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1. Korrekturabzug
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The effect of Tween excipients on expression and activity of P-glycoprotein in Caco-2 cells

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ABSTRACT

Purpose: The present study was designed to investigate the effects of Tween excipients (polysorbates; Tween 20, Tween 40 and Tween 80) on the activity and expression of P-glycoprotein (P-gp). Methods: An MTT ■author: please write the abbreviation out■ assay test was conducted to determine the non-toxic concentration of the excipients. Moreover, the uptake of Rhodamine-123, a P-gp fluorescence substrate, was measured through Caco-2 cell monolayer encountering with excipients overnight to investigate whether sub-toxic concentrations of Tween excipients are able to affect the P-gp activity. Furthermore, Western blotting was performed to investigate the P-gp protein expression. *Results:* The results showed that Tween 20 and Tween 80 at concentrations below 0.01 % (w/v), and Tween 40 at concentrations below 0.05 % (w/v) were non-toxic to Caco-2 cells. Results from the Rho-123 uptake test assay and Western blotting showed that Tween 20 at concentration of 0.01 % (w/v) and Tween 40 at concentration of 0.05 % (w/v) were able to block P-gp significantly. Conclusion: Based on the obtained results it is concluded that Tween excipients at the defined concentrations could be used to inhibit the P-gp efflux transporter resulting in an altered bioavailability of drugs.

ZUSAMMENFASSUNG

Der Effekt von Tween Hilfsstoffen auf die Aktivität und Expression von P-Glycoprotein in Caco-2- Zellen

Ziel: In dieser Arbeit wurde der Effekt von Tween Hilfsstoffen (Tween 20, Tween 40, Tween 80) auf die Aktivität und Expression von P-Glycoprotein (P-GP) untersucht. *Methoden:* Ein MTT Untersuchungstest wurde durchgeführt um die nicht-toxische Konzentration von Hilfsstoffen zu ermitteln. Ferner wurde die Aufnahme von Rhodamin-123, ein P-GP Fluoreszenzsubstrat, gemessen, indem Caco-2-Monolayer-Zellen über Nacht den Hilfsstoffen ausgesetzt wurden. Dabei wurde untersucht, ob die subtoxischen Konzentrationen von Tween Hilfsstoffen die Aktivität von P-GP beeinflussen können. Daraufhin wurde die Western Blot-Methode durchgeführt, um die Proteinexpression von P-GP zu untersuchen. *Ergebnisse und Diskussion:* Die Ergebnisse zeigen, dass Tween 20 und Tween 80 bei Konzentrationen unter 0.01 % (w/v), und Tween 40 bei Konzentrationen unter 0.05 % (w/v) eine nichttoxische Wirkung auf Caco-2-Zellen haben. Die Ergebnisse von Rhodamin-123-Aufnahmen und der Western Blot-Methode zeigen, dass Tween 20 bei einer Konzentration von 0.01 % (w/v) und Tween 40 bei einer Konzentration von 0.05 % (w/v) P-GP erheblich blockieren können. *Fazit:* Anhand der erreichten Ergebnisse ist festzustellen, dass Tween Hilfsstoffe in bestimmten Konzentrationen angewendet werden können, um den Efflux-Transporter P-GP zu blockieren, und damit die Bioverfügbarkeit von Arzneistoffen zu verändern.

KEY WORDS

- Contact angle
- P-gp
- Caco-2
- Excipient
- Bioavailability
- Tween
- Surfactant

Pharm. Ind. 75, No. 10, ■–■ (2013)

1. Introduction

Having many advantages, like enhanced patient compliance and easier chronic administration, 85 % of drugs sold around the world are administered orally. Oral delivery is the most preferred route in drug prescription but, unfortunately, nearly 50 % of orally administrated drugs have low bioavailability (BA) due to their poor and inadequate absorption through the intestinal mucosa [1, 2]. Active efflux of drugs into the gastrointestinal (GI) lumen is generally recognized as a factor for limiting drug absorption and it is responsible for variability in plasma drug concentrations [3].

An efflux pump, P-glycoprotein (P-gp), which has been known to be over-expressed in tumor cells [4], is highly expressed in almost all endothelial tissues and is the most representative in the group of efflux transporters in controlling the drug disposition [4–9]. P-gp, encoded by the multidrug resistance (MDR1) gene in humans, is a 170 kD integral membrane protein associated with the transport of several hydrophobic, amphipathic, cationic and neutral molecules [10] with a molecular weight (M_w) of 200–300

Da [11]. It is expressed in the apical surface of epithelial cells in many tissues of the human body such as mature enterocytes, canalicular membranes of hepatocytes, kidney cells, the placenta barrier and endothelial cells of the brain membrane [12]. P-gp plays a crucial role in the absorption, distribution and elimination of a variety of drugs [13]. In the brain, P-gp prevents the drug molecules from entering through the blood-brain barrier (BBB) into the central nervous system [14]. In the intestines, P-gp pumps cytotoxic drugs out of the cells and into the lumen and reduces the drugs' intracellular concentration and toxicity. Thus, P-gp limits the BA of orally administered drugs.

Hence, P-gp is considered to act as an absorptive barrier for orally administered drugs and drug efflux, by means of membrane proteins, has become an issue. Compounds which can inhibit the P-gp efflux of drugs are important because they can improve the drug BA due to better absorption through the intestines and reduce MDR [15, 16]. P-gp is highly expressed in the lower small intestine [17] and the expression level of P-gp is 2.31-fold higher in the ileum than that in the jejunum [18]. Inhibition of P-gp activity may be an effective way to enhance the oral drug BA. Since the late 1980s, scientists have been interested in designing specific P-gp blockers to administer together with anticancer drugs in order to enhance the anti-cancer drugs' therapeutic effect [19, 20]. Rhodamine-123 (Rho-123) [21], a cationic, lipophilic fluorochrome, which accumulates rapidly in cells, preferentially in the mitochondria membranes of living cells, is a tool that has been vital for the investigation of MDR phenotypes in previous studies [22–25]. This dye is transported out of cells by P-gp and indicates P-gp pumping. Studies have shown that a P-gp blocker can change ■the pumping ability of P-gp ■author:ok?■, increase the Rho-123 uptake, soar cellular accumulation of Rho-123 and ascend Rho-123 fluorescent light from lysed Caco-2 cells [26]. According to recent research a number of excipients/ surfactants commonly used in pharmaceutical formulations may disrupt the efflux caused by P-gp and lead to an increase in drug BA. Vitamin E-TPGS (d-α- tocopherolpolyethylene glycol 1 000 succinate) is a P-gp inhibitor example that increases the absorption flux of amprenavir [27, 28]. As such, „inactivity" of excipients and the idea supporting „excipients are inert" has been reevaluated [29]. Surfactants are ■a common class of excipients ■author: rephrasing ok?■. They are categorized in two groups: ionic and nonionic. Tween surfactants are polysorbate molecules, each containing ethylene glycol (oligo) (OEG) head groups (hydrophilic) and an alkyl tail (hydrophobic) ■[29]■author:please correct reference number■. They consist of a mixture of different molecules rather than a single molecule. Tween excipients are used as emulsifying agents, solubilizing agents, wetting agents and dispersing/suspending agents in pharmaceutical formulations [30].

The Caco-2 cell line, ■which is representative for human carcinoma colorectal cells ■author: rephrasing ok?■, is widely used for *in vitro* studies and has been used as a model for estimating drug permeability and the ability of different substrates for P-gp inhibition since the 1980s [31, 32]. Growing onto trans-well polycarbonate

membranes, cells undergo differentiation and express efflux transporters (e. g., P-gp) [33,34]. In this paper, we have characterized the effects of Tween excipients (Tween 20, Tween 40, Tween 80) on the activity and expression of P-gp.

2. Materials and methods

2.1. Materials

Tween excipients and Dimethylsulfoxide (DMSO) were purchased from Merck, Germany. Rhodamine 123 was bought from Sigma, USA. Anti β-actin Antibody was supplied by GE, USA. X-ray film was purchased from Xoe, USA. Fetal Bovine Serum (FBS) and Trypsin were obtained from Gibco, Invitrogen, USA. Human carcinoma colorectal Caco-2 cell line was provided by the National cell bank of Iran, Pastur institute. All cell culture disposable equipment was provided by Orange, Belgium. The total protein assay kit was obtained from Pars Azmoon, Iran. RPMI 1640 – Powdered Cell Culture Medium was a product of PAA Co, Austria and Trypan blue was purchased from Biosera, France.

2.2 Methods

2.2.1 Cell culture procedures

All operations were carried out using standard sterile conditions under a laminar flow cabinet. The cabinets were routinely sterilized by exposure to ultra-violet radial ion and then washed in 70 % alcohol prior to use. Caco-2 cells were routinely maintained in culture dishes (T75 falkons, Orange, Belgium) at 37 °C in 5 % CO2 atmosphere, using RPMI-1640 supplemented with 10 % FBS, 1 % sodium pyruvate, 1 % non-essential amino acids and 1 % L-alanine-glutamine 200 mM. The medium was exchanged every other day and cells were detached after reaching 90 % ■confluency washing ■author: please check term■ with PBS, detached with 0.25 % trypsin and 0.02 % EDTA.

2.2.2 MTT test assay

Cells were trypsinized and centrifuged at 1 000 rpm for 5 min. After resuspending, the cells were counted and diluted to receive 15 000 cells in 200 µL in each well. 200 µL of cell suspension was added to each well except those within the last column. Fresh medium was added to the last column to maintain humidity and prevent an „edge effect". The well-plate was put into an incubator overnight. On the following day, series of dilutions of Tween excipients were prepared and the medium of columns 2 to 11 was removed and cells were seeded with different dilutions of excipients (triplicate). Fresh medium was added to column 1 (control). Cells were incubated for 24 h and on day 3 of the experiment, media were removed from wells and cells were washed with PBS (phosphate buffered saline); then 50 µL of MTT solution (2 mg/mL) were added and incubated for 4 h. MTT solution was removed and MTT-formazan crystals were dissolved in 200 µL DMSO and 25 µL Sorensen buffer. The absorbance rate was recorded at 570 nm and the cell viability percentage was calculated [35].

2.2.3 Assessing the uptake of Rhodamine-123

For the uptake studies, Caco-2 cells were seeded into 24-well plates and left for 24 h. On the following day, the old medium was removed and cells were washed with PBS. Then new culture media containing different concentrations of excipients and ■0.3 mM■ author: please verify messurements: μM? unify ■ verapamil, as P-gp inhibitor [36], were added and left for another 24 hours. On day 3 of the experiment, the old medium was removed and cells were washed three times with PBS and Rho-123 solution (RPMI containing ■10 mM■ HEPES (pH=7.4) and 5 μM Rho-123) were added and incubated in 37 °C for 3 h. After the incubation period, Rho-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 1 000 rpm for 5 min. Supernatant was used to measure the fluorescence and total protein content. The quantity of Rho-123 was calculated. Then cellular Rho-123 accumulation was normalized to the total protein content determined by protein the assay kit [37].

2.2.4 Western blotting

Cells were moved to a 6-well plate in the density of 10^6 cells per well and treated for 24 h with the culture medium (control) or a culture medium containing Tween 20 (0.01 %, 0.001 % (w/v)), Tween 40 (0.05 %, 0.01 % (w/v)) or Tween 80 (0.01 %, 0.001 % (w/v)). Solutions were removed and cells were washed with PBS, then incubated in 37 °C for 5 min with Trypsin/EDTA 0.25 %. The supernatant was removed and cell sediment was washed twice with PBS. Lysis buffer (Triton X-100 50 mM, Tris-HCl, pH=7.4, NaCl 150 mM, EDTA 5 mM, 1 % protease inhibitor cocktail) was added and cell suspension was centrifuged in 15 000 rpm for 5 min. 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 a Polyvinylidene difluoride (PVDF) membrane using semi-dry Western blotting; 3 % dried skim milk was used to block the membrane for 1 h at room temperature and the membrane was washed 3 times with PBS and 0.1 % Tween 20 (PBS-T) and then incubated overnight with a primary monoclonal antibody (Anti-β-actin), diluted 1/1 000 in PBS-T. After washing with PBS-T, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies for 2 h. Membrane was washed and solution A and B of the Enhanced Chemiluminescence (ECL) kit were added. After that the membrane was exposed to X-ray film. The membrane was washed twice and incubated with a MDR1 antibody (C219) overnight. ■After washing, the membrane was put into horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies for 2 h. The membrane was washed and then solution A and B of ECL kit were added, after which the membrane was exposed to X-ray film.■ author: This paragraph is double, is the procedure actually done twice? If so I would add “again”. Please check!■

3. Results and discussion

The cytotoxicity of excipients on cells was evaluated using an MTT test assay. The MTT test assay of the excipients indicated the proper concentration of excipients which were used in Western blotting and uptake of Rho-123 in the following steps. ■The optical density (OD) value obtained from the ELISA reader was divided to that of the control samples ■author: ok?■ and cell viability for each excipient was calculated after 24 h exposure to different concentrations. Two maximum nontoxic concentrations were selected for Western blotting and the Rho-123 uptake test. According to the results, cell viability was significantly decreased after the treatment with concentrations of 0.05 % to 4 % (w/v) of Tween 80 and Tween 20, but treatment with 0.05 % (w/v) Tween 40 showed no toxicity (Fig. 1).

■Fig. 1■

To investigate the functional activity of P-gp, Caco-2 cells were incubated ins 48-well plates with different concentrations of excipients for 24 h, then cells were washed with PBS and exposed to Rho-123 (5 μM) for 3 h. Cells were lysed and the 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 with the protein assay kit and the cellular Rho-123 accumulation was normalized with respect to the total protein in each well (Fig. 2). Photos taken with the immunofluorescence microscope, (Fig. 3), showed an increase in Rho-123 uptake to cells which had been treated with Tween 40 (0.05 % w/v).

■Fig. 2■

■Fig. 3■

The P-gp expression was measured in Caco-2 cells which were treated for 24 h with excipients and compared to that of the control samples. 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-T and 3 % dried skim milk at room temperature for 1 h and washed three times for 15 min in PBS-T. Encountering primary and secondary antibodies, the bands were visualized using ECL Western blotting detection reagents and exposed to an X-ray film (Fig. 4).

■Fig. 4■

For orally administered drugs, membrane efflux proteins located in intestinal cells are challenges reducing drug bioavailability. The findings of this study proved that there are some excipients which could down regulate the MDR1 gene expression and the P-gp protein expression leading to improvement in drug bioavailability. The present study, characterizes the effects of excipients on the P-gp expression and the activity in the Caco-2 monolayer. Western blotting confirms the Rho-123 uptake data. Those excipients which were able to increase the Rho-123 accumulation showed decrease in P-gp expression as well.■author:ok?■ This study aimed to access a rational drug formulation development strategy for oral dosage forms based on the Caco-2 monolayer as an *in vitro* screening model.

The results showed that Tween 20 and Tween 40 are able to inhibit the P-gp efflux pump, as indicated by an increase in the Rho-123 accumulation and confirmed by lightening in the P-gp band according to the Western blotting.

MDR proteins, belonging to ABC transporters, are membrane transport proteins which detoxify cells from external substrates. These proteins are known to limit absorption through biological membranes such as intestinal, brain and cancer cells [38]. Some ABC transporters seem relatively specific to their endogenous substrates while others such as P-gp, exhibit a broad substrate spectrum [39]. Ligand-based approaches have demonstrated that P-gp substrates and inhibitors are hydrophobic, partitioning into membrane, and many of them have net positive charge [40] containing hydrogen bond acceptors [40, 41].

Knowing P-gp substrates may have advantages whether for finding blockers to enhance drug BA and prevent resistant to anti chemotherapies or for using a P-gp inducer to slow the progression of the Alzheimer's disease [42]. Using equilibrium and kinetic radioligand binding, at least 4 sites are suggested for P-gp to interact with substrates. While sites 1, 2 and 3 are known to be in relation with transport activities and interact with Rho-123, vinblastin and paclitaxel, site 4 is believed to be a regulatory site and modulators could interact with it which prevents substrates from binding to P-gp and results in P-gp inhibition [43]. The choice of an experimental method is a concern in P-gp studies. Data from different labs may also have significant differences [44]. For instance, midazolam has been realized as non-substrate, an inhibitor and a substrate in three different studies. Same as Midazolam, Doxorubicin has been identified both a substrate and a non-substrate [45]. A report claims that Tween 80 decreased the ratio of the serosal-mucosal transport to the mucosal-serosal transport of Rho-123 across the rat jejunal membrane *in vitro* and the Caco-2 cell monolayer suggestive of P-gp inhibition [46]. In addition, the *in vitro* absorption of digoxin across an everted rat gut sac (a P-gp substrate) was increased after the treatment with Tween 20 (0.5 % w/v) and Tween 80 [47]. Co-administration of digoxin with Tween excipients showed an increase in AUC and C_{max} in rats while Tween 80 was not able to increase the Rho-123 uptake and Western blotting bands were not lightened when treated with concentrations 0.01 % (w/v) and 0.001 % (w/v) of Tween 80 either [48]. It should be considered that the concentrations used in this study were less than those of mentioned studies as further concentrations were toxic to cells according to the MTT results.

Although usage of Caco-2 cells is common for P-gp studies[49], P-gp expression in this cells is depended on the time [50], culture conditions [49], passage number and also passage procedure [49]. Therefore variable expression levels must be taken into account and a theory says that Caco-2 cells even over express P-gp [51]. A Western analysis on Caco-2 cells showed P-gp to be expressed earlier than day 7, but verapamil transport study proved that it may not be fully functional until day 17 [52].

In this study, Tween 20 and Tween 40 at concentration of 0.01 % (w/v) and 0.05 % (w/v), respectively, were se-

lected to be evaluated in the Rho-123 accumulation assay. Both excipients increased the Rho-123 uptake by 200 %. Western blotting results confirmed the Rho-123 uptake findings.

Several studies have conducted to investigate the mechanism of inhibition of P-gp by surfactants. Such inhibition of P-gp activity *in vivo* could cause drug–drug interactions, alter the pharmacokinetic profiles of drugs that are P-gp substrates and increase/decrease toxicity. Although the results presented in this study suggest that Tween 20 and Tween 40 are able to inhibit P-gp both activity and expression *in vitro*, the possible mechanism of P-gp inhibition through excipients is, at present, unknown.

Taken together, some factors such as P-gp structure, P-gp environment and substrate partitioning into these tissues should be understood fully to judge the ability of these commonly used excipients to inhibit P-gp activity *in vitro* and to further characterize the effect of commonly used excipients on both activity and expression of P-gp. There should be more data generated on the structure of each binding site, which would then ultimately lead to explain the variability in data and also gives models for each binding site. Additional experiments e. g. gut perfusion studies in rats, pharmacokinetic studies in animals or more specific assays i. e. assays which target specific binding sites on protein, should be carried out to examine the effects of these commonly used excipients *in vivo* and enable us to build more specific models.

4. Conclusion

In this study, Tween 20 (0.01 % w/v) and Tween 40 (0.05 % w/v) had significant inhibitory effects on either the activity or the expression of P-gp. Therefore, the usage of Tween 20 and Tween 40 in above mentioned concentrations may be a reasonable formulating approach to increase drug bioavailability.

Acknowledgements

The authors would like to thank the authorities of the Drug Applied Research Center, Tabriz University of Medical Sciences, for their financial support.

Conflict of Interest

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

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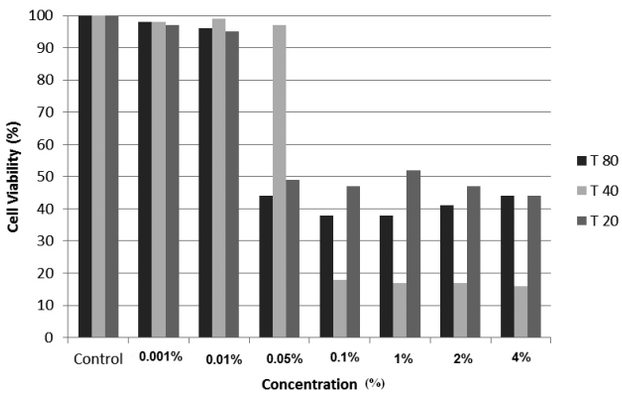

Fig. 1: Effects of Tween 80, Tween 40 and Tween 20 on cell viability in Caco-2 cells. MTT assays were performed to measure the survival rate of Caco-2 cells after treatment with Tweens. Data are expressed by the mean of percent cell viability compared to control after exposure for 24 hours ± standard deviation (n=6). (Source: all figures have been generated by the authors).

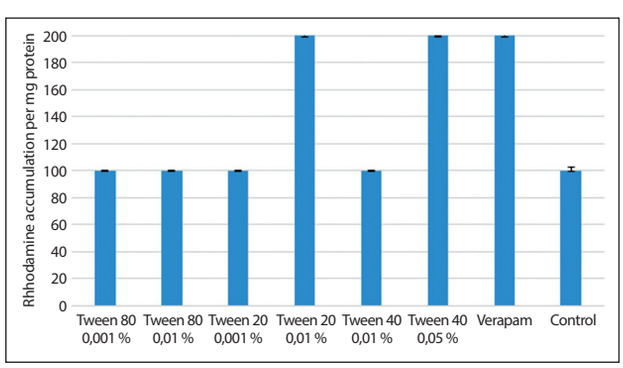

Fig. 2: Tween 20 (0.01 % w/v) and Tween 40 (0.05 % w/v) enhance Rho-123 uptake into Caco-2 cells. Caco-2 cells were treated for 24 hours with different concentrations of excipients and 0.3 mM verapamil as positive control for P-gp inhibition. Data are expressed by the ratio of quantity of Rho-123 (mg×10^6/mL) to total protein (mg/mL) in each well. Values were compared with control group using one way ANOVA with Student-Newman-Keuls post hoc test (P< 0.001).***

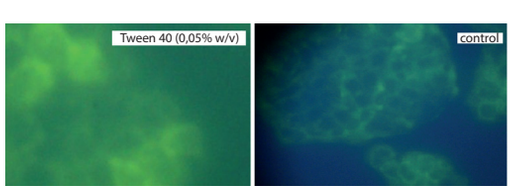

Fig. 3: Photos taken by immunofluorescence microscope showed increase in intercellular accumulation of Rho-123 in cells treated with excipients compared to control.

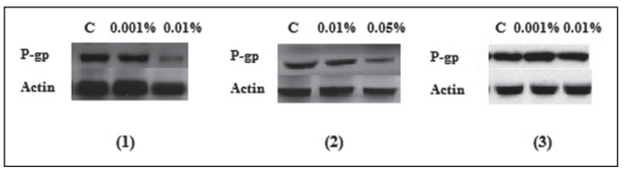

Fig. 4: P-gp protein expression after 24 hour exposure to Tween 20 (1), Tween 40 (2) and Tween 80 (3). Expression in treatment groups were compared with P-gp expression in untreated control cells (C).

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