

## Effects of polyethylene glycols on intestinal efflux pump expression and activity in Caco-2 cells

Darya Hodaiei<sup>1,2</sup>, Behzad Baradaran<sup>3</sup>, Hadi Valizadeh<sup>4</sup>, Parvin Zakeri-Milani<sup>5,\*</sup>

<sup>1</sup>Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran, <sup>2</sup>Student Research Committee, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran, <sup>3</sup>Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, <sup>4</sup>Biotechnology Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, <sup>5</sup>Liver and Gastrointestinal Diseases Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

The present study was planned to investigate the influence of polyethylene glycols (PEGs) on the activity and expression of P-glycoprotein (P-gp). Sub-toxic concentrations of PEGs in Caco-2 cells were determined using the MTT test assay. Then the measurement of Rhodamine-123 (Rho-123) uptake, a P-gp fluorescence substrate, in Caco-2 cells confronting PEG 400 (1% and 2% w/v), PEG 4000 (2% and 4% w/v), PEG 6000 (2% and 4% w/v), PEG 10000 (2% and 4% w/v), PEG 15000 (1% and 2% w/v), and PEG 35000 (2% and 4% w/v) overnight was taken to elucidate whether non-toxic concentrations of PEGs are able to impact P-gp activity. Furthermore, western blotting was carried out to investigate P-gp protein expression. The results showed that PEG 400 at concentrations of 1% (w/v) and 2% (w/v) and PEG 6000 at the concentration of 4% (w/v) are notably capable of blocking P-gp. Based on the obtained results it is concluded that the mentioned excipients could be used to obstruct P-gp efflux transporter in order to increase the bioavailability of co-administered substrate drug.

**Uniterms:** P-glycoprotein/activity. P-glycoprotein/expression. Caco-2. Rhodamine-123. Excipients. Drugs/bioavailability. Polyethylene glycol/effects.

O presente estudo foi planejado para investigar a influência de polietileno glicóis sobre a atividade e expressão da P-glicoproteína (P-gp). Concentrações sub-tóxicas de PGP e em células Caco-2 foram determinadas por meio do ensaio de MTT. Em seguida, efetuou-se a medida de captura de Rodamina-123 (Rho-123), um substrato fluorescente de P-gp, em células Caco-2, confrontando com PEG 400 (1% e 2% m/v), PEG 4000 (2% e 4% m/v) e PEG 6000 (2% e 4% m/v), PEG 10000 (2% e 4% w/v), PEG 15000 (1% e 2% m/v), e PEG 35000 (2% e 4% m/v). Essa medida foi efetuada durante a noite, para saber se as concentrações não tóxicas de excipientes são capazes de influenciar a atividade da P-gp. Além disso, realizou-se o western blotting para investigar a expressão da proteína P-gp. Os resultados mostraram que o PEG 400, nas concentrações de 1% (m/v) e 2% (m/v), e PEG 6000, na concentração de 4% (m/v) são capazes de bloquear P-gp. Com base nos resultados concluiu-se que os excipientes mencionados poderiam ser utilizados para obstruir o efluxo por P-gp, a fim de aumentar a biodisponibilidade de do fármaco co-administrado.

**Unitermos:** Glicoproteína-P/atividade. Glicoproteína-P/expressão. Caco-2. Rodamina-123. Excipientes. Fármacos/biodisponibilidade. Polietilenoglicol/efeitos.

## INTRODUCTION

While oral drug administration has many preferable characteristics, like intensified patient compliance and easier chronic administration as compared with other

pharmaceutical forms, nearly 50% of orally taken drugs bioavailability (BA) is declined owing to their poor and inadequate absorption through intestinal mucosa (Gursoy, Benita, 2004; Shah *et al.*, 2013).

Membrane bound transport systems are one of the physiological factors that impact the intestinal absorption of drugs and therefore could be essential in drug pharmacokinetics and disposition alteration in the body. Among these transporters, P-gp, prevalently

\*Correspondence: P. Zakeri-Milani. Liver and Gastrointestinal Diseases Research Center - Faculty of Pharmacy - Tabriz University of Medical Sciences - East Azerbaijan, University Main St - Tabriz, Iran. E-mail: pzakeri@tbzmed.ac.ir

distributed in small intestine, is a crucial transporter responsible for the efflux of drugs and it is assumed that P-gp is implicated in cell detoxification (Hayeshi *et al.*, 2006; Simon, Schubert, 2012). P-gp is an ATP-dependent product of multi drug resistance gene in human, which is ligand for various substrates from drugs such as verapamil (Bellamy, 1996), cyclosporine A (Simon, Schubert, 2012), vinca alkaloids (Arora, Shukla, 2003), anthracyclines, cardiac glycosides (Sababi, Borga, Hultkvist-Bergtsson, 2001), vinblastine (Shirasaka *et al.*, 2006), venlafaxine (Ehret *et al.*, 2007), R-cetirizine (Shen, He, Zeng, 2007), etoposide (Parsa *et al.*, 2013), quinidine (Fromm *et al.*, 1999), and immunofluorescent dyes such as Rhodamine-123 (Pétriz, García-López, 1997). Moreover, it is believed that drugs such as dexamethasone, rifampicin and some chemotherapeutic agents such as doxorubicin, daunorubicin and vinblastine are able to induce P-gp expression.

P-gp induction is of considerable concern because it can significantly decrease the BA of substrate drugs. In the case of intestinal drug absorption, expression of P-gp can attenuate BA of drugs, which are substrate for P-gp. In a study, co-administration of *Rheum palmatum*, a P-gp inducer, and phenytoin predominantly decreased the oral BA of phenytoin because it initiated P-gp expression (Chi *et al.*, 2012). Additionally, P-gp is considered to overexpress in neoplastic cells. Resistance to chemotherapy is prevalent among treatments and it is attributed to P-gp. It is assumed that P-gp is responsible for cancer treatment failings and it is a major obstacle in chemotherapies. For example, in a study, a P-gp blocker was able to increase paclitaxel concentration in blood (Kang *et al.*, 2001). In addition, Compound K, the major component obtained from ginsenosides metabolism which is being used in treatment of lung cancer, showed reduced plasma  $C_{max}$  and  $AUC_{0-24h}$  when P-gp was inhibited (Yang *et al.*, 2012).

On the other hand, some studies refused the role of P-gp in drug efflux. For example, in an experiment performed by Dickens *et al.*, hypothesis claiming that P-gp is overexpressed at the epileptic focus was rejected as anti-epileptic drugs like carbamazepine and lamotrigine were proven not to be substrate for P-gp (Dickens *et al.*, 2013).

P-gp acts as a barrier for orally used drugs. By restricting the absorption of drugs through intestine, P-gp is involved in the causation of low BA of some drugs. Due to this fact, components, which are P-gp function/expression inhibitors have prominent role in exceeding drug BA and there is an increasing interest in enhancing BA of drugs by inhibiting intestinal P-gp. Reportedly, some synthetic phospholipids and several pharmaceutical excipients may have the potential to modulate this 170

kDa protein, P-gp. (Lo, 2000; Wandel, Kim, Stein, 2013)

Excipients are material used in drug formulations alongside the active compound. They were believed to be an inactive part of a drug formulation; however, studies demonstrated that excipients are not inert and they can have subtle effects on absorption or elimination of concomitant drugs administered (Buggins, Dickinson, Taylor, 2007). Many attempts have been made to evaluate the capability of excipients to augment drug BA. Some excipients, such as Vitamin E TPGS (Collnot *et al.*, 2006), nowadays are used as a P-gp inhibitor with the aim of increasing the BA of co-administered drugs (Guo *et al.*, 2013). In a study previously conducted in our lab, we screened the effect of tween excipients on P-gp expression and activity in Caco-2 monolayers. In the current study, we have extended the range of excipients tested and PEG excipients were selected. PEGs are amphiphilic, non-micelle forming hydrophilic polymers of molecular weights, ranging from 200 to 35000 (Basit *et al.*, 2002). They are commonly used as pharmaceutical excipients to raise the aqueous solubility of drugs that are poorly soluble in water. Therefore, current study was designed to explore whether this subclass of nonionic excipients are able to block P-gp at varied concentrations. By studying the effect of these excipients on both the expression and the activity of P-gp, we aim to present a procedure to increase drug BA.

Derived from human colorectal adeno-carcinoma, Caco-2 cell monolayer is widely used as a representative for human intestinal epithelial cells since it retains many features of small intestine closely mimicking the enterocytes of small intestine (Pang, 2003). Caco-2 cells express P-gp efflux pump and is a well-established cell line to investigate P-gp expression and blockage (Taipalensuu *et al.*, 2001).

Consistent with our previous work in which tween excipients showed a promising inhibitory effect on the expression and activity of P-gp (Hodaei *et al.*, 2013), the aim of this work was to investigate the effect of PEG excipients on expression and activity of P-gp. Analysis of P-gp activity could be performed with an appropriate substrate, which is able to give a measurable amount when P-gp is blocked. To this aim, we utilized Rho-123, which is a fluorescence substrate of P-gp and an ideal probe to explore the effects of excipients on P-gp expressed by Caco-2 cells. Accumulation of Rho-123 in Caco-2 cells were examined both quantitatively and qualitatively; moreover, to elucidate the effects of excipients on P-gp expression, western blotting was conducted. In both experiments, verapamil was selected as relative P-gp inhibitor, as it has been used in frequent studies of P-gp transportation (Sababi, Borga, Hultkvist-Bengtsson, 2001).

## MATERIAL AND METHODS

### Material

Human carcinoma colorectal Caco-2 cell line was from National cell bank of Iran, Pastur institute. Excipients and dimethylsulfoxide (DMSO) were kindly provided by Merck, Germany. Trypsin was provided by Gibco, Invitrogen, USA. Fetal Bovine Serum (FBS) was obtained from Gibco, Invitrogen, USA. RPMI 1640 - Powdered Cell Culture Medium was product of PAA Co, Austria. Trypan blue was obtained from Biosera, France. Rhodamine 123 was purchased from Sigma, USA. Anti  $\beta$ -actin antibody was supplied from GE, USA. MDR1 antibody (C219) was provided from Abcam, Cambridge, UK. X-ray film was purchased from Xoe, USA. Total protein assay kit was obtained from Pars Azmoon, Iran and all cell culture disposable equipment was obtained from Orange, Belgium.

### Caco-2 cell line

Caco-2 cells were routinely maintained in culture dishes (T75 falcons, Orange, Belgium) at 37 °C in a humidified atmosphere incubator containing 5% CO<sub>2</sub> (Memmert, Germany) and maintained in RPMI-1640 supplement with 10% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids and 1% L-alanine-glutamine 200 mM. Medium was exchanged every 2<sup>nd</sup> day and cells were split when they were approximately 90% confluent. Then the cells were washed with PBS (Phosphate Buffered Saline) and detached with 0.25% trypsin and 0.02% EDTA. PBS Solution contains 137 mM sodium chloride, 2.7 mM potassium chloride and 10 mM phosphate buffer.

### MTT test assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) test, as firstly designated by Mosmann (1983), is a delicate test for discovering acute toxicity of materials in an *in vitro* system on a certain type of cells which can determine the toxicity of excipients on Caco-2 cells as well.

After detaching from T-75 flasks, cells were centrifuged at 1000 rpm for 5 minutes (Behdad, Nabziran Inc., Iran). Cells were distributed in 96-well plates by placid sucking (15000 cells in each well). The well plate was incubated overnight. On the other day, 200  $\mu$ L of different series of excipients were pipetted to each well in triplicates. A solution of DMSO was used as positive control (viability 0%) and negative control column

was filled with fresh medium. Cells were incubated for 24 hours and on the third day of experiment (after 48 h cell growing), media were removed from wells and cells were washed by PBS (Sachs-Barrable *et al.*, 2007); subsequently, 50  $\mu$ L of MTT solution (2 mg/mL) (Sigma, St Louis, MO, USA) were added per well and after incubating for 4 hours, MTT solution was removed and 200  $\mu$ L DMSO/25  $\mu$ L Sorensen buffer was added to dissolve MTT-formazan crystals. Identification and quantification of the formazan crystals was accomplished and recorded at 570 nm. All absorbance values calculated as a percent of the negative control, were converted to viability data and the mean value of three determinations was then taken for further calculations (Mohammadzadeh *et al.*, 2014). Concentrations which have not killed 90% of cells are probably non-toxic to cells and were chosen for further investigations (Lonnroth, 2005).

### Assessing uptake of rhodamine-123

For uptake studies, Caco-2 cells were seeded into 24-well plates (10<sup>5</sup> cells/well) and left for 24 hours. After washing with PBS, cells were exposed to solutions containing excipients and incubated for another 24 hours at 37 °C. Standard P-gp inhibitor, verapamil was used as a positive control. After incubation period, Rho-123 solution (RPMI containing 10 mM HEPES (pH=7.4) and 5  $\mu$ M Rho-123) was added and incubated in 37 °C for 3 hours. Rho-123 solution was removed and cells were washed three times with ice-cold PBS. By addition of 1% Triton X-100 cells were lysed and centrifuged at 1000 rpm for 5 minutes. Supernatant was used to measure the fluorescence and total protein content. Quantity of Rho-123 was calculated using a fluorescent microscope reader (Jasco, JAPAN, excitation wavelength: 485 nm, emission: 530 nm). First the calibration curve of Rho-123 for determination of Rho-123 in samples was created (Absorbance= 5.04Conc + 2.07, R<sup>2</sup>=1). After calculating the rhodamine concentration in the cells, the cellular Rho-123 accumulation was normalized with respect to total protein content determined by protein assay kit (Pars Azmoon, Iran), which is based on biuret test. Bovine serum albumin was used as standard (Mohammadzadeh *et al.*, 2014).

### Western blotting

Caco-2 cells were shifted to plates and treated with different concentration of PEGs for 24 hours. Cells were washed with PBS and then incubated for 5 minutes with Trypsin/EDTA 0.25. Cell sediment was washed with PBS and cells were lysed and then centrifuged at 15000 rpm



for 5 minutes. Proteins were separated by electrophoresis through SDS-polyacrylamide gel on 12.5% running gel and 4% stacking gel at 80 V for 120 min and then electro blotted to polyvinylidenedifluoride (PVDF) membrane using semi-dry western blotting; membrane was kept in 3% non-fat dry milk for 1 hour to be blocked and then was washed 3 times with PBS-Tween 20 0.1% and then incubated overnight with primary monoclonal antibody (Anti- $\beta$ -actin), diluted 1/1000 in PBS containing 0.1% tween 20 to detect actin as control. The next day, membrane was washed with PBS-Tween 20 0.1% and incubated with horseradish peroxidase-conjugated Rabbit anti-mouse secondary antibody for two hours. Enhanced chemiluminescence (ECL) kit solutions were added and then membrane was exposed to X-ray film. Membrane was washed twice and incubated with MDR1 Antibody (C219) (Abcam, Cambridge, UK) 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 (Mohammadzadeh *et al.*, 2014).

## RESULTS

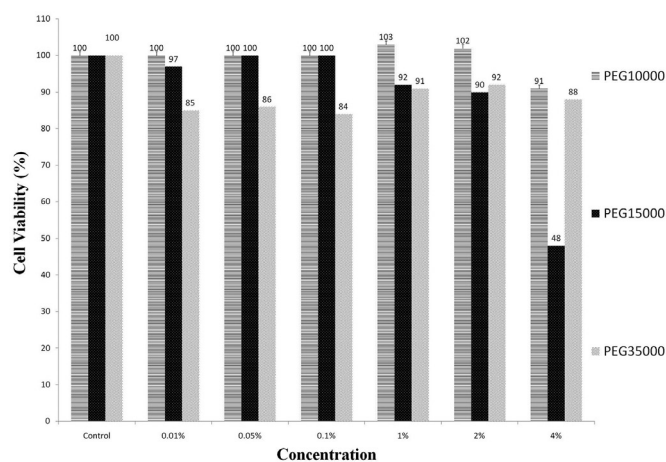
### MTT test results

Different concentrations of PEG 4000, PEG 6000 and PEG 10000 showed no toxic effect on caco-2 cells while PEG 15000 4% (w/v) and PEG 400 4% (w/v) exhibited significant toxic effect on cells as Figure 1 and Figure 2 indicate. PEG 35000 revealed 88±4% cell viability when compared to control well and there was no statistical difference between them ( $p < 0.001$ ). MTT test assay showed the maximum tolerable dose for each excipient on Caco-2 cells which were afterward used to treat cells for the P-gp protein expression, Rho-123 accumulation and transport studies.

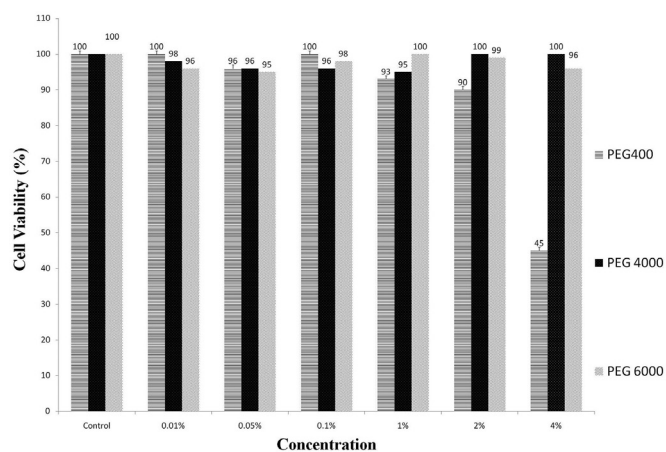
However, we know that the number of examined concentrations is not sufficient to demonstrate the dose-dependent manner. In this view, we only speculate that there may be such a trend in the effects. Further work is needed to confirm this result.

### Rho-123 uptake results

All treatments with PEG 400 and PEG 6000 4% (w/v) resulted in a remarkable increase in Rho-123 accumulation with respect to the control as portrayed in Figure 3. The Rho-123 accumulation in cells treated with PEG 4000, 10000, 15000 and 35000 was not notably different from that of control cells (Figure 4). Observing



**FIGURE 1** - Effects of PEG 10000, 15000, and 35000 on cell viability in Caco-2 cells. MTT assays were performed to measure the survival rate of Caco-2 cells after treatment with PEG excipients. Data were expressed by the mean of percent cell viability compared to control after exposure for 24 hours  $\pm$  standard deviation of at least 3 measurements.

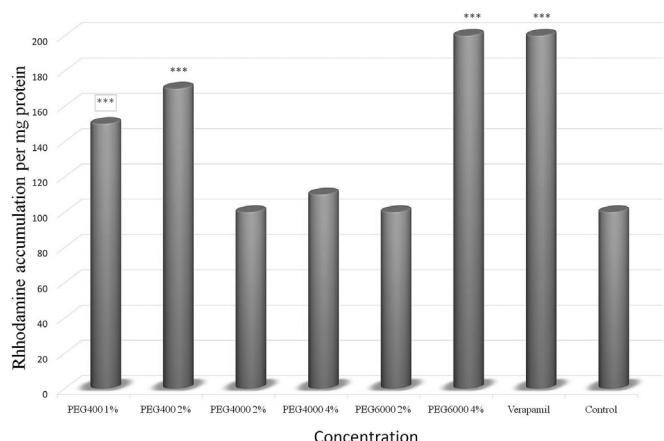


**FIGURE 2** - Effects of PEG 400, 4000 and 6000 on cell viability in Caco-2 cells. MTT assays were performed to measure the survival rate of Caco-2 cells after treatment with PEG excipients. Data were expressed by the mean of percent cell viability compared to control after exposure for 24 hours  $\pm$  standard deviation of at least 3 measurements.

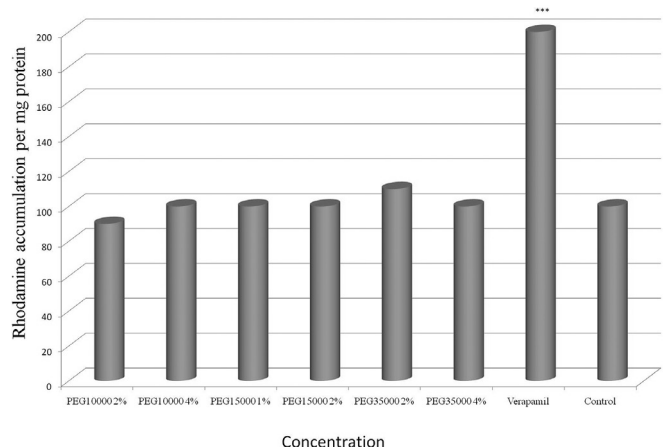
cells under Immuno-fluorescent microscope qualitatively illustrated that PEG 400 and PEG 6000 (4% w/v) presence, subsequently decreased Rho-123 efflux and significantly increased intracellular concentrations when compared to that of control group (Figure 5).

### Western blotting results

P-glycoprotein expression by caco-2 cells was investigated after treating cells with PEGs and verapamil.



**FIGURE 3** - Effect of PEGs on Rho-123 uptake across Caco-2 cells. Caco-2 cells were pretreated for 24 hours with different concentrations of excipients and 0.3 mM verapamil as positive control for P-gp inhibition. Data were expressed by the ratio of quantity of Rho-123 ( $\text{mg} \times 10^6/\text{mL}$ ) to total protein ( $\text{mg}/\text{mL}$ ) in each well (mean $\pm$ S.E). Statistical analysis was performed with analysis of variance (ANOVA) Student-Newman-Keuls post hoc test.

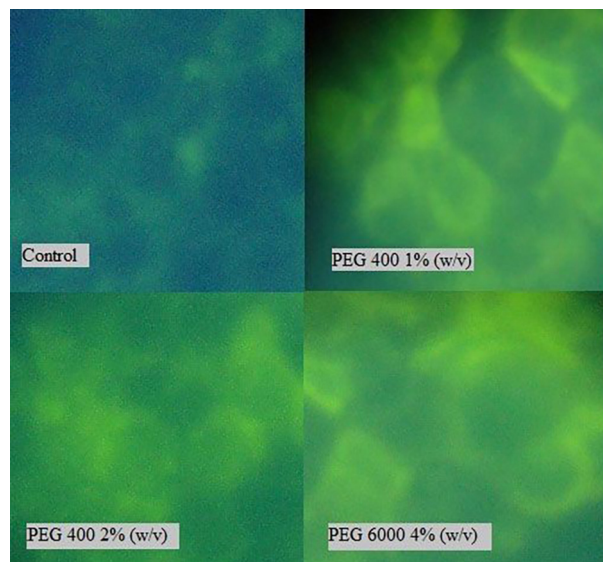


**FIGURE 4** - Effect of PEGs on Rho-123 uptake across Caco-2 cells. PEG 400 1% and 2% (w/v) and PEG 6000 4% (w/v) enhance Rho-123 uptake into Caco-2 cells. Caco-2 cells were pretreated for 24 hours with different concentrations of excipients and 0.3 mM verapamil as positive control for P-gp inhibition. Data were expressed by the ratio of quantity of Rho-123 ( $\text{mg} \times 10^6/\text{mL}$ ) to total protein ( $\text{mg}/\text{mL}$ ) in each well (mean $\pm$ S.E). Statistical analysis was performed with analysis of variance (ANOVA) Student-Newman-Keuls post hoc test.

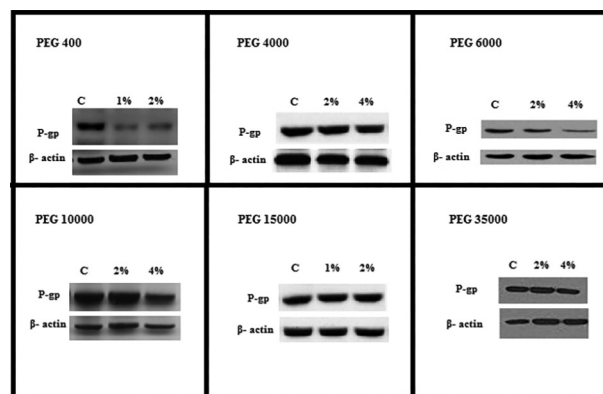
As Figure 6 depicts, among PEGs used in this study, PEG 400 1% and 2% (w/v) and PEG 6000 4% (w/v) decreased the expression of P-gp when compared to control group.

## DISCUSSION

Multidrug resistance (MDR) continues to be a



**FIGURE 5** - Photos taken by immunofluorescence microscope illustrates higher intercellular accretion of Rho-123 in cells treated with excipients when compared to that of control.



**FIGURE 6** - P-gp protein expression level in caco-2 cells treated with PEGs. Expression in groups treated with PEGs were compared with P-gp expression in untreated control cells (C).

challenge in drug delivery since BA of drugs taken orally is being changed by many factors among which low absorption through intestinal barrier cells is of utmost importance. Efflux transporters, such as P-gp, have great preventing effect in drug penetration through intestine; hence, inhibiting P-gp may be an effective way in boosting drug BA. For this purpose, various approaches have been employed. For instance, in our previous study Eudragits were reported to be P-gp inhibitor and increase the BA of co-administered substrate drugs (Mohammadzadeh *et al.*, 2014). Moreover the large molecular weight PEGs, have been reported to hinder the secretory transport (basolateral to apical) of Rho-123 across the isolated rat intestinal membranes at concentrations of 0.1 to 20 %

(w/v), reflective of P-gp inhibition (Shen, *et al.*, 2006). PEG 20000 20% (w/v), a high molecular weight PEG, was also shown to significantly increase the Rho-123 AUC and intestinal absorption in rat *situ* closed loop model (Shen, *et al.*, 2006). However, in the current study, we found no alteration in Rho-123 uptake after treating with high molecular PEGs, except for PEG 6000. The discrepancy between *in vivo* and *in vitro* results was also reported in previous studies (O'Brien *et al.*, 2012). Therefore, there remains a demand for the development of a validated and highly reliable predictive screening model for the accurate identification of P-gp substrates and inhibitors. Figure 3 shows that out of the six PEGs tested only two, PEG 400 1%, PEG 400 2% and PEG 6000 4%, caused an increase in Rho-123 uptake by 150%, 170% and 200% respectively while the transport of Rho-123 was not affected by other PEGs. We can also find a significant difference ( $p < 0.001$ ) between rhodamin uptake in the presence of PEG 400 1% and 2%. Therefore, dose-dependent manner in the case of PEG 400 could be speculated. Moreover the effect of PEG 400 (1% and 2%) and PEG 6000 on rhodamin accumulation was significantly different ( $p < 0.001$ ) from that of verapamil. However, more number of concentrations should be examined to confirm these results. No previous studies have been conducted on the effect of PEGs 4000, 10000, 15000 and 35000 on P-gp while there was a study done on PEG 300; an excipient, which we did not include in our study. Mentioned investigation yielded some data on PEG 300 demonstrating that PEG 300 in relatively high concentrations is able to modulate P-gp substrate drugs such as doxorubicin and taxol across Caco-2 cells; moreover, the inhibitory effect of PEG 300 was claimed to be concentration-dependent (Hugger, Audus, Borchardt, 2002a).

In a study reported by Rege *et al.* (2001) PEG 400 at the concentration of 1.5%, showed no effect on Caco-2 cells in transport of cimetidine and furosemide. In another study it was demonstrated that PEG 400 was able to eminently inhibit P-gp in a dose-dependent manner when concentrations above 1% to 20% was tested on excited rat intestine (Johnson, Charman, Porter, 2002). Corroborating the latter finding, our results showed that PEG 400 was able to inhibit both the activity and expression of P-gp in 1% and 2% (w/v). Although low molecular weight PEGs like PEG 400 could be used in all pharmaceutical formulations, they are typically used in parenteral formulations. The maximum safe concentration of PEG 400 is considered to be 70%. However in parenteral formulations it is considered to be relatively safe in concentrations less than 30% (Hugger *et al.*, 2002b). Moreover, PEG 400

was demonstrated to enhance absorption of ganciclovir in rats; however, the effect was not as significant as that of verapamil (Li *et al.*, 2011). Our study confirms their findings in that the accumulation of Rho-123 was less in cells treated with PEG 400 than that of cells treated with verapamil (200%) which is obvious in Figure 3. Ashiru, Patel, Basit, (2008) and Schulz *et al.* (2003) observed a parabolic increase, with the maximum effect at 1% (w/v), in ranitidine BA in male subjects when co-administered with low doses of PEG 400; however, high doses did not affect ranitidine absorption. The current study did not support their results and failed to concur with their finding because PEG 400 2% (w/v) represented its P-gp inhibitory effect even more than PEG 1% (w/v) and there was no obvious difference in the expression of P-gp between PEG 400 1% and 2% inhibitory effect on P-gp. However it should be noted that their studies were conducted *in vivo*, showing the gender-dependent effect of PEG 400 and also its detrimental effect on drug absorption in high concentrations presumably via a reduction in the small intestinal transit time. Based on our own observations, as can be seen in Figure 3, PEG 400 increased uptake of Rho-123 into Caco-2 cells (Hugger *et al.*, 2002b). Figure 6 shows the effect of PEG 400 on P-gp expression.

The exact mechanism by which P-gp blockage occurs in the presence of PEGs is still unknown but there are some suggested hypotheses. For example, Ming *et al.* proposed that the increase in the absorption is a complex result of P-gp blockage and membrane fluidity (Li *et al.*, 2011), similarly, Rege *et al.* introduced membrane fluidity as an important factor in drug BA amended by excipients (Rege, Kao, Polli, 2002). Compounds that are P-gp substrates are primarily hydrophobic and diffuse passively through the membrane. On the other hand drug-binding domains of P-gp are present in the transmembrane segments. Therefore P-gp will be highly sensitive to the lipid environment. It is suggested that oxyethylene groups existed in the structure of excipients such as PEGs prompts the membrane to change its lipid phase and with this mechanism the P-gp breakdown is resulted (Hugger *et al.*, 2002b). Shen *et al.* remarked that alkyl and unsaturated C–C bond in the chemical structure of PEG derivatives may be essential for interacting with P-gp and influence its function (Shen, *et al.*, 2006). Ferte *et al.* (2000) declared that P-gp is highly sensitive to lipid environment and membrane lipid phase can change when faced with components containing many oxyethylene groups so do the fluidity of the polar head group regions of the cell membrane. However, no general trend has been emerged yet. Further studies with more number of PEGs concentration levels, *in vitro* and *in vivo*, should



be conducted to establish a possible trend. It is also manifestly demanding to build up a logical concept and further biochemical experiments to understand the exact mechanism of this alteration.

## CONCLUSION

Based on the obtained results PEG 400 1% and 2% (w/v) and PEG 6000 4% (w/v) increased Rho-123 uptake and decreased P-gp expression. We also conclude that PEG 400 and PEG 6000 at mentioned concentrations are effective in diminishing P-gp mediated efflux system to increase absorption of drugs that are substrate for P-gp through intestine.

## ACKNOWLEDGMENT

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## CONFLICTS OF INTEREST

The authors report no conflict of interest in the present study.

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