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Inhibitory effect of clemastine on P-glycoprotein expression and function: an *in vitro* and *in situ* study

Mehran Mesgari Abbasi [1, 2], Hadi Valizadeh [1], Hamed Hamishekar [1], Leila Mohammadnejad [3], Parvin Zakeri-Milani [4*]

[1] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Students Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Liver and Gastrointestinal Diseases Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

Objective(s): Transporters have an important role in pharmacokinetics of drugs. Inhibition or induction of drug transporters activity can affect drug absorption, safety, and efficacy. P-glycoprotein (P-gp) is the most important membrane transporter that is responsible for active efflux of drugs. It is important to understand which drugs are substrates, inhibitors, or inducers of P-gp to minimize or avoid unwanted interactions. The aim of this study was to investigate the effects of clemastine on the expression and function of P-gp.

Materials and Methods: The effect of clemastine on P-gp function and expression was evaluated *in vitro* byrhodamine-123 (Rho_{123}) efflux assay in Caco-2 cells and Western blot analysis. Rat *in situ* single pass intestinal permeability model was used to investigate the clemastine effect on digoxin P_{eff}, as a known P-gp substrate. Digoxin levels in intestinal perfusates were assayed by high performance liquid chromatography (HPLC) method.

Results: The Caco-2 intracellular accumulation of Rho_{123} in clemastine and verapamil treated cells was 90.8 ± 9.8 and 420.6±25.4 pg/mg protein, respectively which was significantly higher than that in control cells (50.2±6.0; $P<0.05$). Immunoblotting results indicated that clemastine decreased expression of P-gp in Caco-2 cells *in vitro*. More over effective intestinal permeability (P_{eff}) of digoxin in the presence of clemastine, was significantly increased compare to control group.

Conclusion: Findings of our study suggested dose dependent P-gp inhibition activity for clemastine *in vitro* and *in situ*. Therefore co-administration of clemastine with P-gp substrates may result in unwanted interactions and side effects.

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Introduction

In recent years, transporter-mediated drug-drug and food-drug interactions are being reported with increasing frequency. It is clear that transporters have an important role in absorption, distribution, metabolism, and elimination of drugs. Induction or inhibition of drug transporters can affect drug absorption, safety, and efficacy. Therefore, study of potential interactions during the process of drug development has been noticed (1, 2). Drug transporters are classified into: solute carriers (SLC) and ATP-binding cassettes (ABC) (3).

P-glycoprotein (P-gp) is an ABC transporter and the most important membrane transporter that is responsible for active efflux (secreting) of passively diffused xenobiotics and drugs, out of the cell (4, 5). P-gp is expressed in the epithelia cells of gastrointestinal tract and many other organs. It is involved in the efflux of various kinds of drugs and substances with different chemical structures, out of the cells. After oral administration followed by complete dissolution of a drug, inhibition of P-gp by the drug can result in increased absorption of any co-administered drug that is P-gp substrate (1, 2). Therefore, P-gp-related interactions have critical clinical role and it is important to understand which drugs are substrates, inhibitors, or inducers of P-gp to minimize or avoid unwanted interactions (5, 6).

Clemastine (Figure 1), a competitive antagonist of H_1 histamine receptor, is a widely used, first-generation antihistamine. The drug is an ethanolamine derivative with the following formula: ((+)-2-[2-[(p-chloro-a-methyl-a-phenylbenzyl)oxy]ethyl]-1-ethylpyrrolidine). Clemastine inhibits the vasodilator and vasoconstrictor effects of histamine, and the constriction of respiratory and gastrointestinal smooth muscles; therefore, it is

**Corresponding author:* Parvin Zakeri-Milani. Department of Pharmaceutics, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. 51664. Tel: +98-411- 3392593; Fax: +98-411-3344798; email: pzakeri @tbzmed.ac.ir

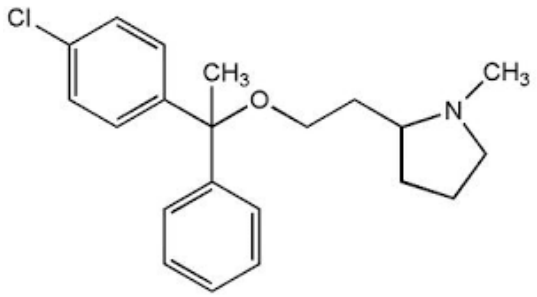

Figure 1. The chemical structure

used for the relief of symptoms associated with allergic rhinitis and skin allergies (7, 8). Clemastine is very slightly soluble in water and is rapidly absorbed from the gastrointestinal tract after oral administration. Clemastine is metabolized mainly by mono- and di-demethylation and glucuronide conjugation. Its main route of elimination is urinary excretion, with the elimination half-life being approximately 21 hr (9) of clemastine ($C_{21}H_{26}C_lNO$) (8).

The current study was designed to investigate the *in vitro* and *in situ* effects of different concentrations of clemastine on the membrane transporter function of P-gp, and its expression. *In vitro* study is performed by assayingrhodamin-123 (Rho_{123})accumulation in Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma) cells after treatment with clemastine and verapamil. Rho_{123} and verapamil are known as P-gp substrate and inhibitor, respectively. Expression of P-gp in Caco-2 cells, after treating with clemastine and verapamil is also assessed by Western blot method. Moreover intestinal effective permeability (P_{eff}) of digoxin, as a well-known P-gp substrate, is investigated *in situ* in the presence and absence of clemastine and verapamil, using single pass intestinal perfusion (SPIP) technique in rats.

Materials and Methods

Materials

Clemastine fumarate, digoxin, verapamil, Rho_{123}, 3-(4,5-dime-thylthiazol-2-thiazolyl)-2,5-diphenyl-tetrazoliumbromide (MTT), penicillin-streptomycin, and protease inhibitor cocktail were obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-P-gp mouse monoclonal antibody (ab80594) and rabbit polyclonal antibody to beta Actin (ab16039) were purchased from Abcam (USA).Horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin (IgG) (AP8036) and anti-rabbit IgG (AP7181) were purchased from Razi Biotech (Iran).Dulbecco's modified Eagle medium (DMEM), trypsin-EDTA (0.25%), fetal calf serum (FCS) were purchased from Gibco (Carlsbad, CA, USA). Tissue culture flasks and other disposable cell culture items were purchased from TPP Co. (Switzerland). Enhanced chemiluminescence (ECL) Western blot detection kit, medical X-ray film, pre-stained protein ladder (10-250kDa), and protein assay kit were purchased from Amersham (GE Healthcare, Chalfont St. Giles, UK)), Fuji (Tokyo, Japan), Cinagen, and Pars Azmoon (Iran), respectively. All other chemicals were purchased from Merck Co. (Darmstadt, Germany).

Cell culture

The Caco-2 cells were obtained from Pasteur Institute of Iran (Tehran, Iran). The cells were routinely cultured in DMEM with 10% heat inactivated fetal bovine serum, 1% penicillin-streptomycin, and 1% non-essential amino acids. Cells were placed in a CO_2-incubator (Memmert, Germany), at 37 °C with 90% relative humidity and 5% CO_2 atmosphere. The culture medium was replaced 2-3times per week. After reaching 80-90% confluency, the cells were detached from the culture flask by adding 0.25% trypsin-EDTA solution to seed them in well-plates or flasks (2).

In vitro cytotoxicity study (MTT assay)

The cytotoxic effect of different concentrations of clemastine (0.01, 0.1, 0.5, 1.5, 5, and 10 µM) on Caco-2 cells were determined, using MTT assay. The Caco-2 cells were seeded into 96-well plates at a density of 15×10^3 cells per well. After 24 hr, the medium was replaced with 200 µl per well of clemastine at different concentrations diluted with complete culture medium. After 24 hr incubation, the cells were washed by PBS, and 50 µl of the MTT solution (2 mg/ml) was added to the wells and incubated for 4 hr at 37 ˚C in a CO_2 incubator. Then MTT solution was removed and the resulting for mazan crystals were solubilized with 200 µl/well of DMSO and 25 µl/well Sorensen's phosphate buffer(mix 80.4 ml of 133 mM Na_2HPO_4 and 19.6 ml of 133 mM KH_2PO_4, pH = 7.4). The optical densities (ODs) were detected with an ELISA microplate reader (Statfax-2100, Awareness, USA) at 570 nm with background subtraction at 630 nm. The following formula was used for calculating the percentage of cell viability (10). The MTT assay was performed in triplicate for control and each concentration of test agents.

$$\text{Cell viability } \% = \frac{\text{OD value of test}}{\text{OD value of control}} \times 100$$

The cell viability was calculated for each concentration and analyzed statistically. The results were expressed as mean ± standard deviation (SD).

In vitro Rho_{123}efflux assay

P-gp function in Caco-2 cells was assayed by measurement of intracellular accumulation of Rho_{123}, a known P-gp substrate, which is inversely proportional to P-gp activity. Caco-2 cells were seeded in 24-well plates and allowed to attach for 24

hr in CO_2-incubator. The old medium was removed and cells were washed with PBS. New culture media containing clemastine (0.01, 0.5, and 10 μM) or verapamil (300 μM) was added to wells. After 24 hr, the old medium was removed and cells were washed three times with PBS. Rho_{123} solution [DMEM containing 10 mM HEPES (pH=7.4) and 5 μM Rho_{123}] was added and incubated for 3 hr at 37 °C, followed by washing three times with ice-cold PBS (pH = 7.4). Cells were lysed in 1% Triton X-100 and centrifuged (3-18k, Sigma, UK) at 1500 g for 10 min. Supernatant was used formeasuringRho_{123} and protein contents. The intracellular accumulation of Rho_{123} in samples were determined quantitatively by fluorescence spectrophotometry at exciting wavelength of 485 nm and emission wavelength of 532 nm (FP-750, Jasco, Japan). Protein content of the samples was evaluated by a commercial protein assay kit (Pars Azmoon, Iran), and Rho_{123} values were normalized by the protein content of each sample (supernatant) (11, 12).

Western blot analysis of P-gp expression in Caco-2 cells

Expression of P-gp in Caco-2 cells was assessed by Western blot after the cells were treated with clemastine, and verapamil. Caco-2 cells were seeded in a 6-well plate at the density of 10^6 cells per well. After 24 hr, the cells were treated with clemastine (10 μM), and verapamil (300 μM) (n = 4). Culture medium alone was considered the control (n = 4). After 48 hr, solutions were discarded, the cells were washed (PBS), and incubated for 5 min with 0.25% trypsin-EDTA at 37 °C. The detached cells were washed twice with PBS. Lysis buffer (Triton X-100 50 mM, NaCl 150 mM, EDTA 5 mM, 1% protease inhibitor cocktail, and Tris-HCl, pH=7.4)was added, and cell suspension was centrifuged at 1500 g for 5 min. Proteins were resolved by 12.5% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE)(80 V, 120 min) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry Western blotting system (Bio-Rad, USA). All membranes were blocked for nonspecific binding, by incubation in 3% nonfat-dried milk for 1 hr, at room temperature. Then, the membrane was washed 3 times with Tris buffered saline with 0.1% Tween 20 (TBS-T), and incubated overnight with mouse monoclonal anti-P-gp antibody (1/1000 in TBS)(ab80594, Abcam, USA). The next day, membranes were washed with TBS, and then incubated with HRP-conjugated rabbit anti-mouse secondary antibody (AP8036, Razi Biotech, Iran), for 2 hr. The membranes were washed and the proteins were then detected using an ECL kit (Amersham, GE Healthcare, Italy). The proteins were visualized by exposing the membrane to a medical x-ray film (Fuji, Japan) for 5 min in a dark room. β-Actin was considered as the internal standard and detected using rabbit polyclonal anti-β-actin as primary antibody (ab16039, Abcam, USA). HRP-conjugated goat anti-rabbit IgG was the secondary antibody (AP7181, Razi Biotech, Iran). P-gp expression is presented as the ratio of intensity of P-gp band to β-actin band in the same blot run (P-gp/ β -actin) (13).

***In situ* study**

Animals

Male Sprague–Dawley rats (200–250 g) were supplied from Pasteur Institute of Iran. The animals were housed in standard cages with a 22±2 °C temperature, a 12/12 hr light/dark cycle, 55**%**–60% relative humidity, and free access to standard rodent chow and clean water. Prior to experimentation, rats were fasted for 12-16 hr (water *ad libitum*). All experiments were conducted in accordance with the humanity and animal ethic protocols. The animal study protocols were approved by the Research Ethical Committee of Tabriz University of Medical Sciences (Ref No: TBZMED. REC.1394.378). In all animal studies "Guide to the care and use of experimental animals" by Canadian Council on Animal Care, was followed.

In situ SPIP method

The animals were anesthetized with an IP injection of thiopantal sodium (60 mg/kg) (LogiChem, UK). A 3-4 cm incision was made in abdominal midline and approximately 9-11cm of the jejunum segment of the intestine was selected and cannulated at both ends with polypropylene tubes. The exposed segment was kept moist with body tempered saline during the experiment. Before injection of the main drug solution, body tempered normal saline was passed through the cannulated segment to wash and clear the lumen of the segment. PBS (pH =7.4) containing 50 mg/l phenol red without drug (blank solution) was pumped through the segment, at a constant flow rate of 0.2 ml/min (Q_{in}). Blank perfused solution samples were collected and used to prepare digoxin calibrator solutions for HPLC and also for stability studies.

The SPIP method was carried out in anesthetized rats, as described above, and the blank solution containing the substance of interest was perfused at a constant flow rate of 0.2 ml/min, using a volumetric infusion pump (Argus Medical AG, Heimberg, Switzerland). After reaching the C_{out}/C_{in} ratio to a steady state (Figure 2), each perfusion experiment lasted for 90 min, and perfusates were quantitatively collected at 10 min intervals (2 ml). Some changes, such as water absorption and secretion, during perfusion may cause errors in the calculated permeability values. Therefore, 50 mg/l phenol red was added as a non-absorbable marker to correct the results. Digoxin (20 μM, a typical substrate of P-gp), verapamil (300 μM, a typical P-gp

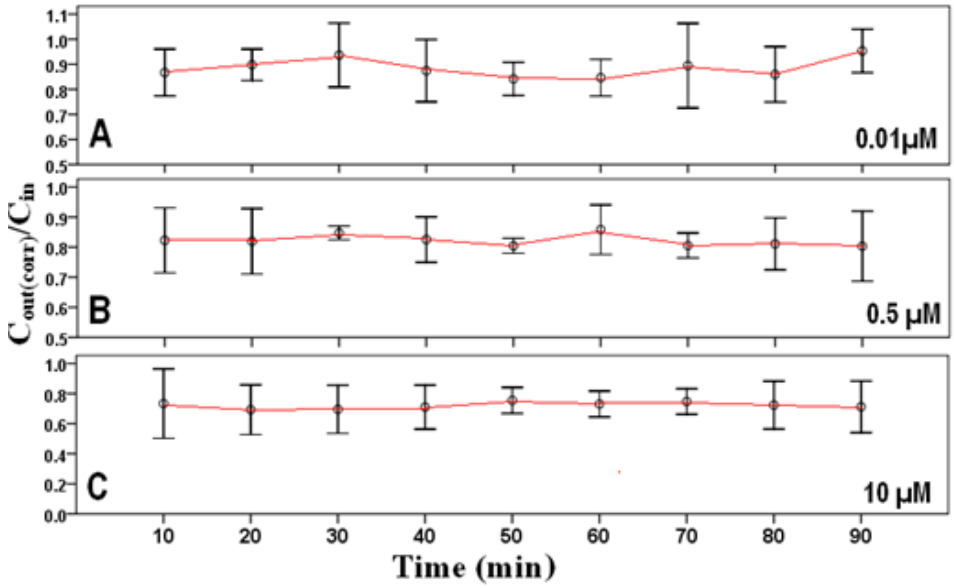

Figure 2. Plots of inlet/outlet concentrations ratio (C_{in}/C_{out}) of digoxin (20 µM) versus time in the presence of (A) 0.01, (B) 0.5, and (C) 10 µM clemastine in single pass intestinal permeability (SPIP) study (n = 3, error bars represent mean ± SD)

inhibitor), and different concentrations of clemastine (0.01, 0.5, and 10 µM), each one in a blank solution with 20 µM digoxin, were administrated, as described above (14, 15). The SPIP method was performed in at least three rats for each concentration of each drug (9 for clemastine, 3 for verapamil, and 3 for digoxin alone) and the blank solution. At the end of procedure, the length of the segment was measured (cm) and the animal was euthanatized. Samples were stored in an ultra-low temperature freezer (JalTajhiz Production, Karaj, Iran), at -70 °C until performing HPLC and phenol red analysis. The concentration of phenol red in the perfused (outlet) samples was measured at 560 nm using an ultraviolet-visible (UV-VIS) spectrophotometer (Ultra-spec 2000, Pharmacia, Pfizer, New York, NY, USA) (16). The amount of digoxin in the perfused samples was detected by HPLC.

HPLC analysis of digoxin in intestinal perfused samples

HPLC system (Smartline manager 5000, Smartline UV Detector 2600, and Smartline Pump 1000, Knauer Advanced Scientific Instruments, Berlin, Germany) was used to detect the amount of digoxin in the perfused samples. The mobile phase was 35% v/v of acetonitrile in water, which was filtered through a sintered glass filter P_5 (1.0-1.6 µm, Winteg, Germany) and degassed in a sonicator. The mobile phase was pumped in isocratic mode at ambient temperature. The flow rate was 2 ml/min. UV detection was performed at 218 nm, and the injection volume of samples was 20 µl. The HPLC column was Knauer-15VE081ESJ-150X 4.6 mm with precolumn-Eurospher 100-5 C8 (14).

Data analysis

P_{eff} values were calculated from concentrations of compounds in the outlet perfused samples, at the steady state. The steady state, as shown in Figure 2, was confirmed by the ratio of the outlet to inlet concentrations (corrected for phenol red concentrations) versus time (14, 15).

Phenol red correction

Corrected outlet concentration of the digoxin (C_{out}) was calculated using the following equation (17).

$$C_{out\,(corr)} = C_{out} \times \frac{CPR_{in}}{CPR_{out}}$$

Where:

$C_{out(corr)}$ = corrected outlet concentration of the drug

C_{out}= outlet concentration of the drug

CPR_{in} = concentration of phenol red entering the intestinal segment.

CPR_{out} = concentration of phenol red leaving the intestinal segment.

Calculations were based on outlet perfusate steady state concentrations after the selected time points. The steady state intestinal$P_{eff}$was calculated according to this equation:

$$P_{eff} = \frac{-Q_{in} \times ln\,(C_{out}/C_{in})}{2 \times 60\pi rl}$$

Where:

P_{eff} = Effective permeability (cm/s)

Q_{in}= perfusion rate (0.2 ml/min)

C_{in}= concentrations of the test drug entering the segment

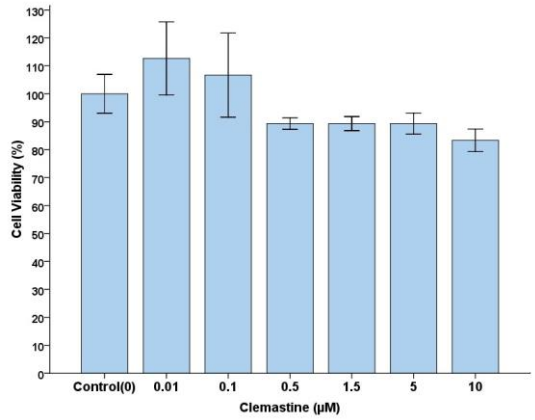

Figure 3. Effects of different concentrations of clemastine on cell viability in Caco-2 cells. Bars show mean ± SD of at least 3 measurements. * *P*<0.05was considered as significance level

C_{out} = concentrations of the test drug leaving the segment
r = the radius of the intestinal segment (≈ 0.18 cm)
l = the length of the intestinal segment (cm)

Statistical analysis

Data are presented as the mean ± S.D. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical tests were done with SPSS13.0 (SPSS Inc., Chicago, IL, USA), where *P*-value (*P*<0.05 and *P*<0.01) were considered statistically significant.

Results

Preliminary experiments showed that no considerable adsorption of compounds on the tubing and syringe took place. The experiments also demonstrated stability of the drugs during the experiments.

In vitro cytotoxicity study (MTT assay)

The results of MTT cytotoxicity tests showed that clemastine had no cytotoxic effect on Caco-2 cells in the range of used concentrations (Figure 3).

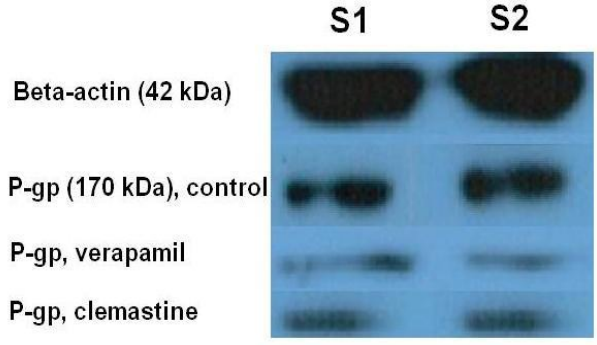

Figure 5. Western blot of P-gp and β-actin in clemastine (10 μM) and verapamil (300 μM) treated, and control (non-treated) Caco-2 cells (bands of 2 samples of each are shown)

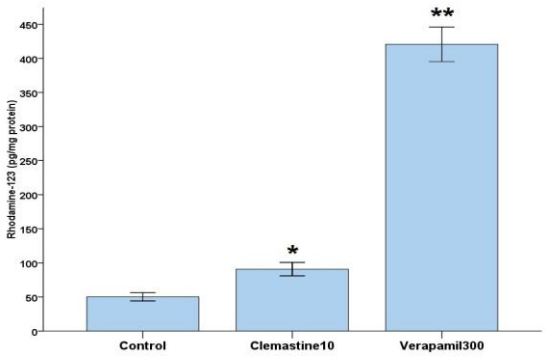

Figure 4. Intracellular concentrations of rhodamine-123 in Caco-2 cells after treating with clemastine (10 μM) and verapamil (300 μM) (n=4). Bars show mean ± SD.
* (*P*<0.05) and ** (*P*<0.01), significantly different from control cells (untreated cells)

Effect of clemastine on the efflux function of P-gp in Caco-2 cells (Rho_{123} uptake)

Rho_{123} efflux activity of P-gp in Caco-2 cells was used to investigate the effect of clemastine on the function of P-gp as an efflux pump. The cells were treated with 10 μM clemastine and 300 μM verapamil, in addition to Rho_{123}. Intracellular concentration of Rho_{123} was significantly increased from 50.2 ± 6.0pg/mg protein (mean±SD) of control (non-treated) to 90.8 ± 9.8 (*P*<0.05) and 420.6±25.4 (*P*<0.01) pg/mg protein of clemastine and verapamil treated cells, respectively (Figure 4).

Immunoblotting

Expression of P-gp in clemastine and verapamil treated Caco-2 cells were investigated using immunoblotting method. The β-actin protein expression was considered as internal immunoblotting control. P-gp expression was evaluated as the P-gp band intensity to β-actin band intensity ratio (P-gp/ β-actin), and was compared with P-gp bands of verapamil treated and non-treated Caco-2 cells bands in the same blot run (14). As illustrated in Figure 5, low density of Western blot bands of clemastine treated cells related to those of untreated cells, is evidence of low expression of P-gp, which indicates the inhibition effect of clemastine on P-gp expression.

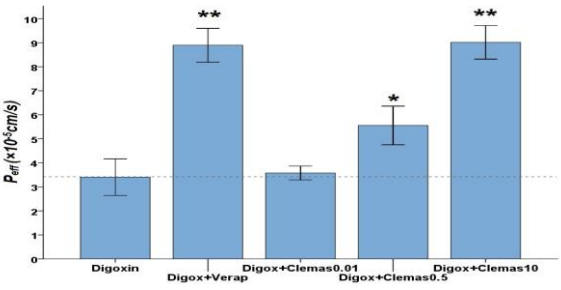

Figure 6. Effects of different concentrations of clemastine (0.01, 0.5, and 10 μM) and verapamil (300 μM) on the effective permeability (P_{eff}) of digoxin compared to the control group (digoxin) (n=3). Bars show mean±SD. * (*P*<0.05) and ** (*P*<0.01) **, significantly different from control (digoxin) group

SPIP study

As shown in Figure 6, the P_{eff} of digoxin did not significantly differ in presence and absence of 0.01 µM clemastine (P>0.05), relative to the control group. Whereas the P_{eff} of digoxin was significantly increased relative to the control group, to 5.6 ± 0.8 ×10^{-5} cm/s (mean ± SD) (P<0.05) and 9.0 ± 0.7 × 10^{-5} cm/s (P<0.01) in presence of 0.5 and 10 µM clemastine, respectively. The P_{eff} of control (digoxin alone) was3.4±0.8×10^{-5}cm/s, and the P_{eff} of digoxin in the presence of 300 µM verapamil, was 8.9 ± 0.7 ×10^{-5}cm/s (P< 0.01).

Discussion

Based on data from the *in vitro* Rho_{123} uptake study, treatment of Caco-2 cells with 10 µM clemastine increased the accumulation of Rho_{123} by 80.9% compared with control (Figure 4), suggesting that clemastine inhibits P-gp *in vitro*. Protein expression of P-gp in clemastine treated Caco-2 cells was lower than the control cells (Figure 5), confirming the inhibition of P-gp by clemastine. We also found *in situ* that, 0.5 and 10 µM clemastine significantly increased digoxin P_{eff} by about 65% and 165%, respectively (Figure 6). The results of the *in situ* study showed a dose dependent effect of clemastine, in the range of 0.01-10 µM, on P-gp. The enhancement can be resulted from inhibition of efflux activity of P-gp.

Putting together, our results strongly suggest that, clemastine is a P-gp inhibitor. This is in agreement with the prediction of Broccatellia *et al* using a reliable *in silico* model (18). The model was established after studying 1200 compounds structure and using various Molecular Interaction Fields (MIF)-based technologies. The pharmacophoric features and membrane partitioning related physico-chemical properties were also considered in creating the model (18). Clemastine was also present in the *in silico* structure-based screening of P-gp inhibitor library of Shityakov and Förster (19). The list was provided by the gradient optimization method, using polynomial empirical scoring functions. In their study the hydrophobic interactions between P-gp and selected drug substances were assessed as the main forces involved in the inhibition effect (19). Clemastine was tested *in vitro* in the MDR-MDCK cell model and *in situ* in the rat brain perfusion model by Obradovic *et al.* The *in vitro* model showed that the presence of cyclosporine-A (a typical inhibitor of P-gp) decreased efflux of clemastine, which indicates that clemastine is a P-gp substrate. But, the *in situ* perfusion results of the study demonstrated that the brain penetration of clemastine was not increased by co-perfusion with cyclosporine-A. Therefore, P-gp-mediated efflux appears not to be a limiting factor to the CNS penetration of clemastine (20). The *in vitro* result of the study was in contrary with the findings of Varma *et al*. In their study, clemastine was placed in non-P-gp-substrate list (21). On the other hand, the most frequent and significant adverse effects of clemastine reported in the literature are those that are related to CNS. Because of strong expression of P-gp in blood-brain barrier cells, it is probable that clemastine is not a P-gp substrate (7). However, a compound may be a substrate or inhibitor of P-gp or both of them (22) and therefore none of the studies are in contrary of our findings.

The therapeutic level of clemastine in blood plasma is 0.7-2 ng/ml (~1.5-4.4 nM) (23).Our findings demonstrated no P-gp inhibition activity for 0.01 µM solution of clemastine (Figure 6). Therefore, clemastine does not have P-gp inhibition activity in its plasma therapeutic levels. On the other hand, the maximum recommended oral dose for clemastine is 2.68 mg. Considering the intestinal fluid volume, after orally receiving the dose, the intestinal concentration of the clemastine is about 7.8 µM, that is more than 0.5 µM and is expected to show P-gp inhibition activity based on our findings (Figure 6).Despite widespread use of clemastine, there are only a few reports on the pharmacokinetics and metabolism of clemastine (8). The P-gp inhibitory effect of clemastine *in vitro* and *in situ*, to our knowledge, has not been previously reported.

In human intestine, P-gp is highly expressed on the brush-border membrane of epithelial cells. Nowadays, the majority of drugs are administered orally and it is important to keep in mind the potential for co-administration of compounds that are the P-gp substrates or inhibitors. The P-gp-mediated drug-drug interaction may result in increasing the permeability and drug concentration in body fluids, followed by unwanted adverse effects (24). Therefore, P-gp inhibition effect of clemastine may provide useful information for clinicians and researchers. However, the molecular mechanisms of the P-gp inhibition effect of clemastine is not clear and further investigations are necessary to find them out.

Conclusion

In conclusion, present study demonstrated that clemastine treatment can down-regulate the expression of P-gp *in vitro* and inhibit function of P-gp *in vitro* and *in situ*. The study provides useful information for predicting potential drug–drug interactions when clemastine is co-administered with drugs transported by P-gp and may reduce unwanted reactions. Further studies are recommended to verify the P-gp inhibition roles of clemastine and clarify the molecular mechanisms of the inhibition.

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Conflict of interest

The authors declare that they have no conflict of interest.

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