period of time. Other important uses are taste and odor masking to increase patient compliance and also protection from external influences like moisture. Eudragit® polymers are copolymers derived from esters of acrylic and methacrylic acid, whose physicochemical properties are determined by functional groups. Eudragit S100 is an anionic polymer showing a pH-dependent solubility and has been utilized for oral drug delivery because of its solubility and consequently drug release at pH above 7. Also Eudragit L100, which is an enteric anionic copolymer, is based on methacrylic acid and methyl methacrylate. On the other hand Eudragit RL100 is a positively charged acrylate polymer which is

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extensively used in pharmaceutical sciences i.e. sustained release film coating, etc. This study has been conducted in order to test the ability of the named excipients, whose influence on the expression of P-gp has not been reported until now, in down regulating the P-gp efflux transporter which would probably lead to enhancement in drug bioavailability.

**Materials and Methods**

**Materials**

Human carcinoma colorectal (Caco-2) cell line was purchased from National cell bank of Iran, Pasteur institute, Iran. All cell culture disposable equipments were obtained from Orange, Belgium. RPMI 1640 – Powdered Cell Culture Medium was from PAA Co, Austria. Fetal Bovine Serum (FBS) was aquired from Gibco, Invitrogen, USA. Dimethylsulfoxide (DMSO) was from Merck, Germany. Penicillin and streptomycin were obtained from Sigma, Germany. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Roche Diagnostics GmbH, Germany. Trypsin was provided from Gibco, Invitrogen, USA.

**Cell culture**

All operations were performed via standard sterile conditions under a laminar flow cabinet. The cabinets were habitually sterilized overnight by exposure to ultra-violet radial ion and then washed in 70% alcohol before use. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were incubated in a humidified incubator having 5% CO2 at 37 °C. Then cells were subcultured into 96-well plates and 6-well plates during various steps of the study.

**MTT assay**

Cytotoxicity of the excipients were measured in Caco-2 cells via the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide according to the manufacturer's procedure. This method is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases. The cells were seeded in 96-well plates with a density of 10^4 cells/well and incubated for 24 h at 37°C and 5% CO2. The cells were treated with various concentrations of solvent extracts (10, 20, 50, 100, 150, 200, 300, 400 μg/ml) and 0.2 % (v/v) DMSO as a negative control. After 12, 24 and 48 h treatment 10 μl of MTT labeling reagent was added to all wells. The plates were incubated at 37°C and 5% CO2 for 4 hours. Then, 100μl of the solubilization solution was added to each well and followed by incubation overnight at 37°C to dissolve formazan crystals. Finally, absorbance was read using an ELISA plate reader (Bio Teck, Germany) at a wavelength of 570 nm. The percentage of cytotoxicity and cell viability were estimated using following equation:

\[
\% \text{ Cytotoxicity} = 1 - \frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of negative control}}
\]

\[
\% \text{ Viability} = 100 - \% \text{ Cytotoxicity}.
\]

**Assessing Uptake of Rhodamine-123**

For the uptake studies Caco-2 cells were seeded into 24-well plates; and left for 24 hours. On the other day old medium was removed and cells were washed by PBS. Then new culture media containing different concentrations of excipients and 0.3 mM verapamil, as P-gp inhibitor, were added and left for another 24 hours. On day 3 of experiment, the old medium was removed and cells were washed three times with PBS and Rh-123 solution (RPMI containing 10 mM HEPES (pH=7.4) and 5 μM Rh-123) were added and incubated in 37 °C for 3 hours. After incubation period, Rh-123 solution was removed and cells were washed three times with ice-cold PBS. Cells were lysed in 1% Triton X-100 and centrifuged in 1000 rpm for 5 minutes. Supernatant was used to measure the fluorescence and total protein content. Quantity of Rh-123 was calculated using the obtained calibration curve (R^2=1). Then cellular Rh-123 accumulation was normalized to total protein content determined by protein assay Kit.

**Western Blotting**

Cells were moved to 6-well plate in density of 10^6 cells per well and treated with excipients for 24 hours. Solutions were removed and cells were washed by PBS then incubated in 37°C for 5 minutes with Trypsin/EDTA 0.25%. Supernatant was removed and cell sediment was washed twice with PBS. Lysis buffer was added and cell suspension was centrifuged in 15000 rpm for 5 minutes. The proteins were separated by electrophoresis through SDS-polyacrylamide gel on 12.5% running gel and 4% stacking gel at 80 V for 120 min. The gel was electro blotted to Polyvinylidene difluoride (PVDF) membrane using semi-dry western blotting. 3% non-fat dry milk was used to block the membrane for 1 hour at room temperature and membrane was washed 3 times with PBS-Tween 20 0.1% and then incubated overnight with primary monoclonal antibody (Anti-β-actin), diluted 1/1000 in PBS containing 0.1% tween 20. After washing with PBS-Tween 20 0.1%, the membrane was incubated with horseradish peroxidase-conjugated Rabbit anti-mouse secondary antibody for two hours. Membrane was washed and solution A and B of Enhanced chemiluminescence (ECL) kit was added, then membrane was exposed to X-ray film. Membrane was washed twice and was incubated with MDR1 Antibody (C219) overnight. After washing, membrane was put into horseradish peroxidase-conjugated Rabbit anti-mouse secondary antibody for two hours. Membrane was washed and then solution A and B of ECL kit was added, then membrane was exposed to X-ray film.
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**Results**

Cytotoxicity of excipients on cells was estimated via MTT test assay. MTT test assay of excipients specified the proper concentration of excipient which should be used in western blotting and Rho-123 uptake experiment. Optical density (OD) value obtained from ELISA reader was divided to that of control and cell viability was calculated for each excipient after 24 hours exposure to different polymer concentrations. Two maximum nontoxic concentrations were selected for western blotting and Rho-123 uptake test. According to the results, Eudragit RL100 in concentrations more than 0.5% was toxic for Caco-2 cells (P<0.001). Therefore we used its lower concentrations (0.25% and 0.1% (w/v)) for the subsequent studies. Eudragit S100 and L100 caused significant decrease in cell viability in concentrations more than 0.1% (w/v) and 0.25% (w/v), respectively. Thus lower concentrations were selected for uptake study and also western blotting. The MTT assay results are shown in Figure 1.

![Figure 1. Effects of Eudragit RL100, L100 and S100 on cell viability in Caco-2 cells. Data are expressed as the mean of percent cell viability compared to control after exposure for 24 hours ± standard deviation (n=3).](image)

In order to investigate the functional activity of P-gp, Caco-2 cells were incubated in 48-well plates with different concentrations of the excipients for 24 hours. Afterwards cells were washed with PBS and then exposed to Rho-123 (5 μM) for 3 hours. Cells were lysed and accumulated Rho-123 in cells was measured (excitation at 485 nm and emission measured at 530 nm) for each sample. The protein content of the aliquots was measured by protein assay and cellular Rho-123 accumulation was normalized with respect to the total protein in each well. Eudragit S100 enhanced Rho-123 uptake significantly into Caco-2 cells as shown in the Figure 2.

![Figure 2. Effect of Eudragit RL100, Eudragit L100 and Eudragit S100 on Rho-123 uptake in Caco-2 cells. Data are expressed by the ratio of quantity of Rho-123 (mg×10^6/mL) to total protein (mg/mL) in each well. Values are versus control as compared with control group using one way ANOVA with Student-Newman-Keuls post hoc test (*P<0.001).](image)
P-glycoprotein expression was measured in Caco-2 cells which had been treated for 24 hours with excipients and compared to that of control. The protein was separated by electrophoresis on 12.5% running gel and 4% stacking gel. Electrophoretic transfer of separated proteins in gel was transferred to a PVDF membrane using semi-dry blotting. The membrane was blocked in PBS containing 0.1% tween-20 and 3% dried skim milk at room temperature for 1 h and washed three times for 15 min in PBS containing 0.1% tween-20. Encountering with primary and secondary antibody, the bands were visualized using ECL Western Blotting Detection Reagents and exposed to an X-ray film. As shown in Figure 3 Eudragit S100 had inhibited the P-gp expression which leads to the inhibition of the efflux pump. Other excipients used in the current work had a similar effect as the control group.

**Figure 3.** P-gp protein expression after 24 hours exposure to the excipients. Expression in treatment groups were compared with P-gp expression in untreated control cells. 20 μg of total protein were separated by SDS polyacrylamide gel electrophoresis and immunoblotted with monoclonal antibody C219 for P-gp and I-19 for actin.

**Discussion**

The results of this study showed that there are excipients which could down regulate MDR1 gene and P-gp protein expressions which can lead to enhancement in drug bioavailability. The present study characterizes the effects of the polyacrylate excipients on P-gp expression and activity in Caco-2 monolayer. Sub-toxic concentrations of excipients were prepared using MTT test assay and in the accumulation studies a known P-gp substrate, Rho-123, uptake was tested. Furthermore western blotting confirmed Rho-123 uptake data. Eudragit S100 that was able to increase Rho-123 accumulation decreased P-gp expression either. This study aimed to access a rational drug formulation development strategy for oral dosage forms based on Caco-2 monolayer as an in vitro screening model.

The oral delivery of drugs is generally the most suitable route to administer drugs, as it is painless and easy to use, and therefore it is followed by high patient compliance. On the other hand, MDR proteins belonging to ABC transporters are a part of membrane transport proteins that detoxify cells from external substrates. These proteins are identified to limit absorption through biological membranes such as intestinal, brain and cancer cells. Several papers have reported data on the effects of different agents and excipients other than Eudragits on the P-gp. For instance a report states that tween 80 can decrease the percentage of serosal-mucosal transport to mucosal-serosal transport of Rho-123 across rat jejunal membrane in vitro and caco-2 cell monolayer, suggestive of p-gp inhibition. In addition, the in vitro absorption of digoxin across an everted rat gut sac (a p-gp substrate) showed a deep increase after treating with 0.5% (w/v) tween 20 and tween 80. Also, digoxin given with tween showed an increase in AUC and Cmax in rats. Another study has claimed that cremophor EL (0.1%, w/v) only partially inhibits P-gp activity in Caco-2 cells. Lipid excipients Pecquel and Gelucire 44/14 decrease P-glycoprotein mediated efflux of rhodamine 123 partially due to modifying P-glycoprotein protein expression within Caco-2 cells. On the other hand there are many other reports on the effects of inhibition of P-gp by co administered drugs on intestinal permeability of drugs. Granting these findings, there was no direct study to be conducted on the changes of the expression of MDR1 gene or P-gp induced by Eudragits. In this study, concentrations of 0.1% and 0.25% (w/v) for Eudragit RL100 had no toxic effect on Caco-2 cells so used in the Rho-123 uptake assay. When compared to control group (Intracellular Rho = 2612 pg/mL; total protein = 26 mg/mL), Eudragit RL100 led to a very slight increase in the accumulation of Rho in cells which was not significant (Intracellular Rho = 2754 pg/mL; total protein = 96 mg/mL). Moreover from the results of MTT test, Eudragit L100 at 0.25% (w/v) and 0.5% (w/v) concentrations and Eudragit S100 at 1% (w/v) and 2% (w/v) were found to be non-toxic to Caco-2 cells and were subjected to Rho-123 uptake assay and western blotting analysis. Treatment of cells with the Eudragit L100 caused to 7%, decrease in Rho-123 accumulation. (Intracellular Rho = 2429 pg/mL; total protein = 93 mg/mL). Eudragit S100 showed significant increase in Rho-123 accumulation in cells. This increase was 61%. (Intracellular Rho = 4205 pg/mL; total protein = 89 mg/mL). According to the obtained results, the present study demonstrated the efficacy of Eudragit S100 as an excipient in decreasing the P-glycoprotein expression and consequently its possible role as an inhibitory factor in the efflux process in different absorption regions. Using this polymer as an efflux pump inhibitor would be novel and it apparently will improve the bioavailability and help both substrate drug intestinal permeability and its absorption. Although the study is fulfilled in vitro, the data leave no doubt that Eudragit S100 has beneficial effects in inhibiting P-gp efflux activity.

**Conclusion**

Taken together, this paper suggests that using proper concentrations of the Eudragit S100 excipient would probably advance the bioavailability and help drug intestinal permeability and absorption which can plausibly have a significant impact on both drug efflux.
process and metabolism. Of course some other factors including P-gp structure, P-gp environment and substrate partitioning into the tissues have to be understood fully to decide on the ability of these commonly used excipients to inhibit P-gp activity in vitro and to further describe the effect of them on both activity and expression of P-gp. Therefore further tests for example, gut perfusion or pharmacokinetic studies in animals or more specific assays which target specific binding sites on P-glycoprotein should be carried out, later.

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Conflict of Interest
The authors report no conflict of interests in the present study.

References


