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Research Paper

Assessment of the First and Second Generation Antihistamines Brain Penetration and Role of P-Glycoprotein

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Purpose. The sedating effect of first generation H_1-antihistamines has been associated with their ability to penetrate the blood-brain barrier (BBB) and lack of efflux by P-glycoprotein (Pgp). Second generation H_1-antihistamines are relatively free of sedation and their limited brain penetration has been suggested to arise from Pgp-mediated efflux. The objective of this work was to evaluate the role of Pgp in brain penetration of first and second generation antihistamines.
Methods. Potential of antihistamines to be Pgp substrates was tested *in vitro* using Madin Darby canine kidney cells transfected with human Pgp. The role of Pgp in limiting brain penetration of antihistamines was tested by using the *in situ* brain perfusion technique.
Results. Majority of antihistamines were Pgp substrates *in vitro*. Following *in situ* brain perfusion, the first generation antihistamines substantially penetrated into rat brain independently from Pgp function. The second generation antihistamines terfenadine and loratadine, achieved substantial brain penetration, which was further enhanced by Pgp inhibition by cyclosporin A (CSA). In contrast, fexofenadine and cetirizine, penetrated brain poorly regardless of CSA administration.
Conclusions. Antihistamines greatly differ in their ability to cross the BBB as well as in the role of Pgp in limiting their transport into the CNS *in vivo*.

KEY WORDS: antihistamine; blood-brain barrier; P-glycoprotein; efflux; brain perfusion.

INTRODUCTION

Histamine H_1-receptor antagonists, or antihistamines, are commonly used for treatment of allergies such as rhinitis, urticaria and asthma (1). Once side effect associated with the administration of first generation antihistamines is diminished central nervous system (CNS) arousal, resulting in sedation (2). This sedation results from the inhibition of central histamine H_1 receptors upon the blood-brain barrier (BBB) (3). In contrast to first generation antihistamines, second generation antihistamines are largely devoid of significant sedative effects (1,2). The difference between first and second generation antihistamines in their CNS effects and brain penetration cannot be attributed to profoundly different physicochemical properties, plasma protein binding, or affinity for central H_1 receptors between the two generations of compounds (3–6). Recently, it has been proposed that the limited brain penetration of second generation antihistamines may be the result of their efflux from CNS via the P-glycoprotein (Pgp) pump located at BBB (7,8). Numerous reports have identified Pgp as a pump responsible for removal of xenobiotics out of the brain resulting in an amelioration of undesired CNS side effects (9–12). Recent studies found that several second generation antihistamines are substrates for P-glycoprotein (Pgp) and that Pgp-mediated efflux at the BBB results in low brain penetration of these compounds (7,8,13–16). However, it is unlikely that all second generation antihistamines have equally low potential to cross the BBB. Some second generation antihistamines, such as terfenadine, cetirizine, and loratadine can cause dose-dependent sedation (2,17,18), which is indicative of their potential to cross BBB. In contrast, another second generation antihistamine, fexofenadine, does not occupy high levels of central H_1 receptors and is devoid of sedative effects even at high doses (19,20). The objective of this work was to evaluate the Pgp-mediated efflux of first and second generation antihistamines and to determine if and to what extent Pgp can limit the brain penetration of antihistamines *in vivo*. First, we investigated whether the antihistamines are Pgp substrates *in vitro* using Madin Darby canine kidney cells transfected with human Pgp (MDR-MDCK cell monolayers), a model often employed as a screening tool for estimation of the drug CNS penetration potential and Pgp affinity (21–23). The penetration of antihistamines into the CNS and the Pgp role as a CNS permeability barrier were determined using the *in situ* rat brain perfusion method. To date, no report has investigated the transport of antihistamines into the brain using this technique. All studies investigating the role of Pgp in the brain disposition of antihistamines *in vivo* have relied on comparisons of brain-to-plasma (B/P) ratios between wild-type and Pgp-knockout

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ABBREVIATIONS: BBB, blood-brain barrier; CSA, cyclosporin A; CNS, central nervous system; K_{in}, unidirectional transfer constant; Papp, apparent permeability coefficient.

mice (8,13,14,24). Determination of B/P ratios for Pgp substrates can often result in ambiguous results where the role of Pgp in limiting brain permeation can be overestimated (25). In addition, recent results demonstrated that knocking out Pgp in the mouse results in changes in the expression of other transporters as well as the level of drug transport across the BBB (26,27). Thus, in Pgp knock-out animals, the brain penetration of antihistamines may be affected by the alteration of other transport systems at the BBB. In this study, by performing *in situ* brain perfusion experiments in wild-type rats, it is possible to compare the ability of first and second generation antihistamines to cross the BBB and the contribution of Pgp to the resistance of the BBB permeability. The first generation antihistamines, clemastine, pyrilamine, chlorpheniramine, diphenhydramine, hydroxyzine, and brompheniramine, and the second generation antihistamines, terfenadine, loratadine, fexofenadine and cetirizine were tested *in vitro* in the MDR-MDCK cell model, and in the *in situ* rat brain perfusion model which maintains the physical integrity of the normal rat brain.

MATERIALS AND METHODS

Materials

Cell culture media, bovine serum albumin, medium supplements (L-glutamine, 20 mM sodium bicarbonate, non-essential amino acids, sodium pyruvate, colchicine), Hanks' balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Krebs Ringer Bicarbonate buffer and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Transwells (12-well, 1.13 cm^2 area, 0.4 μm pores) were purchased from Costar (Cambridge, MA).

Drugs

Clemastine, terfenadine, loratadine, fexofenadine, pyrilamine, chlorpheniramine, brompheniramine, diphenhydramine cetirizine, and hydroxyzine were purchased from Sequoia Research Products (Pangbourne, UK). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals used were of reagent grade.

In Vitro Cell Culture Studies

Multi-Drug Resistance Madin-Darby Canine Kidney (MDR-MDCK) cells were obtained from NIH (National Institute of Health) (Bethesda, MD). Cells were maintained in minimum essential Eagle's medium containing 2 mM L-glutamine, 20 mM sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% bovine serum albumin supplemented with 0.2 mM colchicine to maintain Pgp expression. For permeability experiments, cells with passage numbers 24–33 were seeded at a density of 60,000 cells/cm^2 on rat type I collagen-coated polycarbonate membranes in 12-well transwell plates. The experiments were performed on the eighth day after seeding. Prior to the permeation assay, the transepithelial electrical resistance (TEER) was measured on each cell monolayer using an Endohm Voltameter (World Precision Instruments, Sarasota, FL). The MDR-MDCK cells used in transport experiments had a high TEER value ranging from 1,800 to 2,000 Ω cm^2. The permeability assay buffer was Hank's balanced salt solution containing 10 mM HEPES and 15 mM glucose at pH 7.4. The cells were dosed on the apical side and basolateral side, incubated at 37°C with 5% CO_2 and 90% relative humidity under shaking conditions (150 rpm). The test compounds were first prepared in stock solutions of 10 mM in 100% DMSO. Then, compounds were added to a permeability assay buffer at final concentration of 10 μM with solvent DMSO concentration of 0.1% for all experiments. In the Pgp inhibition assays, the cells were pre-incubated with 10 μM cyclosporin A (CSA), a known Pgp inhibitor, for 30 min and same concentration of CSA was present in the apical and basolateral assay buffer throughout the experiments. Drug permeation was tested in two directions, apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A), in triplicate. Sampling was done every 30 min for 2 h. The apparent permeability coefficient (Papp), was calculated as follows:

$$\text{Papp} = (dC_r/dt)/A \times C_0$$

where, dC_r/dt is the slope of the cumulative concentration in the receiver compartment *versus* time in $\mu M/s^{-1}$; A is the area of the cell monolayer; C_0 is the initial concentration in the donor chamber. The linear portion of the cumulative receiver compartment concentration *versus* time was used to calculate the slope.

Brain Perfusion

The *in situ* rat brain perfusion procedure used was similar to that previously described (28,29). Adult male Sprague–Dawley rats (300–350 g) were purchased from Hilltop Labs (Scottdale, PA, USA). Animals were housed in a temperature controlled animal facility at West Chester University, PA, USA. All the procedures performed were approved by the Institutional Animal Care and Use Committee of West Chester University, PA, USA, and conducted in accordance with approved standards for laboratory animal care.

The rats were anesthetized with a solution containing 50 mg/kg ketamine and 3 mg/kg xylazine. The left common carotid artery was cannulated with a polyethylene-60 catheter (BD Biosciences, Sparks, MD), which was inserted into the left internal carotid artery for perfusion. The perfusion fluid consisted of Krebs Ringer Bicarbonate buffer (KRB) pH 7.4 and was oxygenated with a mixture air of 95% O_2 and 5% CO_2 before starting the perfusion. The perfusion was started immediately after the cardiac blood supply was cut-off. The perfusion flow rate was 20 ml/min and perfusate contained one of the antihistamines (15 μM) in the absence and presence of Cyclosporin A (10 μM). In addition, the perfusate also contained the intravascular space marker atenolol (50 μM) (30) and a moderate brain permeability marker, antipyrine (5 μM) (23). The perfusion with KRB containing drugs lasted 30 s and was followed by 30 s of post perfusion wash (KRB only) to remove drug from the intravascular compartment. Upon completion of the post perfusion wash, brains were quickly removed from the skull and the left cerebral hemisphere was excised.

Table I. Transport of First Generation Antihistamines across MDR-MDCK Cell Monolayers

Compound	Treatment	Average Papp value ± STD ($\times 10^{-6}$ cm/s)	B-A/A-B P_{app} ratio
Clemastine	No CSA	A-B: 40.4±2.4 B-A: 156.4±27.7	3.9
	With CSA	A-B: 31.8±5.4 B-A: 49.5±8.7	1.5
Pyrilamine	No CSA	A-B: 18.0±3.4 B-A: 100.4±7.2	5.6
	With CSA	A-B: 34.1±2.2 B-A: 73.8±13.5	2.2
Chlorpheniramine	No CSA	A-B: 11.6±1.3 B-A: 93.7±21.6	8.1
	With CSA	A-B: 25.9±3.9 B-A: 66.2±8.1	2.6
Diphenhydramine	No CSA	A-B: 27.9±3.3 B-A: 103.7±11.7	3.7
	With CSA	A-B: 21.2±2.1 B-A: 77.8±5.7	3.7
Hydroxyzine	No CSA	A-B: 26.6±1.2 B-A: 38.1±8.3	1.4
	With CSA	A-B: 30.1±1.1 B-A: 32.0±3.6	1.1
Brompheniramine	No CSA	A-B: 11.6±0.6 B-A: 118.8±20.6	10.26
	With CSA	A-B: 26.2±0.7 B-A: 76.8±1.8	2.9

The isolated left brain tissue was homogenized using a Polytron in 4 ml of a methanol/water mixture. The resulting homogenates were stored at −80°C until analysis.

Sample Analysis

Prior to the LC/MS/MS analysis, the brain samples were treated with acetonitrile using a Tomtec Quadra 96 Model 320 liquid handler (Hamden, CT) and Sirocco™ protein precipitation plates from Waters Corp (Milford, MA). A 200 µl aliquot of the brain homogenate was mixed with 400 µl of 100 ng/ml warfarin in pure acetonitrile. After mixing the supernatants were vacuumed through the protein precipitation plate and collected in a 96-well plate. These samples were then evaporated to dryness under N_2 at 37°C using a Zymark TurboVap 96 (Hopkinton, MA). The resulting residue was then reconstituted with 200 µl of 20% acetonitrile in water after drying. A 70 µl aliquot of the samples was then transferred into cromacol vials for LC/MS/MS analysis. The mass spectrometry was conducted on a Sciex API2000 triple quadrupole. Mass spectrometer response for each compound was individually optimized to result in best sensitivity. The LC consisted of one series 200 autosampler and two Perkin-Elmer series 200 micropumps.

Calculations

The unidirectional transfer constants K_{in} (ml/min/g) were determined using the following equation for the single-point perfusion assay (31):

$$K_{in} = \left[Q_{br}/C_{pf}\right]/t$$

where Q_{br}/C_{pf} is the apparent brain distribution volume, Q_{br} is the amount of drug in the brain tissue (nanograms per gram of brain tissue), C_{pf} is the drug concentration in the perfusion fluid (nanograms per milliliter) and t is the net perfusion time (minutes). To exclude the drug contained in the capillary space from the brain concentration values, the apparent brain distribution volume of atenolol was subtracted from the drug values in each animal. The validity of this approach is supported by observations that atenolol does not cross BBB and the similarity between the brain distribution volumes of atenolol and the intravascular space marker mannitol (30,32).

RESULTS

In Vitro Efflux Studies

All of the first generation antihistamines, with the exception of hydroxyzine, underwent efflux in MDR-MDCK cells. The B-to-A /A-to-B Papp ratios for clemastine, pyrilamine, chlorpheniramine, diphenhydramine and brompheniramine were higher than 3 (Table I), a value previously suggested to indicate the existence of efflux in the MDR-MDCK cell model (23). In addition, the A-to-B Papp values of the first generation antihistamines in the absence of CSA ranged from 11.6 to 40.4×10^{-6} cm/s, which suggests that these compounds have a high intrinsic A-to-B permeability across the cell barrier despite undergoing net secretion (Table I). The addition of CSA caused a substantial decrease in the B-to-A/A-to-B Papp ratio of all the first generation antihistamines tested, with the exception of diphenhydramine (Table I). The diphenhydramine efflux

Table II. Transport of Second Generation Antihistamines Across MDR-MDCK Cell Monolayers

Compound	Treatment	Average Papp value ± STD ($\times 10^{-6}$ cm/s)	B-A/A-B P_{app} ratio
Terfenadine	No CSA	A-B: 0.62±0.08 B-A: 11.43±0.68	18.5
	With CSA	A-B: 1.51±0.61 B-A: 2.39±0.52	1.6
Loratadine	No CSA	A-B: 4.75±1.58 B-A: 35.0±4.3	7.4
	With CSA	A-B: 15.52±4.9 B-A: 39.64±1.53	2.6
Fexofenadine	No CSA	A-B: 0.34±0.14 B-A: 1.34±0.72	3.9
	With CSA	A-B: 0.13±0.05 B-A: 0.27±0.12	2.0
Cetirizine	No CSA	A-B: 0.29±0.02 B-A: 18.3±3.2	63
	With CSA	A-B: 2.30±0.73 B-A: 2.46±0.74	1.1

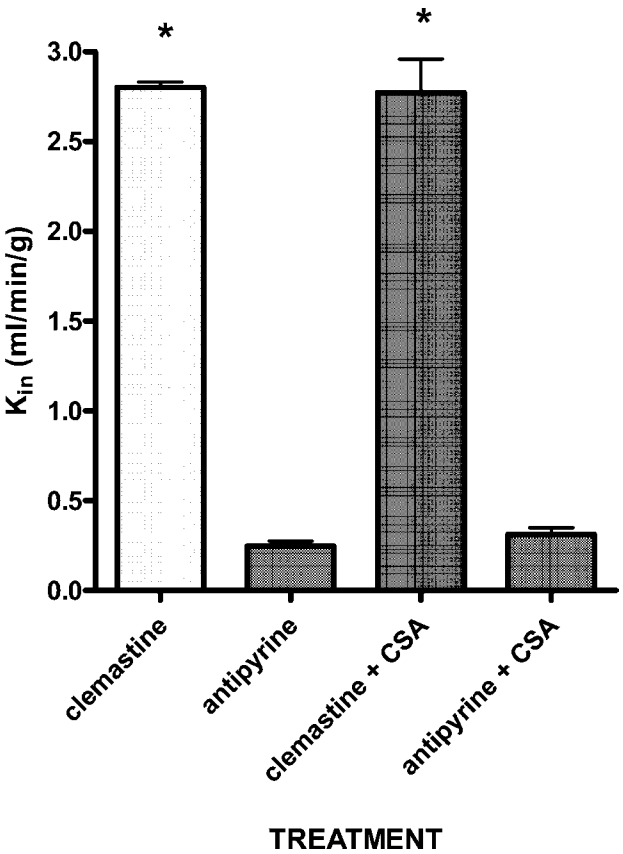

Fig. 1. The unidirectional transfer constants (K_{in}) of clemastine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean±SEM from three animals in each treatment group. *Clemastine K_{in} value is significantly higher than corresponding antipyrine K_{in} value ($P<0.05$, paired Student's t-test). There was no statistical difference between K_{in} values of clemastine in absence or presence of CSA. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

ratio of 3.7 remained unaltered by Pgp inhibition in the presence of CSA (Table I).

In contrast to the first generation antihistamines, the second generation compounds had relatively low A-to-B Papp in the absence of CSA (A-to-B Papp ranged from 0.19×10^{-6} cm/s to 4.75×10^{-6} cm/s, Table II). The B-to-A/A-to-B Papp ratios for the second generation antihistamines in the absence of CSA ranged from 3.9 for fexofenadine to 63 for cetirizine (Table II). These results in MDR-MDCK cells suggest that terfenadine, fexofenadine and cetirizine can be expected to have a low brain penetration potential since they have A-to-B Papp across MDR-MDCK cells lower than 1×10^{-6} cm/s and a substantial efflux in absence of CSA (23). In contrast to terfenadine, fexofenadine and cetirizine, loratadine had high intrinsic permeability across MDR-MDCK cells despite undergoing efflux (A-to-B Papp value 4.75×10^{-6} cm/s and B-to-A/A-to-B Papp ratio of 7.4, Table II). In CSA treated cells, the B-to-A/A-to-B Papp ratios of second generation antihistamines terfenadine and cetirizine were 1.6 and 1.1, respectively (Table II). The addition of CSA did not completely diminish efflux of loratadine and fexofenadine since their efflux ratio remained at 2.6 and 2.0, respectively (Table II). These results may reflect additional transport mechanisms, responsible for the persistent *in vitro* efflux of loratadine and fexofenadine, distinct from Pgp. While loratadine interaction with other transporters is still not known, the fexofenadine biliary excretion is mediated by uncharacterized transporter other than Pgp (24).

Brain Perfusion Results

The unidirectional transfer constants (K_{in}) of all first generation antihistamines were higher than 1 ml/min/g of brain tissue. Furthermore, the K_{in} values of all the first generation antihistamines were significantly higher than the associated K_{in} value of the co-perfused moderate brain permeability marker antipyrine, which had a K_{in} of approximately 0.2 ml/min/g brain tissue (Figs. 1, 2, 3, 4, 5 and 6). The addition of CSA to the perfusate did not change the K_{in} values of either the first generation antihistamines or antipyrine (Figs. 1, 2, 3, 4, 5 and 6).

When perfused without CSA, fexofenadine and cetirizine average K_{in} values were 0.0003 ml/min/g brain tissue and 0.005 ml/min/g of brain tissue, respectively (Figs. 7 and 8). These values were significantly lower than the K_{in} value of co-perfused antipyrine (Figs. 7 and 8). Under conditions of

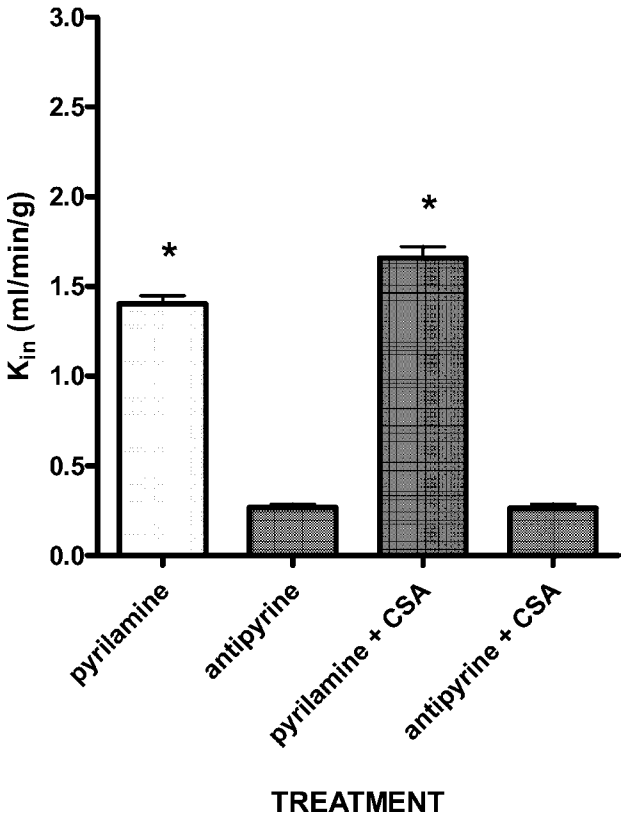

Fig. 2. The unidirectional transfer constants (K_{in}) of pyrilamine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Pyrilamine K_{in} value is significantly higher than corresponding antipyrine K_{in} value ($P<0.05$, paired Student's t-test). There was no statistical difference between K_{in} values of pyrilamine in absence or presence of CSA. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

no Pgp inhibition by CSA, the second generation antihistamine, loratadine, had a K_{in} value somewhat lower than antipyrine (Fig. 9) but substantially higher than those of fexofenadine and cetirizine (Figs. 7 and 8). The K_{in} value of terfenadine, when this antihistamine was perfused alone, was comparable to the antipyrine K_{in} value, with average of approximately 0.3 ml/min/g of brain tissue (Fig. 10) indicating moderate brain penetration in spite of the Pgp activity at the BBB. For all of the second generation antihistamines, co-perfusion with CSA resulted in a significant increase in the K_{in} value (Figs. 7, 8, 9 and 10). However, even in the presence of CSA, fexofenadine and cetirizine still had K_{in} values lower more than tenfold than antipyrine (Figs. 7 and 8). In contrast, loratadine and terfenadine exhibited brain penetration rates that significantly surpassed that of antipyrine in the presence of CSA (Figs. 9 and 10). The CSA application did not increase the K_{in} value of the co-perfused antipyrine in any of the treatments tested (Figs. 7 and 10). Under all experimental conditions antipyrine K_{in} was approximately 0.2 ml/min/g brain tissue (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10), a value close to that previously reported using *in situ* brain perfusion technique by Youdim *et al.* (33).

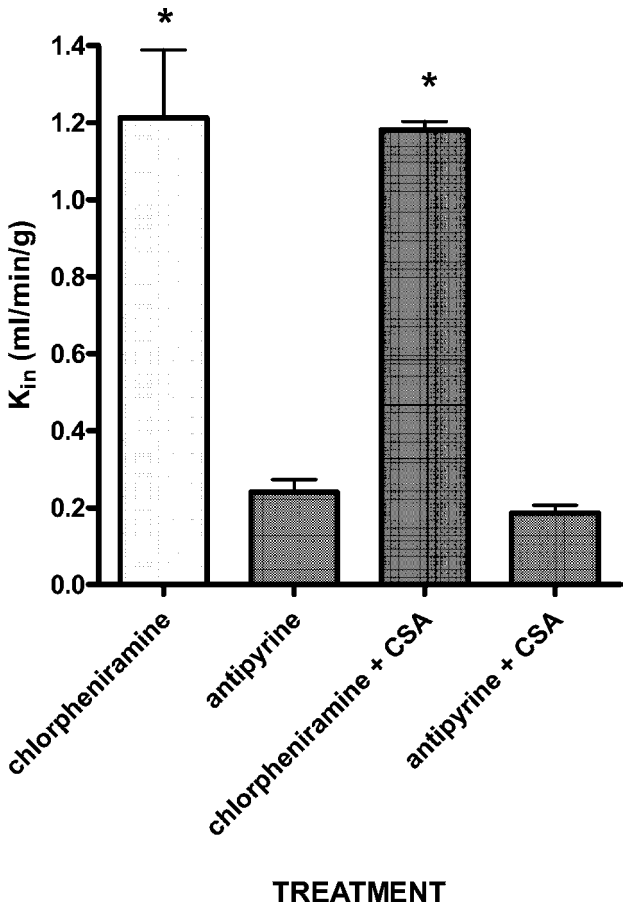

Fig. 3. The unidirectional transfer constants (K_{in}) of chlorpheniramine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Chlorpheniramine K_{in} value is significantly higher than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). There was no statistical difference between K_{in} values of chlorpheniramine in absence or presence of CSA. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

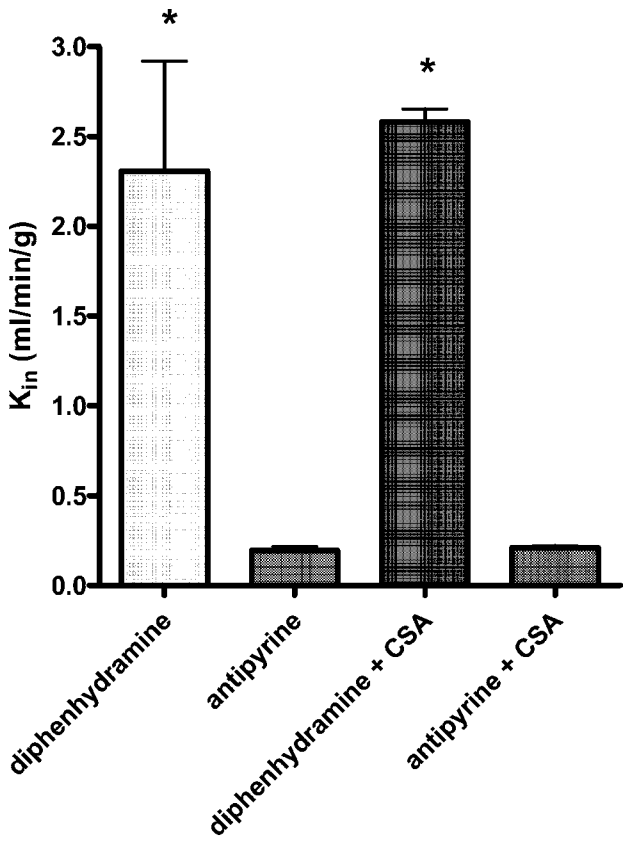

Fig. 4. The unidirectional transfer constants (K_{in}) of diphenhydramine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Diphenhydramine K_{in} value is significantly higher than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). There was no statistical difference between K_{in} values of diphenhydramine in absence or presence of CSA. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

DISCUSSION

First generation antihistamines such as clemastine, pyrilamine, chlorpheniramine, diphenhydramine, hydroxyzine and brompheniramine have been associated with a high incidence of sedation (34). Second generation antihistamines such as terfenadine, loratadine, cetirizine, and fexofenadine, tend to be less sedating than first generation antihistamines (1,2). Since sedation of antihistamines is mediated via centrally located H_1 receptors, it has been hypothesized that differences in sedating effects between first and second generation antihistamines arise from their ability to penetrate into the CNS (5). Optimal CNS penetration depends on drug physicochemical properties, binding to serum proteins, and interaction with efflux/uptake transporters present at the BBB (35). The differences in brain penetration and sedating effects between the fist and second generation antihistamines could not be explained by differences in physicochemical characteristics, serum protein binding, or affinity for central H_1 receptors (4–6). Therefore, it has been hypothesized for some of the second generation antihistamines to be devoid of

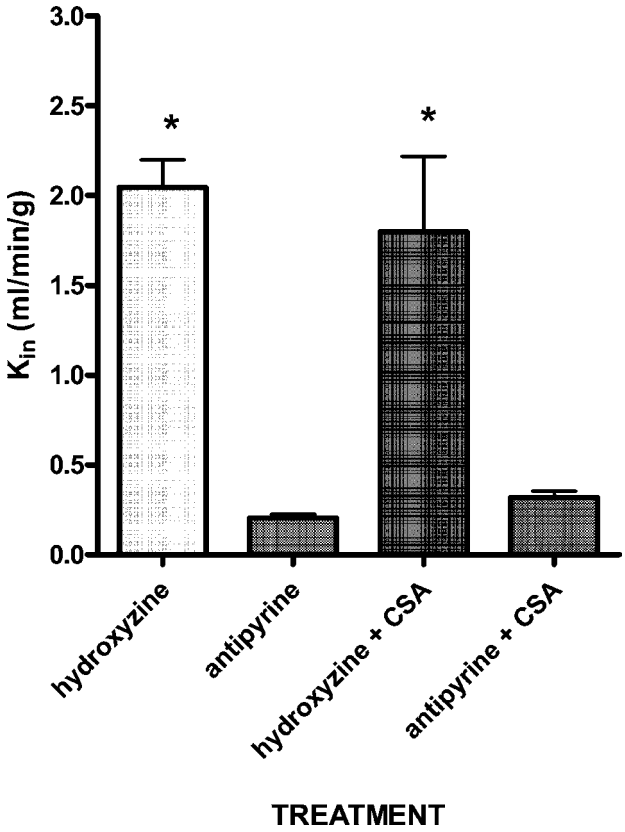

Fig. 5. The unidirectional transfer constants (K_{in}) of hydroxyzine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Hydroxyzine K_{in} value is significantly higher than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). There was no statistical difference between K_{in} values of hydroxyzine in absence or presence of CSA. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

sedating effects due to their active efflux from the CNS mediated by Pgp. A significantly reduced brain penetration in wild-type mice compared to Pgp-knockout mice has been reported for second generation antihistamines cetirizine, loratadine and fexofenadine (8,14,24). In addition, cetirizine, loratadine, and fexofenadine, were found to be Pgp substrates *in vitro* (7,8,14–16). In contrast, the first generation antihistamines hydroxyzine and diphenhydramine were not classified as Pgp substrates *in vitro* and their brain penetration was similar in the wild-type and Pgp-knockout mice (8,14). This study expands the evaluation of Pgp role in brain penetration of additional first generation and second generation antihistamines to make a more comprehensive comparison between two classes.

Overall, *in vitro* results on MDR-MDCK cells did not demonstrate clear distinction between first and second generation antihistamines in their potential to be effluxed by Pgp. The majority of compounds in both antihistamine classes exhibited Pgp-mediated efflux. In agreement with a previous report of Polli *et al.* (14), first generation antihistamine hydroxyzine was not found to be effluxed across MDR-MDCK cell monolayers because it had a B-to-A/A-to-B Papp ratio of approximately 1 in the absence and presence of CSA. However, all the other first generation antihistamines tested had B-to-A/A-to-B Papp ratio higher than 3, a value that in this cell model indicates the existence of efflux (23). The presence of CSA in the transport buffer resulted in decreased efflux of clemastine, pyrilamine, chlorpheniramine and brompheniramine, which indicates that Pgp plays a role in the efflux of these compounds. The exception was diphenhydramine whose B-to-A/A-to-B Papp ratio was greater than 3, and it was unaffected by CSA, indicating that diphenhydramine is not a likely Pgp substrate but may be transported by other, Pgp independent mechanism. Similarly to present findings, diphenhydramine is actively transported across Caco-2 cell monolayers but was found not to be an Pgp substrate, and is likely transported via pH-dependent tertiary amine transport system (36,37).

The *in vitro* results of this study indicate that chlorpheniramine has the potential to interact with Pgp. The chlorpheniramine efflux was decreased by CSA, which is in accordance with recent findings on the isolated bovine nasal tissue where the Pgp inhibitors quinidine and verapamil significantly decreased chlorpheniramine efflux (38). Polli *et al.* (21), however, did not observe chlorpheniramine efflux in MDR-MDCK cells. The discrepancy between chlorpheniramine results in

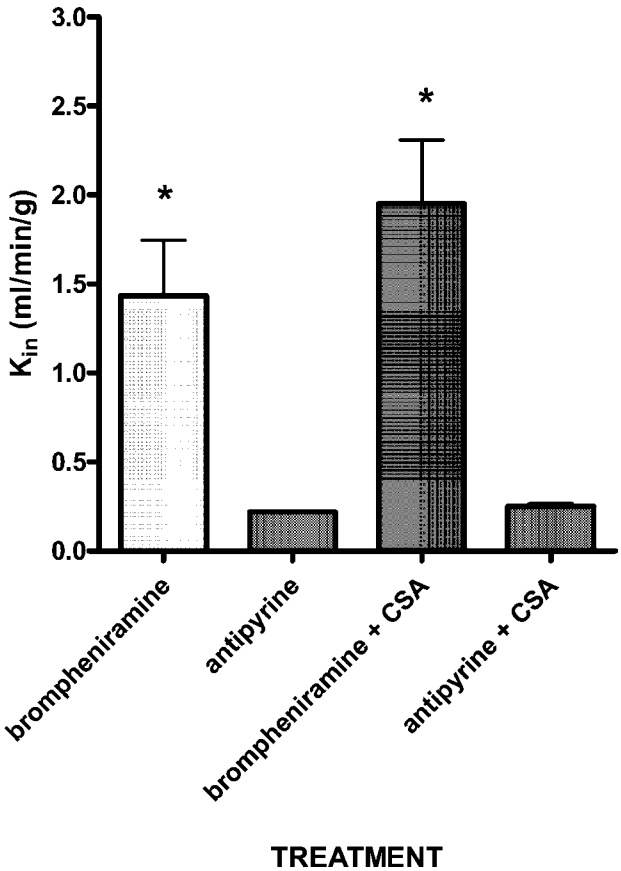

Fig. 6. The unidirectional transfer constants (K_{in}) of brompheniramine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Brompheniramine K_{in} value is significantly higher than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). There was no statistical difference between K_{in} values of brompheniramine in absence or presence of CSA. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

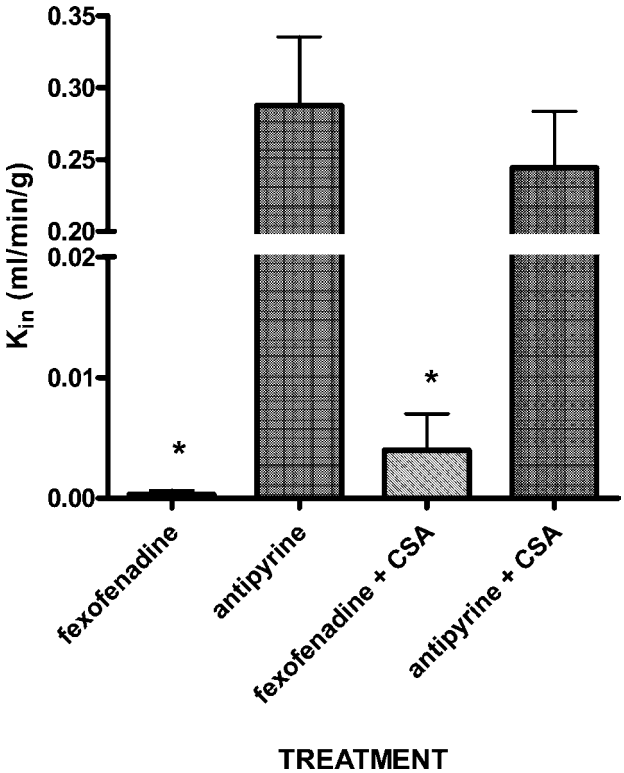

Fig. 7. The unidirectional transfer constants (K_{in}) of fexofenadine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Fexofenadine K_{in} value is significantly lower than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). Despite higher average K_{in} value of fexofenadine in presence of CSA the difference did not reach statistical significance. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

this study and those previously reported may arise from different MDR-MDCK cell culture conditions. For example, the cell seeding density in the present study was 60,000 cells/cm^2 and permeability assays were initiated on the seventh day post-seeding, while in the previous study (21), cells were seeded at a density of 300,000 cells/cm^2 and assays performed 3 days after seeding. Furthermore, unlike the previous work, cells used in experimentation were cultured in the presence of colchicine, a selection agent to maintain Pgp expression. In addition, another reason for discrepancy may be application of different concentrations since current chlorpheniramine assay was done at 10 μM whereas previously tested concentration was 20 μM (21), which may have saturated Pgp resulting in no observation of efflux.

The substantial Pgp-mediated efflux of all of the second-generation antihistamines tested in this study in MDR-MDCK cells resembles previous *in vitro* findings in other cell models (7,8,15). However, despite similarities in efflux, there was a clear distinction between loratadine and other second generation antihistamines in intrinsic permeation across MDR-MDCK cells. The A-to-B Papp value of loratadine exceeded that of terfenadine several-fold in the absence of CSA. The high loratadine permeation across MDR-MDCK cells *in vitro*, despite efflux, indicates that this second generation antihistamine can be expected to have substantial brain penetration *in vivo* and higher than that of terfenadine (23). The low A-to-B permeation across MDR-MDCK cells (less than 1×10^{-6} cm/s) accompanied with high efflux in the absence of CSA, suggests poor permeation of terfenadine as well as cetirizine and fexofenadine across the BBB (23).

Despite Pgp involvement in the transport of the majority of the first generation antihistamines *in vitro*, the results from the in situ perfusion results demonstrated that the brain penetration of these compounds is not increased by co-perfusion with CSA. Therefore, Pgp-mediated efflux appears not to be a limiting factor to the CNS penetration of clemastine, pyrilamine, chlorpheniramine, diphenhydramine, hydroxyzine and brompheniramine. It is possible that, despite undergoing Pgp-mediated efflux, the first generation antihistamines may reach the CNS as a result of high intrinsic permeation. This is supported by reports that, *in vivo*, diphenhydramine and hydroxyzine had similar brain penetration in wild type and Pgp knock-out mice (8,14) and by numerous examples of compounds that are centrally active and have high intrinsic permeability while undergoing Pgp-mediated efflux *in vitro* (22,23). However, in the case of second generation antihistamine loratadine, a high intrinsic permeability despite efflux *in vitro*, did not translate into high brain penetration since K_{in} value of loratadine was significantly lower than that of antipyrine, a moderate brain penetrant (28,39). In addition, there

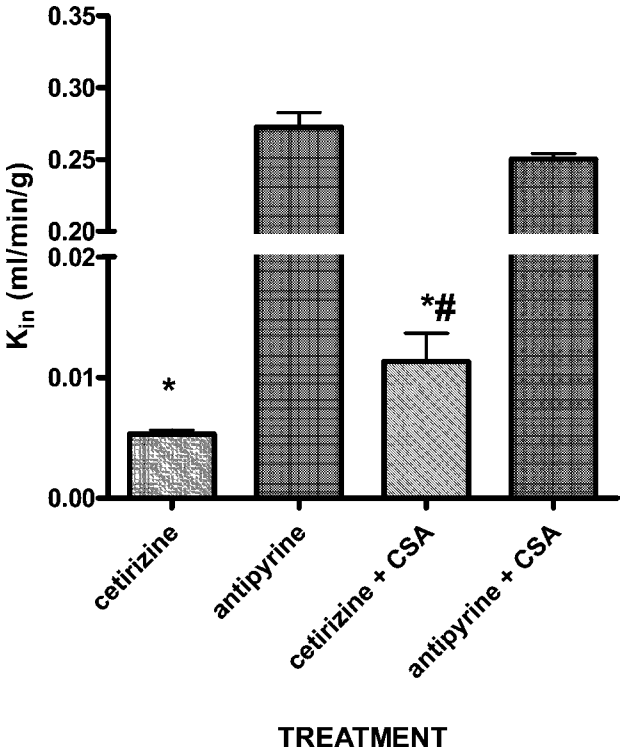

Fig. 8. The unidirectional transfer constants (K_{in}) of cetirizine and antipyrine in absence and presence of cyclosporine A (CSA). Values present mean ± SEM from three animals in each treatment group. *Cetirizine K_{in} value is significantly lower than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). #The cetirizine K_{in} value in presence of CSA significantly higher than cetirizine K_{in} value in absence of CSA ($P < 0.05$, paired Student's t-test). In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

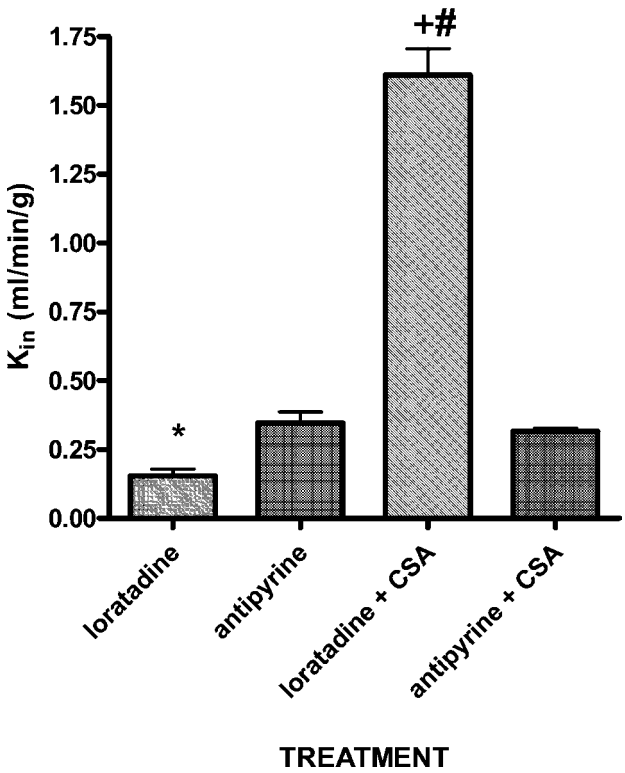

Fig. 9. The unidirectional transfer constants (K_{in}) of loratadine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Loratadine K_{in} value is significantly lower than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). $^{+}$Loratadine K_{in} value in presence of CSA significantly higher than antipyrine K_{in} value under same conditions ($P < 0.05$, paired Student's t-test). $^{\#}$Loratadine K_{in} value in presence of CSA significantly higher than loratadine K_{in} value in absence of CSA ($P < 0.05$, paired Student's t-test). In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

is a discrepancy between high terfenadine brain permeation (K_{in} similar to antipyrine, Fig. 10) and the expected results based on low intrinsic permeability and high Pgp-mediated efflux across MDR-MDCK cells *in vitro*. These findings support recent observations by Summerfield *et al.* (40), where on a large set of CNS compounds efflux in MDR-MDCK cells was found to be a poor predictor of *in vivo* brain penetration. In numerous cases of Pgp substrates, *in vitro* results on MDR-MDCK cells had a tendency to overestimate drug efflux, thus resulting in underestimation of drug potential to reach CNS as evidenced by substantial drug brain-to-blood (B/P) ratio *in vivo* (25,40). In addition, some compounds with low intrinsic A-to-B permeation and high Pgp-mediated efflux across MDR-MDCK cells, still exhibit substantial brain penetration (40), a similar profile as the one observed for terfenadine. Therefore, evaluation of drug potency to penetrate brain based solely on *in vitro* results using MDR-MDCK cell model, may lead to misclassification of the compound potential to reach CNS *in vivo*. The discrepancy between *in vitro* and *in vivo* observations indicates a role for other parameters in addition to intrinsic permeability and interaction with Pgp. Recent studies demonstrated significant roles of plasma and brain tissue biding in drug CNS disposition (25,41). For compounds such as terfenadine, which are Pgp substrates with poor *in vitro* permeability, a sufficiently high brain tissue binding may provide a powerful driving force surpassing effects of the efflux or plasma binding (40,41). Furthermore, an active uptake across BBB can contribute to substantial drug CNS penetration. For H1 antihistamines mepiramine and epinastine, an uptake by a cation-transporting peptide expressed in the rat brain capillary endothelial cells has been found (13). However, the extent of terfenadine brain tissue binding or existence of the active uptake across BBB, remains to be elucidated.

When assayed for CNS penetration using brain perfusion method in the absence of CSA, cetirizine had a K_{in} value more than ten times lower than terfenadine and loratadine. Similarly, fexofenadine brain concentration following perfusion in absence of CSA was very low, resulting in a K_{in} value close to 0. This negligible brain penetration of fexofenadine and cetirizine is in accordance with reports of very low or non-existent binding of these compounds to cortical H_1 receptors following oral ingestion in humans (20,42). Furthermore, fexofenadine and cetirizine are devoid of sedation at therapeutic or higher doses (17–19,43,44). In contrast, terfenadine and loratadine that exhibited substantial brain penetration potential even under conditions of efflux have been associated with relatively frequent reports of motor and psychological functions impairment in patients (17,34).

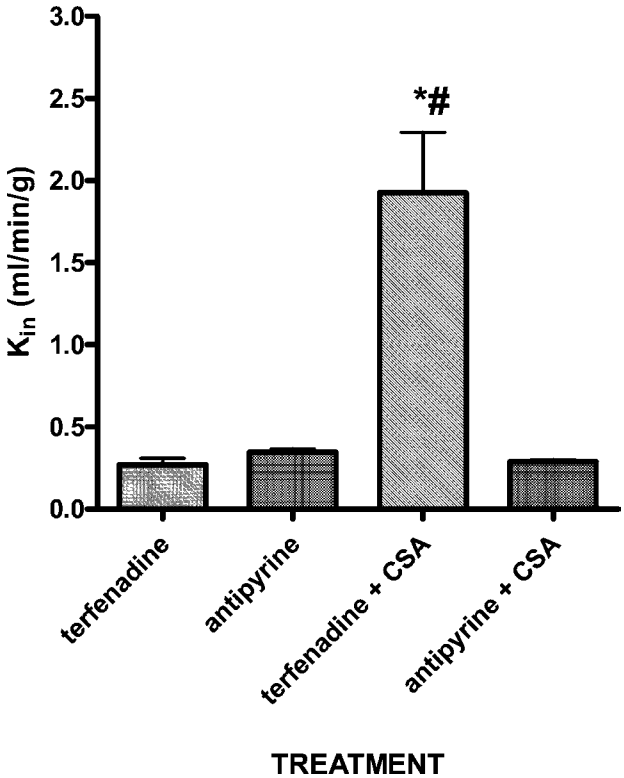

Fig. 10. The unidirectional transfer constants (K_{in}) of terfenadine and antipyrine in absence and presence of cyclosporine A (*CSA*). Values present mean ± SEM from three animals in each treatment group. *Terfenadine K_{in} value in presence of CSA is significantly higher than corresponding antipyrine K_{in} value ($P < 0.05$, paired Student's t-test). $^{\#}$Terfenadine K_{in} value in presence of CSA significantly higher than terfenadine K_{in} value in absence of CSA ($P < 0.05$, paired Student's t-test). In absence of CSA terfenadine and antipyrine K_{in} values were not different. In addition, antipyrine K_{in} value in absence of CSA was similar to antipyrine K_{in} value in presence of CSA.

Under conditions of inhibited Pgp-mediated efflux by CSA, K_{in} values of fexofenadine, certirizine, loratadine, and terfenadine significantly increased by several fold, which demonstrates that potential of these second generation antihistamines to cross BBB is limited by their interaction with Pgp. The brain penetration of fexofenadine and cetirizine remained poor even under conditions of Pgp inhibition. In contrast, brain delivery of terfenadine and loratadine surpassed that of antipyrine when Pgp was inhibited. These results suggest that a primary limiting factor for terfenadine and loratadine CNS penetration is likely Pgp-mediated efflux, whereas fexofenadine and cetirizine have low potential to cross BBB independent from Pgp function.

In summary, the present study demonstrated that the majority of antihistamines, including both first and second generation compounds, are Pgp substrates *in vitro*. However, Pgp-mediated efflux is not a limiting factor for brain penetration of first generation antihistamines. Regarding the transport of second generation compounds, Pgp plays a minor role in limiting terfenadine and loratadine disposition into the CNS. In contrast, brain permeation of fexofenadine and cetirizine is intrinsically low and further suppressed by Pgp-mediated efflux.

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REFERENCES

1. F. Estelle and R. Simons. Advances in H1-antihistamines. *N. Engl. J. Med.* **351**:2203–2217 (2004).
2. I. Hindmarch, S. Johnson, R. Meadows, T. Kirkpatrick, and Z. Shamsi. The acute and sub-chronic effects of levocetirizine, cetirizine, loratadine, promethazine and placebo on cognitive function, psychomotor performance, and wheal and flare. *Curr. Med. Res. Opin.* **17**:241–255 (2001).
3. H. Timmerman. Factors involved in the absence of sedative effects by the second-generation antihistamines. *Allergy* **55** (Suppl. 60):5–10 (2000).
4. A. M. ter Laak, G. J. Bijloo, M. J. E. Fisher, K. G. M. Donne-Op, P. A. Carrupt, B. Testa, and H. Timmerman. Serum protein binding of histamine H1-antagonists: a comparative study on the serum protein binding of a sedating (^{3}H-mepyramine) and a non-sedating H1-antagonist (^{3}H-loratadine). *Eur. J. Pharm. Sci.* **4**:307–319 (1996).
5. A. M. ter Laak, K. G. M. Donne-Op, A. Bast, and H. Timmerman. Is there a difference in the affinity of histamine H1-receptors agonists for CNS and peripheral receptors? *Eur. J. Pharm. Sci.* **232**:199–205 (1993).
6. A. M. ter Laak, R. S. Tsai, K. G. M. Donne-Op, and G. M. Kelder. Lipophilicity and hydrogen-bonding capacity of H1-antihistaminergic agents in relation to their central sedative side-effects. *Eur. J. Pharm. Sci.* **2**:373–384 (1994).
7. M. Chishty, A. Reichel, J. Siva, N. J. Abbot, and D. J. Begley. Affinity for the P-glycoprotein efflux pump at the blood brain barrier may explain the lack of CNS side-effects of modern antihistamines. *J. Drug Target.* **9**:223–228 (2001).
8. C. Chen, E. Hanson, J. W. Watson, and J. S. Lee. P-Glycoprotein limits the brain penetration of nonsedating but not sedating H1-antagonists. *Drug Metab. Dispos.* **31**:312–318 (2003).
9. A. H. Schinkel. P-glycoprotein: a gatekeeper in the blood-brain barrier. *Adv. Drug Del. Rev.* **36**:179–194 (1999).
10. A. H. Schinkel, J. J. M. Smit, O. van Tellingen, J. H. Beijnen, E. Wagenaar, L. van Deemter, C. A. A. M. Mol, M. A. van der Valk, E. C. Robanaus-Maandag, H. P. J. te Riele, A. J. M. Berns, and P. Borst. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**:491–502 (1994).
11. H. Sun, H. Dai, N. Shaik, and W. F. Elmquist. Drug efflux transporters in the CNS. *Adv. Drug Deliv. Rev.* **55**:83–105 (2003).
12. H. A. Volk and W. Loscher. Multidrug resistance in epilepsy: rats with drug-resistant seizures exhibit enhanced brain expression of P-glycoprotein compared with rats with drug-responsive seizures. *Brain* **128**:1358–1368 (2005).
13. N. Ishiguro, T. Nozawa, A. Tsujihata, A. Saito, W. Kishimoto, K. Yokoyama, T. Yosumoto, K. Sakai, T. Igarashi, and I. Tamai. Influx and efflux transport of H1-antagonist epinastine across the blood-brain barrier. *Drug Metab. Dispos.* **32**:519–524 (2004).
14. J. W. Polli, T. M. Baughman, J. E. Humphreys, K. H. Jordan, A. L. Mote, J. A. Salisbury, T. K. Tippin, and C. J. Serabjit-Singh. P-glycoprotein influences the brain concentration of cetirizine (Zyrtec), a second-generation non-sedating antihistamine. *J. Pharm. Sci.* **92**:2082–2089 (2003).
15. M. Cvetkovic, B. Leake, M. F. Fromm, G. R. Wilkinson, and R. B. Kim. OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metab. Dispos.* **27**:866–871 (1999).
16. K. M. Mahar Doan, S. A. Wring, L. J. Shampine, K. H. Jordan, J. P. Bishop, J. Kratz, E. Yang, C. J. Serabijit-Singh, K. K. Adkison, and J. W. Polli. Steady-state brain concentrations of antihistamines in rats: interplay of membrane permeability, P-glycoprotein efflux and plasma protein binding. *Pharmacology* **72**:92–98 (2004).
17. F. Bonifazi, L. Provinciali, L. Antonicelli, M. B. Bilo, S. Pucci, M. Signorino, B. Franciolino, B. Censori, P. Pagelli, and A. Iudice. Comparative study of terfenadine and cetirizine in hay fever: assessment of efficacy and central nervous system effects. *J. Investig. Allergol. Clin. Immunol.* **5**:40–46 (1995).
18. A. Shamsi and I. Hindmarch. Sedation and antihistamines: a review of inter-drug differences using proportional impairment ratios. *Hum. Psychopharmacol.* **15**(Suppl)1:S3–S30 (2000).
19. I. Hindmarch, Z. Shamsi, and S. Kimber. An evaluation of the effects of high-dose fexofenadine on the central nervous system: a double-blind, placebo-controlled study in healthy volunteers. *Clin. Exp. Allergy* **32**:133–139 (2002).
20. M. Tashiro, Y. Sakurada, K. Iwabuchi, H. Mochizuki, M. Kato, M. Aoki, Y. Funaki, M. Itoh, R. Iwata, D. F. Wong, and K. Yanai. Central effects of fexofenadine and cetirizine; measurement of psychomotor performance, subjective sleepiness, and brain histamine H1-receptor occupancy using ^{11}C-doxepin positron emission tomography. *J. Clin. Pharmacol.* **44**:890–900 (2004).
21. J. W. Polli, S. A. Wring, J. E. Humphreys, L. Huang, J. B. Morgan, L. O. Webster, and C. J. Serabjit-Singh. Rational use of *in vitro* P-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **299**:620–628 (2001).
22. K. M. Mahar Doan, J. E. Humphreys, L. O. Webster, S. A. Wring, L. J. Shampine, C. J. Serabjit-Singh, K. K. Adkison, and J. W. Polli. Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *J. Pharmacol. Exp. Ther.* **303**:1029–1037 (2002).
23. Q. Wang, J. D. Rager, K. Weinstein, P. S. Kardos, G. L. Dobson, J. Li, and I. J. Hidalgo. Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. *Int. J. Pharm.* **288**:349–359 (2005).
24. H. Tahara, H. Kusuhara, E. Fuse, and Y. Sugiyama. P-glycoprotein plays a major role in the efflux of fexofenadine in the small intestine and blood-brain barrier, but only a limited role in its biliary excretion. *Drug Metab. Dispos.* **33**:963–968 (2005).
25. A. Doran, R. S. Obach, B. J. Smith, N. A. Hosea, S. Becker, E. Callegari, C. Chen, X. Chen, E. Choo, J. Cianfrogna, L. M. Cox, J. P. Gibbs, M. A. Gibbs, H. Hatch, C. E. C. A. Hop, I. N. Kasman, J. LaPerle, J. Liu, X. Liu, M. Logman, D. Maclin, F. M. Nedza, F. Nelson, E. Olson, S. Rahematpura, D. Raunig, S. Rogers, K. Schmidt, D. K. Spracklin, M. Szewc, M. Troutman, E. Tseng, M. Tu, J. W. Van Deusen, K. Venkatakrishnan, G. Walens, E. Q. Wang, D. Wong, A. S. Yasgar, and C. Zhang. The impact of P-glycoprotein on the disposition of drugs targeted for indications in the central nervous system: evaluation using

MDR1A/1B knockout mouse model. *Drug Metab. Dispos.* **33**:165–174 (2005).
26. S. Cisternino, C. Mercier, F. Bourasset, F. Roux, and J. M. Schermann. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. *Cancer Res.* **64**:3296–3301 (2004).
27. N. N. Salama, E. J. Kelly, T. Bui, and R. J. Ho. The impact of pharmacologic and genetic knockout of P-glycoprotein on nelfinavir levels in the brain and other tissues in mice. *J. Pharm. Sci.* **94**:1216–1225 (2005).
28. Y. Takasato, S. I. Rapaport, and Q. R. Smith. An *in situ* brain perfusion technique to study cerebrovascular transport in the rat. *Am. J. Physiol.* **247**:H484–H493 (1984).
29. Q. R. Smith. Brain perfusion systems for studies of drug uptake and metabolism in the central nervous system. *Pharm. Biotechnol.* **8**:285–307 (1996).
30. J. A. Street, B. A. Hemsworth, A. G. Roach, and M. D. Day. Tissue levels of several radio labeled beta-adrenoceptor antagonists after intravenous administration in rats. *Arch. Int. Pharmacodyn. Ther.* **237**:180–190 (1979).
31. B. V. Zlokovic, D. J. Begley, B. M. Djuricic, and D. M. Mitrovic. Measurement of solute transport across the blood-brain barrier in the perfused guinea pig brain: method and application to N-methyl-alpha-aminoisobutyric acid. *J. Neurochem.* **46**:1444–1451 (1986).
32. J. E. Preston, H. Al-Sarraf, and M. B. Segal. Permeability of the developing blood-brain barrier to 14C-mannitol using the rat *in situ* brain perfusion technique. *Brain Res. Dev. Brain Res.* **87**:69–76 (1995).
33. K. A. Youdim, M. Z. Qaiser, D. J. Begley, C. Rice-Evans, and N. J. Abbott. Flavonoid permeability across an *in situ* model of the blood-brain barrier. *Free Radic. Biol. Med.* **36**:592–604 (2004).
34. M. J. Mattila and I. Paakkari. Variations among non-sedating antihistamines: are there real differences? *Eur. J. Clin. Pharmacol.* **55**:85–93 (1999).
35. D. E. Clark. Prediction of intestinal absorption and blood-brain barrier penetration by computational methods. *Comb. Chem. High Throughput Screen.* **4**:477–496 (2001).
36. H. Mizuuchi, T. Katsura, H. Saito, Y. Hashimoto, and K. I. Inui. Transport characteristics of diphenhydramine in human intestinal epithelial Caco-2 cells: contribution of pH-dependent transport system. *J. Pharmacol. Exp. Ther.* **290**:388–392 (1999).
37. H. Mizuuchi, T. Katsura, K. Ashida, Y. Hashimoto, and K. I. Inui. Diphenhydramine transport by pH-dependent tertiary amine transport system in Caco-2 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**:G563–G569 (2000).
38. K. K. Kandimalla and M. D. Donovan. Carrier mediated transport of chlorpheniramine and chlorcyclizine across bovine olfactory mucosa: implications on nose-to-brain transport. *J. Pharm. Sci.* **94**:613–624 (2005).
39. S. Lundquist, M. Renftel, J. Brillault, L. Fenart, R. Cecchelli, and M. P. Dehouck. Prediction of drug transport through the blood-brain barrier *in vivo*: a comparison between two *in vitro* cell models. *Pharm. Res.* **19**:976–981 (2002).
40. S. G. Summerfield, A. J. Stevens, L. Cutler, M. C. Osuna, B. Hammond, S. P. Tang, A. Hersey, D. Spalding, and P. Jeffrey. Improving the *in vitro* prediction of *in vivo* CNS penetration: integrating permeability, Pgp efflux and free fractions in blood and brain. *J. Pharmacol. Exp. Ther.* **316**:1282–1290 (2006).
41. X. Liu, B. J. Smith, C. Chen, E. Callegari, S. L. Backer, X. Chen, J. Cianfrogna, A. C. Doran, S. D. Doran, J. P. Gibbs, N. Hosea, J. Liu, F. R. nelson, M. A. Szewc, and J. V. Deusen. Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: an experimental analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. *J. Pharmacol. Exp. Ther.* **313**:1254–1262 (2005).
42. K. Yanai, J. H. Ryu, T. Watanabe, R. Iwata, T. Ido, Y. Sawai, K. Ito, and M. Itoh. Histamine H1 receptor occupancy in human brains after single oral doses of histamine H1 antagonists measured by positron emission tomography. *Br. J. Pharmacol.* **116**:1649–1655 (1995).
43. J. C. Verster and E. R. Volkerts. Antihistamines and driving ability: evidence from on-the-road driving studies during normal traffic. *Ann. Allergy Asthma Immunol.* **92**:294–303 (2004).
44. R. D. Mann, L. G. Pearce, N. Dunn, and S. Shakir. Sedation with "non-sedating" antihistamines: four prescription-event monitoring studies in general practice. *BMJ* **320**:1184–1187 (2000).