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The verapamil transporter expressed in human alveolar epithelial cells (A549) does not interact with β_2 -receptor agonists

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Summary

Affinity of different organs for verapamil is highly variable and organ-specific. For example, the drug exhibits high levels of accumulation in lung tissues. A transporter recognising verapamil as substrate has previously been identified in human retinal pigment epithelial (RPE) and in rat retinal capillary endothelial (TR-iBRB2) cells. This transporter is distinct from any of the cloned organic cation transporters. Therefore, we hypothesised that the verapamil transporter is also functionally expressed in the human respiratory mucosa. Moreover, we tested the hypothesis that this transporter interacts with pulmonary administered cationic drugs such as β_2 -agonists. The uptake of [^3H]-verapamil was studied in A549 human alveolar epithelial cell monolayers at different times and concentrations. The influence of extracellular proton concentration and various organic cations on verapamil uptake was determined. Verapamil uptake into A549 cells was time- and concentration-dependent, sensitive to pH and had a K_m value of $39.8 \pm 8.2 \mu\text{M}$. Verapamil uptake was also sensitive to inhibition by amantadine, quinidine and pyrilamine, but insensitive to other typical modulators of organic cation and choline transporters. Whilst we demonstrated functional activity of the elusive verapamil transporter at the lung epithelium, our data suggest that this transporter does not interact with β_2 -agonists at therapeutic concentrations.

Keywords:

Organic cation transporters; respiratory epithelium; drug-transporter interactions; inhalation biopharmaceutics; bronchodilators

1. Introduction

Whilst extensive research has been carried out identifying the underlying mechanisms of water and solute transport in the lung, uptake, secretion and accumulation of organic molecules has largely remained unstudied. In recent years, however, it has increasingly been recognised that the actions of membrane transporter proteins are pivotal in lung physiology as well as in pulmonary drug disposition.¹⁾ The functional expression of several members of the ATP-binding cassette (ABC) and solute carrier (SLC) transporter super families has previously been confirmed in the epithelia of proximal and distal lung.^{1,2)} These transporters might be involved in absorption and secretion of drugs administered by oral inhalation. In addition, a number of xenobiotics are accumulated within the lung tissue as shown, for example, for paraquat, amiodarone, imipramine, chlorpromazine and propranolol, and an involvement of membrane transporters in this lung retention has been discussed.^{3,4)}

The aim of our study was to investigate if active transport processes are responsible for the accumulation of verapamil.^{5,6)} A transporter for organic cations has been previously described in human retinal pigment epithelial and in rat retinal capillary endothelial (TR-iBRB2) cells, which recognises the calcium channel inhibitor, verapamil, as its substrate.^{7,8)} Verapamil is also a substrate and inhibitor of several transporters including P-glycoprotein and organic cation transporters.⁹⁾ Although the verapamil transporter has not yet been cloned, we hypothesised that this elusive transporter is functionally expressed in the human respiratory mucosa. In this context, it is important to study if inhaled therapeutics compete with verapamil for this uptake pathway. This type of drug-drug interaction might potentially lead to altered pharmacokinetics of either compound or even lung toxicity. Complementary to evidence emanating from *in vitro* studies, Gnadt and colleagues recently reported cation transporter interactions during the pulmonary absorption of β_2 -agonists using an isolated perfused human lung lobe model.¹⁰⁾ In a different study, increases in $AUC_{(0-\tau)}$ after co-administration of verapamil with the novel long-acting muscarinic receptor antagonist,

GSK573719 (umeclidinium bromide) and the long-acting β_2 -agonist, GSK642444 (vilanterol) were observed in healthy subjects to be 38% and 36%, respectively. ¹¹⁾

2. Methods

2.1. Materials

All chemicals, cell culture media and supplements were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest commercially available grade. Cell culture plastics were obtained from Greiner BioOne (Frickenhausen, Germany). [N-methyl-³H] verapamil hydrochloride ([³H]-verapamil; 80 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

2.2. Cell culture

Human alveolar epithelial A549 cells (American Type Culture Collection, CCL-185) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and cultured at a seeding density of 40,000 cells/cm² in 24-well plates. Cells were maintained at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium:Ham's F-12 (1:1 mix) (DMEM:F12) supplemented with 5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was exchanged every other day, until confluent monolayers were formed.

2.3. Uptake studies

Uptake experiments were carried out using A549 monolayers after 5 days in culture in extracellular fluid buffer (ECF; composed of 122 mM NaCl, 3 mM KCl, 0.4 mM KHPO₄, 25 mM NaHCO₃, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 10 mM HEPES and 10 mM D-glucose; pH 7.4). Cell monolayers were washed three-times with pre-equilibrated ECF solution. To initiate organic cation uptake, 200 µl ECF buffer containing [³H]-verapamil (3 nM) were added to each well. Verapamil uptake was followed for 5 min to investigate time-dependent saturation. Concentration dependency and (self)-inhibitory effects on uptake were assessed by incubation of A549 monolayers for 3 min with the radioisotope in the presence of increasing

concentrations of the unlabelled compound. Sodium-dependence of [³H]-verapamil uptake was determined by exchanging all sodium salts in the buffer with equimolar concentrations of the relevant potassium salts. Furthermore, pH-dependence of [³H]-verapamil translocation was studied by performing uptake studies at various pH values (i.e., 5.5, 6.5 and 8.5). Uptake of [³H]-verapamil was also studied in the presence of modulators of cation transporter function (i.e., amantadine, L-carnitine, choline, decynium 22, 1-methyl-4-phenylpyridinium (MPP⁺), pyrilamine, quinidine and tetraethylammonium (TEA)). In addition, the inhibitory potential of two β₂-agonists, salbutamol sulphate and formoterol fumarate on [³H]-verapamil uptake was determined. Uptake experiments were stopped by washing A549 cell monolayers three times with ice-cold ECF, followed by 12 h of incubation with 400 μl of 1N NaOH to lyse the cells, before 400 μl of 1N HCl were added for neutralisation. Five-hundred microlitres of lysate were used to measure the cell-associated radioactivity in a liquid scintillation counter (LSC5200, Aloka, Tokyo, Japan). In parallel, the total cell protein content was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

2.4. Data analysis

The uptake of [³H]-verapamil was expressed as the cell-to-medium ratio calculated according to the following equation:

$$\text{Cell/medium ratio} = \frac{[\text{^3H}] \text{ dpm per cell protein}}{[\text{^3H}] \text{ dpm per } \mu\text{l buffer}} \quad [\text{Eq.1}]$$

To determine the kinetics of verapamil uptake, including saturable and non-saturable compartments, the initial uptake rates were fitted to Michaelis-Menten kinetics by means of non-linear least-squares regression analysis using WinNonlin (Pharsight, Sunnyvale, CA). Results were expressed as mean ± SD. The significance of differences between the mean

values was determined by unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered significant. All experiments were carried out at least in triplicate.

3. Results and Discussion

3.1. Time course

Verapamil uptake into A549 cell monolayers rapidly increased over time (Figure 1). The cell-medium ratio reached 94.4 $\mu\text{l}/\text{mg}$ protein at 30 s and increased to 307.5 $\mu\text{l}/\text{mg}$ protein after 5 min. By extrapolating the uptake data to time-point zero, a surface binding of 66.2 $\mu\text{l}/\text{mg}$ protein was calculated. When verapamil uptake was studied at 4°C, the cell-medium ratio was significantly reduced and only reached values of 29.7 $\mu\text{l}/\text{mg}$ protein at 30 s and 111.3 $\mu\text{l}/\text{mg}$ protein at 5 min. The extensive accumulation of verapamil in A549 cells can therefore on the one hand be attributed to high levels of passive diffusion due to the drug's lipophilicity ($\log P = 3.45$), on the other hand, an involvement of a transporter, similar to the one reported by Han *et al.* in RPE cells⁷⁾ and Kubo *et al.* in TR-iBRB2 cells, is likely.⁸⁾

3.2. Concentration dependence

As shown in Figure 2, uptake of verapamil reached saturation over the concentration range studied (i.e., 0-200 μM), indicating a carrier-mediated process. Eadie-Hofstee transformation showed one dominant transporter site with an apparent K_m of 39.8 ± 8.2 μM and a V_{\max} of 2.2 ± 0.32 $\text{nmol}/\text{min}/\text{mg}$ of protein, suggesting a major contribution to the saturable uptake. The passive diffusion constant K_d was 2.7 ± 1.1 $\mu\text{l}/\text{min}/\text{mg}$ of protein. These kinetic parameters are quite similar to those published by other groups (7.2 ± 0.7 μM and 61.9 ± 3.9 μM , respectively),^{7,8)} when lab-to-lab variability and differences in cell type are considered. These results clearly underline an involvement of a carrier-mediated uptake mechanism into A549 cells.

3.3. Extracellular conditions

Verapamil uptake was not sodium-dependent (Figure 1). Decreasing extracellular pH (i.e., pH 5.5), however, lead to a significant decrease of verapamil uptake, whereas at pH 6.5 and pH

8.5 no significant differences were observed (data not shown). Again, these characteristics are consistent with observations in retinal epithelial and endothelial cells.^{7,8)}

3.4. Pharmacological inhibition

In order to investigate if the uptake of the organic cation, verapamil, by A549 cell monolayers was specific, the inhibitory potential of a number of compounds was evaluated that have been reported to interact with organic cation transporter function (Table 1). Generally, our data demonstrate similar specificity of the verapamil transporter in alveolar epithelial cells, when compared with the ones in RPE cells⁷⁾ and at the inner blood-retinal barrier.⁸⁾ Amantadine, pyrilamine and quinidine significantly ($P < 0.01$) inhibited the uptake of verapamil into A549 cells, whereas typical substrates of the cloned organic cation transporters (i.e., TEA, MPP⁺, decynium 22 and L-carnitine) only marginally interfered with the uptake of verapamil. Choline did not show an effect, ruling out an involvement of choline transporter (CHT) or choline transporter-like proteins (CTL). Amantadine, pyrilamine and quinidine are all of very different chemical structure, but all three are quite lipophilic with respective log P values of 2.53, 2.89 and 2.6. Pyrilamine, quinidine and verapamil are transported by OCTN1. The *bona fide* OCTN inhibitor, L-carnitine at 500 μ M, however, did not attenuate verapamil uptake. Therefore, it is unlikely that any of the five cloned OCT/Ns was involved in verapamil uptake into A549 cells. Pyrilamine also has been suggested to be translocated by a polyspecific organic cation-sensitive transporter, different from the known OCT/Ns or multidrug and toxin extrusion transporter (MATE1), in TR-BBB13 cells¹²⁾, whereas quinidine is known to be an inhibitor of OCT1, OCT2 and for P-gp.⁹⁾ Whilst P-gp is expressed in many biological barriers including the alveolar epithelium, an involvement of this efflux pump in drug uptake is unlikely.

In order to determine a role for the verapamil transporter in the disposition of inhaled bronchodilators, we investigated if the two β_2 -agonists, formoterol and salbutamol, interacted

with verapamil uptake at the respiratory epithelial barrier. Formoterol decreased verapamil uptake slightly but significantly to $71.7 \pm 10.2\%$ at $500 \mu\text{M}$, whereas salbutamol had even less inhibition potential ($85.5 \pm 3.1\%$). Thus, it is unlikely that this inhibition is responsible for drug-drug interactions as the clinically relevant concentrations of drug reaching the pulmonary epithelium are considerably lower. A recently published study from our lab showed that formoterol reduced uptake the cationic fluorophore, 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+ ; $500 \mu\text{M}$) into A549 cells by over 90%, suggesting a common binding site for β_2 -agonists and organic cations at the alveolar epithelium¹³⁾, whilst in the case of verapamil uptake the binding site seems to be distinct.

In conclusion, additional studies are needed to molecularly identify this elusive transporter that mediates verapamil uptake at the alveolar epithelial barrier. Whilst we can only speculate about possible clinical implications of this transporter in the lung, it is unlikely that it is the molecule responsible for drug-drug interactions involving bronchodilators observed previously.^{10,11)}

References

- 1) Salomon, J.J., Ehrhardt, C.: Organic cation transporters in the blood-air barrier: Expression and implications for pulmonary drug delivery. *Ther. Deliv.*, **3**:735-7 (2012).
- 2) Endter, S., Francombe, D., Ehrhardt, C., Gumbleton, M.: RT-PCR analysis of ABC, SLC and SLCO drug transporters in human lung epithelial cell models. *J. Pharm. Pharmacol.*, **61**: 583-91 (2009).
- 3) Foth, H.: Role of the lung in accumulation and metabolism of xenobiotic compounds-implications for chemically induced toxicity. *Crit. Rev. Toxicol.*, **25**:165-205 (1995).
- 4) Dinis-Oliveira, R.J., Remião, F., Duarte, J.A., Ferreira, R., Sánchez Navarro, A., Bastos, M.L., Carvalho, F.: P-glycoprotein induction: an antidotal pathway for paraquat-induced lung toxicity. *Free Radic. Biol. Med.*, **41**:1213-24 (2006).
- 5) Schwartz, J.B., Todd, E., Abernethy, D.R., Mitchell, J.R.: Steady state verapamil tissue distribution in the dog: differing tissue accumulation. *Pharmacology*, **32**:307-12 (1986).
- 6) Todd, E.L., Abernethy, D.R.: Physiological pharmacokinetics and pharmacodynamics of (+/-)-verapamil in female rats. *Biopharm. Drug Dispos.*, **8**:285-97 (1987).
- 7) Han, Y.H., Sweet, D.H., Hu, D.N., Pritchard, J.B.: Characterization of a novel cationic drug transporter in human retinal pigment epithelial cells. *J. Pharmacol. Exp. Ther.*, **296**:450-7 (2001).
- 8) Kubo, Y., Kusagawa, Y., Tachikawa, M., Akanuma, S.I., Hosoya, K.I.: Involvement of a Novel Organic Cation Transporter in Verapamil Transport Across the Inner Blood-Retinal Barrier. *Pharm. Res.*, **30**:847-56 (2013).
- 9) Koepsell, H., Lips, K., Volk, C.: Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm. Res.*, **24**:1227-51 (2007).

- 10) Gnadt, M., Trammer, B., Freiwald, M., Kardziej, B., Bayliss, M.K., Edwards, C.D., Schmidt, M., Friedel, G., Högger, P.: Methacholine delays pulmonary absorption of inhaled $\beta(2)$ -agonists due to competition for organic cation/carnitine transporters. *Pulm. Pharmacol. Ther.*, **25**:124-34 (2012).
- 11) Mehta, R., Kelleher, D., Preece, A., Hughes, S., Crater, G.: Effect of verapamil on systemic exposure and safety of umeclidinium and vilanterol: a randomized and open-label study. *Int. J. Chronic Obstr. Pulm. Dis.*, **8**:159-167 (2013).
- 12) Okura, T., Hattori, A., Takano, Y., Sato, T., Hammarlund-Udenaes, M., Terasaki, T., Deguchi, Y.: Involvement of the pyrilamine transporter, a putative organic cation transporter, in blood-brain barrier transport of oxycodone. *Drug Metab. Dispos.*, **36**:2005-13 (2008).
- 13) Salomon, J.J., Endter, S., Tachon, G., Falson, F., Buckley, S.T., Ehrhardt, C.: Transport of the fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺) in human respiratory epithelial cells. *Eur. J. Pharm. Biopharm.*, **81**:351-9 (2012).

Figure 1. Time course of [³H]-verapamil uptake into A549 cells at 37°C (●) and 4°C (○). Uptake of [³H]-verapamil was measured for 5 min. The impact of low extracellular sodium concentration on [³H]-verapamil uptake was studied after 3 min at 37°C (▲). Each point represents mean ± SD (*n* = 3). ** *P* < 0.01 significantly different from control at 37°C.

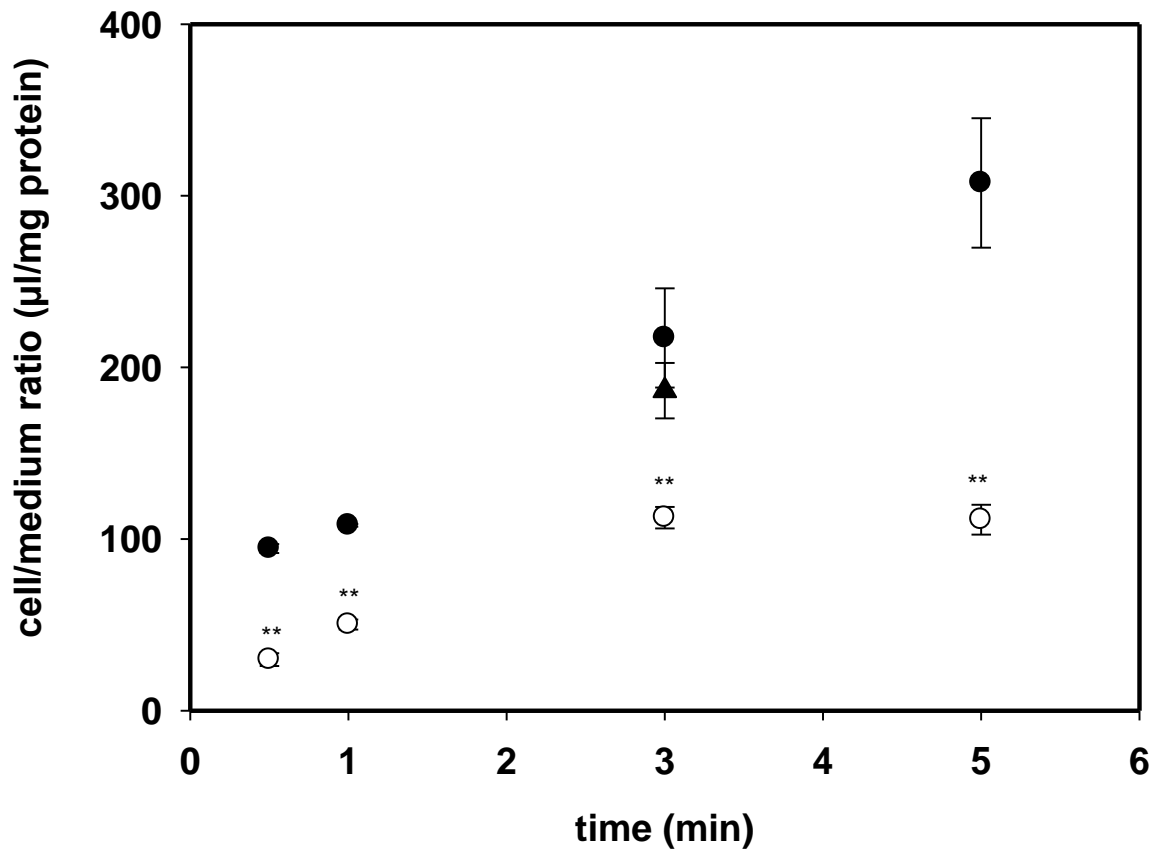


Figure 2. Concentration dependence of [³H]-verapamil uptake by A549 cell monolayers. Cells were incubated for 3 min at concentrations ranging from 1 μM to 200 μM. Total uptake (●) was analysed by Michaelis-Menten kinetics, transporter-mediated uptake (▲) and passive diffusion (dotted line) were calculated and represent the best fit. The inset shows the Eadie-Hofstee transformation indicating a single transporter site: *v* (uptake rate; nmol/min/mg protein), *v/s* (μl/min/mg protein), *s* (concentration of unlabelled verapamil). Each point represents mean ± SD (*n* = 3).

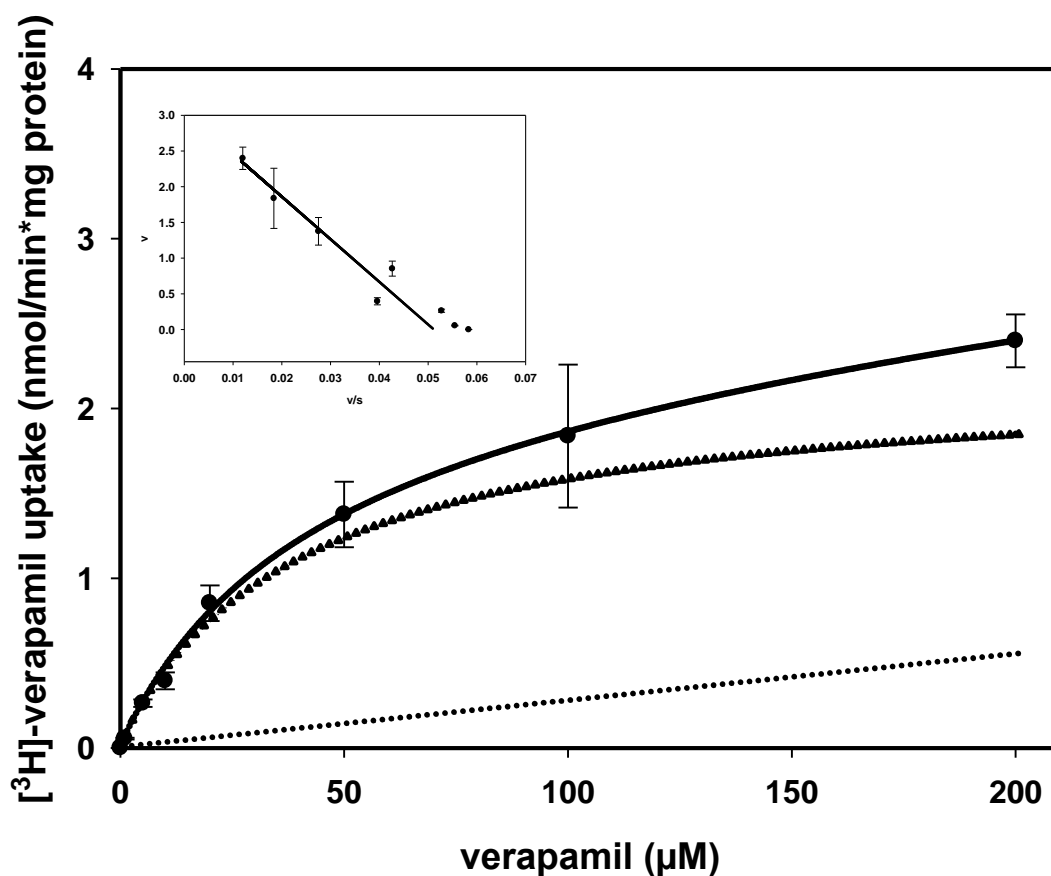


Table 1. Effect of extracellular cations on the uptake of [³H]-verapamil into A549 cell monolayers. Uptake was measured for 3 min at pH 7.4 in ECF buffer. Data represents means \pm SD ($n = 3$). *Indicates a significant difference from control (** $P < 0.01$, * $P < 0.05$).

Compound	Concentration (mM)	[³ H]-verapamil uptake (% of control)
Amantadine	0.5	29.8 \pm 4.1**
L-Carnitine	0.5	92.4 \pm 17.4
Choline	0.5	99.2 \pm 9.4
Decynium 22	0.5	79.7 \pm 11.2
Formoterol	0.5	71.7 \pm 10.2*
MPP ⁺	0.5	85.0 \pm 17.2
Pyrilamine	0.5	32.6 \pm 6.8**
Quinidine	0.5	26.4 \pm 1.4**
TEA	20	93.7 \pm 12.7
Salbutamol	0.5	85.5 \pm 3.1
Verapamil	0.5	30.6 \pm 4.7**