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Transport of the fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) in human respiratory epithelial cells

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ABSTRACT

Organic cation/carnitine transporters (OCT/N) mediate uptake of positively charged molecules. Their role in lung epithelium, however, is not well understood. OCT/N expression and activity was studied in cell lines of human alveolar (A549), bronchial (16HBE14o- and Calu-3) and intestinal (Caco-2) epithelium. Protein levels were largely comparable for all OCT/Ns in the respiratory epithelial cell lines studied, however, OCT2 was exclusively observed in A549 cells. OCT1 and -2 were present at significantly higher levels in Caco-2 cells, compared with the pulmonary epithelial cell types. OCTN1 and -2 were also more abundant in Caco-2. Only OCT3 was expressed evenly across all cell lines investigated. Uptake of 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) was dependent on concentration, temperature, membrane potential and pH. In 16HBE14o-, Calu-3 and Caco-2 monolayers substrate saturation of ASP^+ uptake was not reached. Alveolar A549 cells showed saturable ASP^+ uptake via two transporter sites with K_m values of 12.5±4.0 μM and 456.9±164.5 μM, respectively. This uptake was sensitive to organic cations, but insensitive to carnitine and lysine. We conclude that uptake of organic cations is facilitated by distinct pathways in different regions of lung mucosa. Luminally localised OCT2 appears to be exclusively involved in the alveolar epithelium, whereas basolateral localised OCT3 might play a role in alveolar as well as in bronchial epithelial cells.

KEYWORDS: Organic cation transporters; Bronchial epithelium; Alveolar epithelium; Pulmonary drug disposition; Inhalation biopharmaceutics

1. INTRODUCTION

Although the body of literature on disposition of inhaled medicines is increasing, there is still a surprising lack of mechanistic information on the processes involved (1). To further complicate matters, a broad range of different epithelial cell types, which change significantly in phenotype depending on their localisation, is found along the mucosal surface of the lung (2). It is now widely accepted that membrane transporters impact on the absorption and extrusion of drugs resulting in alterations of their pharmacokinetics and pharmacodynamics, potentially leading to organ toxicity. The study of drug transporters, however, has been mainly focused on the liver, kidneys, gut and also the blood-brain barrier, with the lung remaining largely unchartered terrain (3, 4).

The largest group of transporters are the SoLute Carrier (SLC) proteins with approximately 320 genes in 43 families (5). SLC transporters are often found in epithelial membranes and mediate uptake and secretion of (among many other substrates) organic cations (6). Numerous drug molecules, because of their structural similarity to endogenous compounds, are also substrates of SLC transporters. Transporters involved in organic cation translocation are electrogenic cation transporters OCT1-3 (SLC22A1-3), cation and carnitine transporters OCTN1 and OCTN2 (SLC22A4-5), proton/cation antiporters of the MATE family (SLC47A1-2), monoamine neurotransmitter transporters (SLC6 family), cationic amino acid transporters (SLC7 family), nucleoside transporters (SLC28A1-3 and SLC29A1-4) and several choline transporters (SLC5A7 and SLC44A1-4) (7, 8).

Evidence is now emerging that organic cation transporters are involved in transport processes in various cell types of the lung (9, 10). Epithelial surface cells (11), but also airway smooth muscle cells (12) have been suggested to functionally express members of the SLC22A family of proteins. Hence, it was one of the aims of this work to investigate the expression

levels of the five human OCT/N members in alveolar epithelial A549 and the bronchial epithelial 16HBE14o- and Calu-3 cell lines; all well established *in vitro* models used in biopharmaceutical sciences (13, 14).

The second aim of this paper was to use 4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide (ASP^+) as a non-radioactive OCT/N probe (15, 16), and to study the mechanisms of organic cation transport across distinct lung epithelia, with particular emphasis on OCT/N-mediated drug-drug interactions. Commonly prescribed inhalation medicines such as β_2-agonists and anti-cholinergic drugs carry a cationic charge at physiological pH, but have to cross the epithelial barrier in order to reach the β_2-adrenoceptor localised to the airway smooth muscles. Data from our laboratory and others suggest that β_2-agonists are transported by OCT/N, or at least interfere with OCT/N function (17, 18). The well characterised human intestinal epithelial cell line, Caco-2, was also included in our study, in order to investigate if any differences in transporter expression and function exist between the epithelia of the gut and the lung.

2. MATERIAL AND METHODS

2.1 Chemicals. Cell culture plastics (i.e., Cellstar® 75 cm^2 flasks [cat. no. 658175] and 24-well tissue culture plates [cat. no. 662160]) were obtained from Greiner BioOne (Frickenhausen, Germany), with the exception of Lab-Tek chamber slides (Nunc, Roskilde, Denmark). 4-(4-(Dimethylamino)-styryl)-N-methylpyridinium iodide (ASP^+) was supplied by Invitrogen (Bio-Sciences, Dun Laoghaire, Ireland). Enantiomerically pure S-salbutamol (S-Sal), R-salbutamol (R-Sal), SS-formoterol (SS-For) and RR-formoterol (RR-For) were kind gifts from Sunovion (Marlborough, MA). Polyclonal rabbit anti-OCT1, -OCT2 and -OCT3 antibodies were purchased from Sigma-Aldrich (Dublin, Ireland) and polyclonal goat anti- OCTN1 and -OCTN2 antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Horseradish peroxidase-conjugated anti-rabbit and anti-goat antibodies were purchased from Promega (Medical Supply Company, Dublin, Ireland) and Sigma-Aldrich (Dublin, Ireland), respectively. All other chemicals and reagents were either of analytical grade or of the highest purity available and purchased from Sigma-Aldrich unless stated otherwise.

2.2 Cell culture. Human alveolar epithelial A549 cells (ATCC CCL-185) were obtained from LGC Promochem (Teddington, UK). Cells of passage numbers 79 to 95 were grown at 40,000 cells/cm^2 in an 1:1 mixture of Dulbecco´s modified Eagle´s medium and Ham's nutrient mixture F-12 medium (DMEM/F-12) supplemented with 5% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

Passages of 2.51 to 2.79 of the 16HBE14o- human bronchial epithelial cell line, a gift from Dr. Dieter C. Gruenert (University of California, San Francisco, CA), were cultured at 100,000 cells/cm^2 in minimum essential medium (MEM) supplemented with 10% (v/v) FBS,

100 U/ml penicillin and 100 µg/ml streptomycin, 1% non-essential amino acid solution, 1% sodium pyruvate solution and 0.5% glucose solution.

Calu-3 cells (ATCC HTB-55, LGC Promochem) are derived from serous cells of human lung submucosal glands. Passage numbers 44 to 59 were grown at 75,000 cells/cm^2 in an identical medium as used for 16HBE14o- cultures.

The human colon carcinoma Caco-2 cell line (ATCC HTB-37, LGC Promochem) at passages between 36 and 56 was grown to monolayers at a density of 66,000 cells/cm^2 in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1% non-essential amino acid solution and 1% sodium pyruvate.

All cell types were cultured at 37°C in 5% CO_2 atmosphere on cell culture treated plastics and the culture medium was exchanged every 48 h. Cells were grown to confluent monolayers for 5 (A549), 8 (16HBE14o-), 12 (Calu-3) and 21 (Caco-2) days, before being used in studies as described below.

2.3 Western blot analysis. Cell cultures were lysed with cell extraction buffer (Invitrogen Karlsruhe, Germany) on ice and sonicated (10 s, 2 cycles, 2 Watts) using a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY). Protein sample concentrations were determined using a standard protein concentration assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. Samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gels via electrophoresis (SDS-PAGE) and transferred to immunoblot polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (pH 7.4) for 1 h at room temperature. Incubation with the relevant primary antibody (1:500 for OCT1 and OCT2, 1:2000 for OCT3 and 1:200 for OCTN1 and OCTN2, respectively) was carried out overnight at 4°C, followed by incubation with HRP-conjugated

secondary antibody (1:12,500 [anti-rabbit] and 1:22,500 [anti-goat], respectively) at room temperature for 1 h. Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). Relative levels of protein abundance were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system (Bio-Rad). When appropriate, blots were stripped and analysed for β-actin (Sigma-Aldrich) as internal control.

2.4 Uptake studies. Uptake of the cationic fluorescent probe, ASP^+ was investigated at different concentrations, temperatures, pH values and sodium ion concentrations. The effects of several organic cations (i.e., amantadine, D- and L-carnitine, hemicholinium-3 (HC-3), tetraethylammonium (TEA) and verapamil) on ASP^+ uptake into cell monolayers were studied. Furthermore, the inhibitory potential of β_2-agonists (i.e., formoterol, salbutamol and salmeterol) as well as inhaled glucocorticosteroids (i.e., budenoside and beclomethasone) on ASP^+ uptake was investigated. All experiments were performed in bicarbonated Krebs-Ringer buffer (KRB) composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, and 5.55 mM glucose, pH 7.4, unless stated otherwise.

Time-dependence of ASP^+ uptake was studied by incubating cell monolayers with the fluorophore (at 10 μM final concentration) for up to 90 min at 37°C. Five, 10, 15, 30, 45 and 60min after the beginning of the experiment, monolayers were rinsed twice with ice-cold KRB, solubilised with 1% (w/v) Triton X-100 and ASP^+ activity of the cell lysate was measured (see below). Concentration-dependence of ASP^+ uptake was studied from 10 to 1000 μM at 37°C and 4°C for 20 min. Uptake experiments were also performed in buffers of varying pH values (i.e., pH 5.5, 6.5 and 8.5). In addition, the effect of membrane depolarisation on ASP^+ uptake was investigated. This was achieved by increasing the K^+

concentration of the KRB to intracellular levels with a concurrent reduction of Na^+ by replacing NaCl, $NaHCO_3$ and NaH_2PO_4 with equimolar concentrations of KCl, KH_2PO_4 and $KHCO_3$ (i.e., Na^+ concentration, 5.4 mM; K^+ concentration, 142 mM).

Uptake inhibition experiments were carried out in the presence of amantadine (100 μM and 500 μM), D-carnitine (10 μM and 100 μM), L-carnitine (100 μM and 500 μM), verapamil (10 μM and 200 μM), TEA (1 mM and 20 mM) and HC-3 (100 μM and 500 μM). Concentrations of these organic cations were chosen according their published K_i or IC_{50} values (19). The two β_2-receptor agonists, salbutamol and salmeterol were used as racemic mixtures (RS-salbutamol in all cell lines studied; salmeterol only in A549 cells) and in addition, as pure enantiomers in A549 and 16HBE14o- cells. In the case of formoterol, the RR- and SS-enantiomers were studied in A549 and 16HBE14o- cells. Salbutamol and formoterol were used at final concentrations of 100 μM and 500 μM; salmeterol only at 100 μM (due to the drug's poor solubility). The inhibitory potential of the inhaled corticosteroids (ICS), budesonide (10 - 30 μM) and beclomethasone (10 μM), and additionally selected cationic substrates, acetylcholine (1 mM and 5 mM), corticosterone (100 μM and 500 μM), L-ergotheoneine (100 μM), L-lysine (1 mM) and 1-methyl-4-phenylpyridinium (MPP^+) at final concentrations of 100 - 1000 μM, was studied in A549 cell monolayers. All drugs and compounds were dissolved in KRB (pH 7.4). Salmeterol as well as the ICS drugs were freshly prepared from ethanolic stock solutions. A similar amount of co-solvent was used in the relevant control experiments. In all cases cell monolayers were incubated for 20 min with the compound of interest.

To indirectly investigate the localisation of transporters involved in ASP^+ uptake, studies in Calu-3 monolayers were carried out in the presence of 5 mM EDTA in KRB for 15 min. EDTA will form complexes with multivalent cations, such as, calcium ions and thus lead to

an opening of the tight junctional complexes, allowing ASP^+ access to transporter sites localised to the basolateral membranes.

The fluorescence activity of samples was analysed in 24-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 and 590 nm, respectively. Samples were diluted with KRB, where appropriate. Total cell protein was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, USA).

2.5 Kinetic analysis. Half-saturation constant (K_m) and maximum uptake or release rates (V_{max}) of ASP^+ was calculated by fitting the uptake (or release) rate (v) to the following equation by means of non-linear least squares regression analysis according to the Michaelis-Menten equation (Eadie-Hofstee plot):

$$v = V_{max} \times (S) / [K_m + (S)] \qquad \text{(eq. 1)}$$

where (S) was $[ASP^+]$.

2.6 Statistical analysis. All experiments were carried out at least in triplicate using cells from at least three different passages (n = 3-9). Results, expressed as mean ± S.D., were compared using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test. $P < 0.05$ was considered as significant.

3. RESULTS

3.1 OCT protein abundance in human respiratory epithelial and Caco-2 cell monolayers.

Protein abundance of all five known human organic cation/carnitine transporters was analysed by Western blot in three respiratory epithelial cell lines and Caco-2 cell monolayers (Figure 1). Densitometric analyses revealed that protein levels were more or less equivalent for all transporters across the respiratory epithelial cell types (Figure 2). The notable exceptions were OCT2, which was only detected in A549 cells at relevant levels, and OCTN2, which was found at lower levels in A549 compared to the bronchial cell types. In comparison with signal intensities obtained from Caco-2 cells, it was noted that OCT1, OCT2, OCTN1 for 15 min OCTN2 were synthesised at significantly ($P < 0.05$) lower levels in the lung-derived cell lines. OCT3 was observed at similar levels in all cell types studied (Figure 2).

3.2 ASP^+ uptake into human respiratory epithelial and Caco-2 cell monolayers.

Transport mechanisms of organic cations across membranes of A549, Calu-3, 16HBE14o- and Caco-2 cell monolayers were studied by performing uptake experiments with the fluorescent probe, ASP^+. When studying the time course of ASP^+ uptake, A549 monolayers exhibited the highest intracellular concentration after 60 min by a significant ($P < 0.05$) margin, i.e., 16.54±1.59 nmol/mg protein (Figure 3A). ASP^+ concentrations in Caco-2, 16HBE14o- and Calu-3 monolayers were lower at 2.43±0.79, 1.79±0.44 and 1.01±0.23 nmol/mg protein, respectively (Figure 3B). The uptake increased linearly up to 45 min in A549 and up to 60 min in 16HBE14o-, Calu-3 and Caco-2 cells. Consequently, all further uptake studies were carried out over 20 min.

ASP^+ translocation across cellular membranes at 4°C was significantly reduced, when compared to data obtained at 37°C (data not shown).

3.3 Kinetics of ASP^+ uptake. In Figure 4 the concentration dependence of ASP^+ uptake into cell monolayers is depicted. ASP^+ uptake was observed to be a saturable process only in A549 cells (Figure 4A). An Eadie-Hofstee transformation, corrected for the non-saturable component of ASP^+ uptake into A549 cells, identified two distinct transporter sites (Figure 4C). The calculated kinetic parameters, K_m and V_{max}, are shown in Table 1. Of note, substrate saturation was not reached for ASP^+ uptake into 16HBE14o-, Calu-3 and Caco-2 cell layers (Figure 4B).

3.4 Effect of extracellular conditions on ASP^+ uptake. To further characterise the mechanisms involved in organic cation transport at the respiratory epithelium, the effects of extracellular pH and membrane depolarisation on ASP^+ uptake were studied. The uptake of ASP^+ was significantly ($P < 0.05$) decreased in acidic conditions in all cell lines (Figure 5A). There was, however, no further uptake observed at pH 8.2. In fact, uptake at high pH was reduced when compared to control values, i.e., pH 7.4 (Figure 5A). Membrane depolarisation, achieved by replacement of extracellular Na^+ with equimolar K^+, also resulted in a significantly ($P < 0.05$) reduced ASP^+ uptake into human respiratory epithelial cells. This effect was most pronounced in the case of A549 cell monolayers (Figure 5B).

3.5 Effect of organic cations on ASP^+ uptake. The structural analogue, MPP^+ reduced ASP^+ uptake into A549 monolayers almost completely at 1 mM (Figure 6A). TEA, resulted in a concentration-dependent decrease of ASP^+ uptake and also verapamil significantly diminished ASP^+ uptake to <10% of control (at 200 μM). Both substances have the potential to inhibit OCT1-3 and OCTN1 and -N2 at the concentration range used (19). The steroids, corticosterone, beclometasone and budesonide showed unequal effects. Corticosterone, which

is known to inhibit OCT1, -2 and -3 reduced ASP^+ uptake by almost 80%, whilst both inhaled corticosteroids (ICS) caused a much lower, albeit significant, reduction. Amantadine, like the ICS, an inhibitor of mainly OCT2, had a more pronounced effect, but the concentrations used were also higher. . The OCTN substrates, ergothioneine and D- and L-carnitine reduced ASP^+ uptake to 70-80%. ASP^+ uptake was also inhibited by ~35%, when the choline transporter substrate HC-3 or acetylcholine was present in the transport buffer. L-lysine at 1 mM had no effect on ASP^+ accumulation.

A concentration-dependent attenuation of ASP^+ uptake into A549 cells was achieved with a rank order salmeterol (>90% at 100 μM) > formoterol (>80% at 500 μM) > salbutamol (>40% 500 μM) (Figure 6A). R-salbutamol proved to be a slightly, but not significantly more potent inhibitor of ASP^+ uptake than S-salbutamol ($P = 0.06$), whilst in the case of the formoterol, the SS-enantiomer was significantly ($P < 0.05$) more effective than the RR-enantiomer. Cromolyn sodium did not show a concentration dependency, but both tested concentrations (100 μM and 500 μM) were able to reduce ASP^+ uptake by >20%.

A more limited set of compounds was tested in 16HBE14o- (Figure 6B), Calu-3 (Figure 6C) and Caco-2 (Figures 6D) cell monolayers. Whilst some inhibition of ASP^+ uptake was observed, the effects were generally much less pronounced when compared to A549 monolayers. In all three cell types amantadine significantly ($P < 0.05$) inhibited ASP^+ uptake at the higher concentration (i.e., 500 μM). A similar, but more pronounced effect was also observed with the universal inhibitor, verapamil. Intriguingly, TEA inhibition was not very distinct (i.e., 20 - 25%), and inversely correlated to concentration in the case of Calu-3 and Caco-2 cells. The choline transporter blocker, HC-3, was only significantly ($P < 0.05$) effective in 16HBE14o- cells, and only at the lower concentration used. D- and L-carnitine did not inhibit ASP^+ uptake in 16HBE14o- cell layers. In Caco-2 cells only D-carnitine had a significant ($P < 0.05$) effect, whilst L-carnitine did not attenuate ASP^+ uptake. In Calu-3

monolayers, only the lower concentration of D-carnitine and the higher concentration of L-carnitine exhibited significance, albeit the difference between values obtained for the respective concentrations was not significantly different from each other. Racemic salbutamol showed a concentration dependent inhibition only in the bronchial cell types. Therefore, the enantiomers of salbutamol and formoterol were only tested in 16HBE14o- cells. In all cases a concentration dependent inhibition of ASP^+ uptake was observed. No distinction, however, could be made between the relevant enantiomers.

3.6 Contribution of tight junctions to ASP^+ uptake. Uptake of ASP^+ into Calu-3 monolayers was studied after the tight junctional complex was opened using EDTA complexation of calcium ions. When transporter sites localised to the basolateral membranes were accessible to ASP^+, uptake was significantly increased (Figure 7).

4. DISCUSSION

This work demonstrated the presence of specific transporters for the uptake of organic cations in continuously growing cell lines of respiratory epithelium. Moreover, it confirmed organic cation/carnitine transporter abundance in intestinal Caco-2 cell monolayers. Of note, previous literature on OCT/N expression in these *in vitro* models of pulmonary origin was almost entirely limited to genomic studies (9, 20-23). For example, in a gene expression study of human whole lung tissue based on a reference set of 19,000 genes, OCTN1 and OCTN2 appeared to be strongly expressed transcripts at the 50-75% quartile of gene expression intensity, whereas OCT1 and OCT3 were in the 25-50% quartile and OCT2 even lower at 0-25% (10).

Transporter expression. We confirmed by immunoblot that all five known isoforms of the human SLC22 organic cation/carnitine transporter family, i.e., OCT1, -2, -3 and OCTN1 and -N2 were present in the alveolar epithelial cell line, A549 as well as in intestinal epithelial Caco-2 cells. This is consistent with previous observations of OCT1 (23), OCT3 (20, 21) and OCTN1 and OCTN2 protein expression (21) in the A549 cell line. Müller *et al.* by RT-PCR found mRNA encoding OCT1, -2 and -3 in Caco-2 cell monolayers, however, immunocytochemistry in their study showed only faint staining for hOCT1 (24). Immunoreactivity for hOCT2 and hOCT3 in Caco-2 cells was more pronounced and predominantly localised to the cytoplasm and to the lateral cell membranes. In a separate study, Lamhanwah and colleagues (25) demonstrated the presence of OCTN1 and -2 in Caco-2 cells. Of note, whilst OCTN1 was observed by confocal laser scanning microscopy being predominantly subcellularly localised, OCTN2 was found at both the apical and basolateral cell membranes of Caco-2 cells cultured on Transwell filters. Data from another study showed that Caco-2 cells when grown to differentiated monolayers on glass coverslips,

localise OCTN2 protein exclusively to the brush-border membrane, whereas no signal was detected in basolateral membrane fractions by Western blot (26).

By Western analysis, we found all OCT/N gene products present in the bronchial epithelial cell lines, 16HBE14o- and Calu-3, with the exception of OCT2. Lips and co-workers (11) previously observed immunoreactivity of OCT1 mainly within human bronchial epithelial cells *in situ*, however, weak staining of the apical membrane of ciliated cells was also detected. hOCT2 was mainly located in apical membranes of ciliated cells and to a lesser extent also in basal cells. Immunoreactivity of hOCT3 was localised to plasma membranes of basal cells, basolateral membranes of intermediate cells and apical membranes of ciliated cells. To our knowledge, no data is available on the spatial expression pattern of OCT1, -2 and -3 in human distal lung. In another study, Horvath *et al.* (18) identified OCTN1 and OCTN2 in human airway epithelium *in situ* and freshly isolated airway epithelial cells in primary culture. Whilst OCTN1 was primarily localised to the apical surface of tracheal epithelia, expression was also observed in alveolar type II epithelial cells, albeit at lower levels. OCTN2 was found in apical membranes of airway epithelial cells and also in alveolar type I and type II cells in the lung parenchyma.

Quantitatively, OCT1 and OCT2 were detected at significantly higher levels in Caco-2 cells compared to the respiratory cell types. Only OCT3 was expressed at equivalent levels in all cell lines studied. Abundance of OCTN1 and OCTN2 was lower in the lung derived cells, but these differences were not as pronounced as in the case of OCT1 and -2.

Dependence of ASP^+ uptake on extracellular conditions. ASP^+ has been used as a non-radioactive substrate for monoamine and organic cation/carnitine transporters (15, 16, 18, 27-

30). In our hands, ASP^+ uptake was concentration and temperature-dependent and sensitive to extracellular pH and membrane potential in all cell lines studied.

Horvath *et al.* (18) previously reported that ASP^+ uptake into human airway epithelial cells in primary culture on collagen-coated coverslips had a pH-profile, but was independent of extracellular Na^+ concentration. ASP^+ uptake into placental BeWo cells was mediated by a low-affinity, carrier-associated process exhibiting a K_m of 580±110 μM, and asymmetric transport was observed, with greater permeability in the apical to basolateral direction (16). Whilst the authors of that study did not test for the presence of OCT3, OCTN1/2 or indeed any of the monoamine transporters, they concluded that ASP^+ uptake in their hands was not facilitated by OCT1 or OCT2. Bleasby and co-workers (10) reported that absorption of 1-methyl-4-phenylpyridinium (MPP^+; a structural analogue to ASP^+) by Transwell-grown Caco-2 cells was mediated by a Na^+-dependent transport mechanism, which was inhibited at acidic pH values. Another study from the same year, however, described uptake of [^{3}H]-MPP^+ by Caco-2 monolayers to be metabolic energy-dependent and Na^+-, pH- and potential-independent (31). Of note, Caco-2 monolayers in the latter study were cultured on tissue-treated plastic. The differences observed between published studies reporting organic cation uptake by cultured epithelial cells might be explained by the choice of culture conditions or indeed by clonal variation between the cells used by various groups.

Kinetics and inhibition of ASP^+ uptake. Eadie-Hofstee transformation of ASP^+ uptake into alveolar epithelial-like A549 cell layers indicated two differential transporter sites; one of which exhibited a relatively high affinity (K_m = 12 μM), whereas the other one had a lower affinity (K_m = 450 μM). ASP^+ uptake into the bronchial (i.e., 16HBE14o-, Calu-3) and the intestinal (i.e., Caco-2) cell models did not reach substrate saturation. However, concentrations above 1 mM for ASP^+ could not be dissolved in the buffer system used. These

observations are consistent with a previous study which likewise concluded that total initial uptake rates for ASP^+ in an OCT expression system (at 1 mM) were not saturable, and only time-dependent saturation for maximal cellular fluorescence could be measured (32). In contrast, Horvath *et al.* (18) reported airway epithelial cells in primary culture to exhibit a single transporter site for ASP^+ uptake with an estimated K_m of 394 μM.

Inhibition of ASP^+ uptake into A549 monolayers was most pronounced with MPP^+, TEA, verapamil and corticosterone. A common trait shared by these compounds is their capacity to interact with OCT1-3 (33). TEA and verapamil also have a reported affinity for OCTN1 and –N2 (19). OCTN substrates, ergothioneine (OCTN1), D-carnitine (OCTN2) and L-carnitine (OCTN2 > OCTN1), also attenuated ASP^+ uptake, but only by ~20%. This effect was similar to that observed with budesonide and beclomethasone, both known OCT2 inhibitors (19). A slightly higher inhibition was observed for choline analogues, HC-3 and acetylcholine, and the NMDA-receptor antagonist, amantadine (OCT2 > OCT1). Moreover, β_2-receptor agonists formoterol and salmeterol exerted very strong inhibitory effects. Previous reports from our laboratory and others already hypothesised an interaction between β_2-adrenoceptor agonists and organic cation/carnitine transporters (17, 18, 34). Of note, the inhibitory potential closely followed the lipophilicity of the substances (log *P* salbutamol -2.15; formoterol 0.4; salmeterol 3.88).

Interestingly, in the two bronchial cell lines and Caco-2 monolayers, inhibition of ASP^+ uptake by organic cations was much less pronounced. In 16HBE14o- cells, verapamil, TEA (20 mM), amantadine (500 μM) as well as salbutamol and formoterol (both at 500 μM) showed significant effects. Comparable results were obtained with Calu-3 and Caco-2 cells. In these two cell types, an interesting, yet unexplainable, inverted concentration dependence was observed in the case of TEA.

This study identified clear differences between the processing of ASP^+ by A549 cell monolayers and bronchial 16HBE14o- and Calu-3 as well as the intestinal Caco-2 cell models. Our observations suggest variations in organic cation transporter expression level and localisation between the various cell types. Whilst OCT1, -3, -N1 and -N2 were ubiquitously found, OCT2 was only present in A549 and Caco-2 cells. In the latter cell type Müller *et al.* (24) previously reported hOCT2 and -3 localised to the lateral cell membranes. No information on the cellular localisation of any of the OCT in A549, Calu-3 and 16HBE14o- cells is currently available. We have evaluated several commercially available antibodies and in our hands it was difficult to obtain reproducible results in confocal laser scanning microscopical analysis (data not shown). From the immunohistochemistry data of Lips and colleagues, however, it can be speculated that OCT2, if present, is localised to the apical membrane, whilst OCT3 is found in the basolateral membranes (11).

Our results showed that in alveolar A549 cells significantly higher OCT-mediated ASP^+ uptake occurred than in the other cell types. Sensitivity of this uptake to MPP^+, TEA, verapamil, corticosterone, amantadine and the ICS compounds indicated an involvement of OCT2 and probably OCT3 (19, 34). Interestingly, uptake rates in Calu-3 monolayers reached almost comparable levels, after opening the tight junctions with EDTA (35). This strongly supports the notion that at least one of the responsible transporter sites is localised at the basolateral cell aspect. Under normal conditions, ASP^+ diffusion to the basolateral membrane will be restricted by the tight and adherens junctions, hence, it is unlikely that sufficiently high concentrations are achieved to saturate the transporter site. This is consistent with our observation that no saturation of ASP^+ absorption was observed in polarised Calu-3, 16HBE14o- and Caco-2 monolayers. The observation that cellular junctions in A549 cells are compromised, resulting in an inability of this cell line to form electrically tight monolayers

when cultured on semi-permeable membranes, offers further support to this hypothesis (13). Most pharmacological inhibitors showed only relatively weak activities in 16HBE14o-, Calu-3 and Caco-2 monolayers. The exception to this was the highly lipophilic compound, verapamil. It is therefore possible that passive diffusion of verapamil into the cells occurred, resulting in the inhibition of these basolaterally localised transporters. The exact nature of the transporters involved, however, remains to be elucidated. OCT1 was found in all cell lines studied, but with pronounced differences in protein abundance. In addition, several studies have described the protein predominantly localised to intracellular domains in both Caco-2 cells as well as human bronchial epithelium (11, 24). OCT3, on the other hand, was equivalently expressed across all cell types and has previously been associated with basolateral expression (11, 24). It might hence be argued that OCT3 is the low affinity, high throughput transporter for ASP^+ in our study.

Cis-inhibition by ergothioneine and D- and L-carnitine only weakly attenuated ASP^+ uptake, suggesting that organic carnitine transporters had only a minor involvement in the process. Horvath and colleagues (18) previously proposed OCTN2 as the responsible transporter for ASP^+ uptake into the human airway epithelium. Regretfully, their report fails to demonstrate direct evidence of ASP^+ transport by OCTNs. In addition, a recent publication by Grigat and co-workers (36) comes to the conclusion that OCTN2 is not a general drug transporter and hence, does not translocate ASP^+ in HEK-293 cells. Moreover, the data on the structural ASP^+ analogue, MPP^+ reports only a very weak affinity to OCTN transporters (19). We therefore believe that OCTNs are not involved in ASP^+ uptake by the cell lines investigated in our work.

CONCLUSIONS

In summary, respiratory epithelial cells functionally express a complex array of organic cation transporters. These transporters facilitate ASP^+ uptake into human respiratory epithelial cell monolayers. The major findings of this work were that alveolar A549 cell monolayers showed an absorption pattern different from bronchial 16HBE14o- and Calu-3 as well as intestinal Caco-2 monolayers. Figure 8 shows the hypothetical absorption pathways in the different cell types. The uptake of ASP^+ into A549 cells was consistent with active uptake of ASP^+ across the apical membrane mediated by organic cation transporters, most likely OCT2. Beta-2 agonists and ICS were able to attenuate this process, highlighting possible drug-drug interactions at the respiratory epithelial barrier. In addition, concentration- and temperature-dependent uptake of ASP^+ across the basolateral membrane was observed. It can be speculated that this transport was mediated by OCT3. Studying drug transport processes in an organotypic *in vitro* model is far more complex than in expression systems transfected with only one or two transporters. Particularly, in a case where the transporter sites in question show a high degree of substrate overlap, and where no specific inhibitors are available. Ultimate proof might be obtained using RNAi methods, but the paucity of good commercially available antibodies renders this approach inadequate.

ACKNOWLEDGEMENTS

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Table 1. Kinetic parameters of ASP^+ uptake into A549 cell monolayers.

	K_m (μM)	V_{max} (nmol/min/mg protein)
A549 (high affinity)	12.50±4.00	1.51±0.12
A549 (low affinity)	456.88±164.47	4.50±0.69

FIGURE LEGENDS

Figure 1. Protein abundance of OCT1 (66 kDa), OCT2 (65 kDa), OCT3 (70 kDa), OCTN1 (65 kDa) and OCTN2 (70 kDa) in human respiratory epithelial cells. Representative Western blot of cell lysates isolated from A549 alveolar epithelial cells, 16HBE14o- and Calu-3 bronchial epithelial cells and intestinal epithelial cells, Caco-2. Beta-actin was used as loading control.

Figure 2. Densitometric analysis of organic cation transporter abundance. Protein levels are shown as mean peak intensities relative to the amount found in Caco-2 cells ± SD, $n = 3$.

Figure 3. Time-dependent uptake of ASP^+ into human respiratory epithelial cells and Caco-2 monolayers. (**A**) Time profile of ASP^+ uptake in A549 (●) and (**B**) time profile of ASP^+ uptake in 16HBE14o- (○), Calu-3 (▼) and Caco-2 (∇) monolayers. Cell layers were incubated with ASP^+ (10 µM) as indicated. Data are represented as means ± SD, $n = 3 - 9$.

Figure 4. Concentration-dependent uptake of ASP^+ in human respiratory epithelial cells and Caco-2 monolayers. (**A**) A549 (●) cells and (**B**) 16HBE14o- (○), Calu-3 (▼) and Caco-2 (∇) cell layers were incubated at pH 7.4, 37°C and 4°C for 20 min as indicated. Uptake rates were calculated as the difference in ASP^+ uptake between 37°C and 4°C. The inset in **A** shows the specific (carrier-mediated) uptake by the two distinct transporter sites. (**C**) Eadie-Hofstee transformations of the data: ν, uptake rate (nmol/min/mg protein); S, ASP^+ concentration in mM, ν/S (µl/min/mg protein). In A549 monolayers two distinct transporter sites were identified. Data are represented as means ± SD, $n = 3 - 6$.

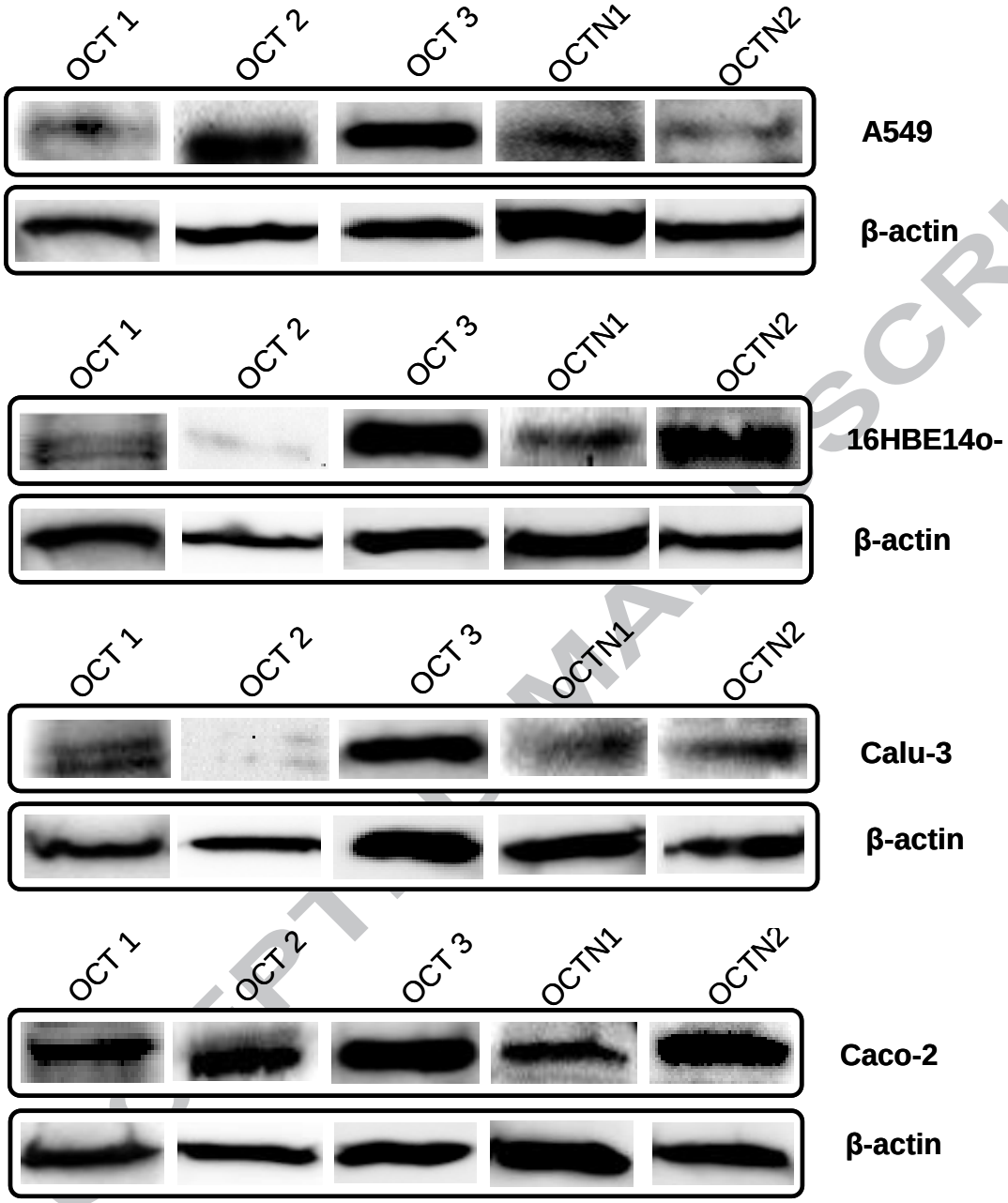
Figure 5. Effect of extracellular pH (**A**) and membrane potential (**B**) on uptake of ASP^+ in human respiratory epithelial cells and Caco-2 monolayers. A549, 16HBE14o-, Calu-3 and Caco-2 monolayers were incubated with ASP^+ (10 µM) at pH 5.7 - 8.2, 37°C for 20 min as indicated. ASP^+ uptake was measured in A549, 16HBE14o-, Calu-3 and Caco-2 monolayers in the presence of increasing and decreasing concentrations of KCl and NaCl, respectively, pH 7.4, 37°C for 20 min. Data are represented as means ± SD, n = 3 - 6.* $P < 0.05$, ** $P < 0.01$ versus control.

Figure 6. Inhibitory effects of organic compounds including β_2-receptor agonists on ASP^+ uptake in human respiratory epithelial cells and Caco-2 monolayers. ASP^+ uptake (10 µM) was measured in the presence of amantadine (100 µM and 500 µM), D-carnitine (100 µM and 500 µM), L-carnitine (10 µM and 100 µM), hemicholinium-3 (100 µM and 500 µM), tetraethylammonium (1 mM and 20 mM) and verapamil (10 µM and 200 µM) in A549 (**A**), 16HBE14o- (**B**), Calu-3 (**C**) and Caco-2 (**D**) cell monolayers at pH 7.4, 37°C for 20 min as indicated. Additionally, ASP^+ uptake (10 µM) was measured in the presence of racemic salbutamol (RS-salbutamol, 100 µM and 500 µM) in A549, 16HBE14o-, Calu-3 and Caco-2 cell monolayers. Furthermore, the effect of salbutamol enantiomers (R-salbutamol and S-salbutamol; 100 µM and 500 µM) and formoterol enantiomers (RR-formoterol and SS-formoterol; 100 µM and 500 µM) was studied in A549 and 16HBE14o- cells at pH 7.4, 37°C for 20 min as indicated. Moreover, ASP^+ uptake was characterised in the presence of acetylcholine (1 mM and 5 mM), beclomethasone (10 µM), budesonide (10, 20 and 30 µM), corticosterone (100 µM and 500 µM), ergotheoneine (100 µM), lysine (1 mM) and MPP^+ (100 µM, 500 µM and 1 mM) in A549 cells. Values are represented as means (% of control) ± SD, n = 3 - 9.* $P < 0.05$, ** $P < 0.01$ versus control.

Figure 7. Contribution of the tight junctional barrier to uptake of ASP^+ in human respiratory epithelial cells. Calu-3 cell monolayers were incubated with ASP^+ as described previously (●) or in the presence of 5 mM EDTA (○). Data are represented as means ± SD, $n = 3$. * $P <$ 0.01 versus control.

Figure 8. Proposed absorption pathways for ASP^+ in alveolar, bronchial and intestinal epithelial cells.

Figure 1.



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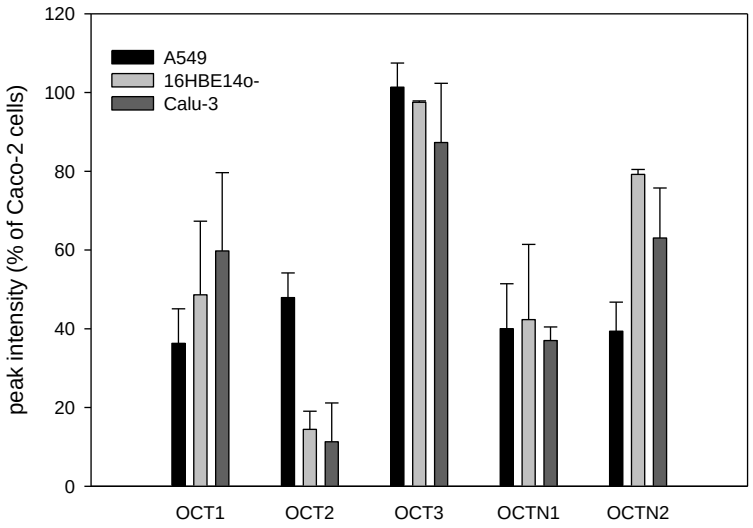

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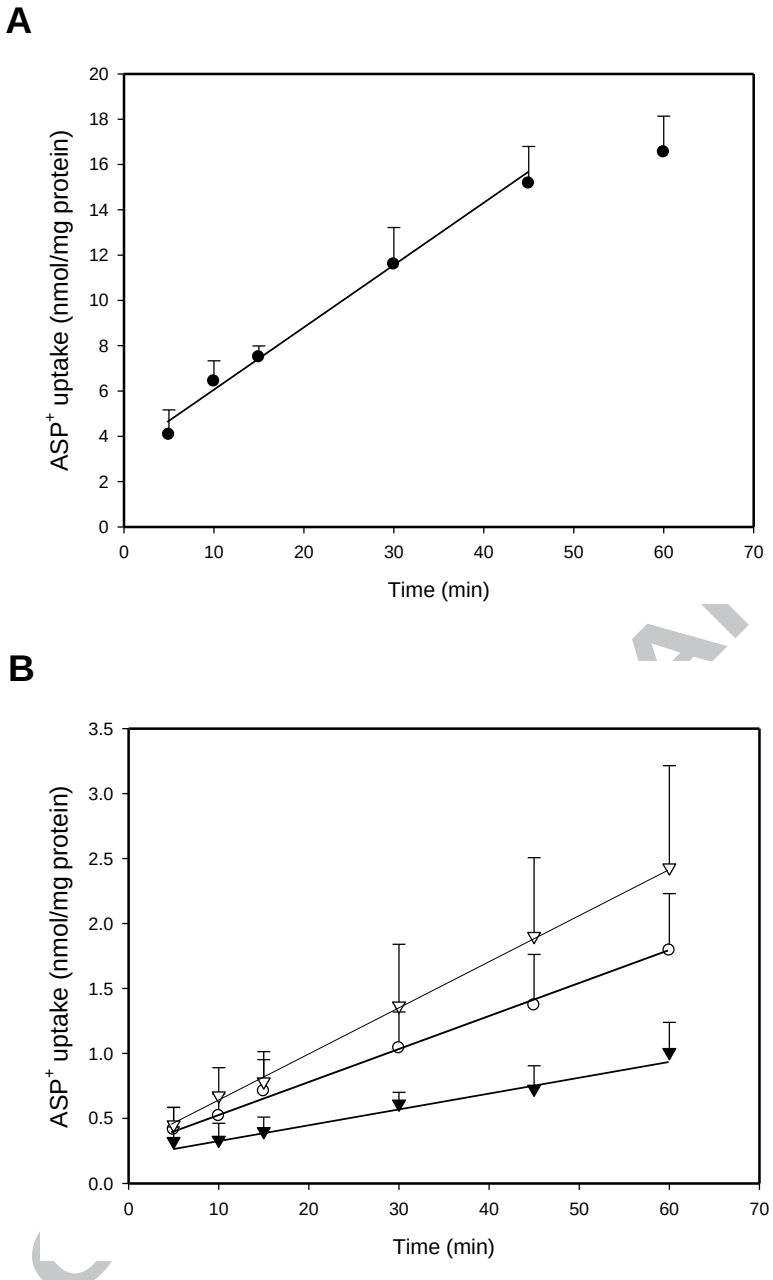

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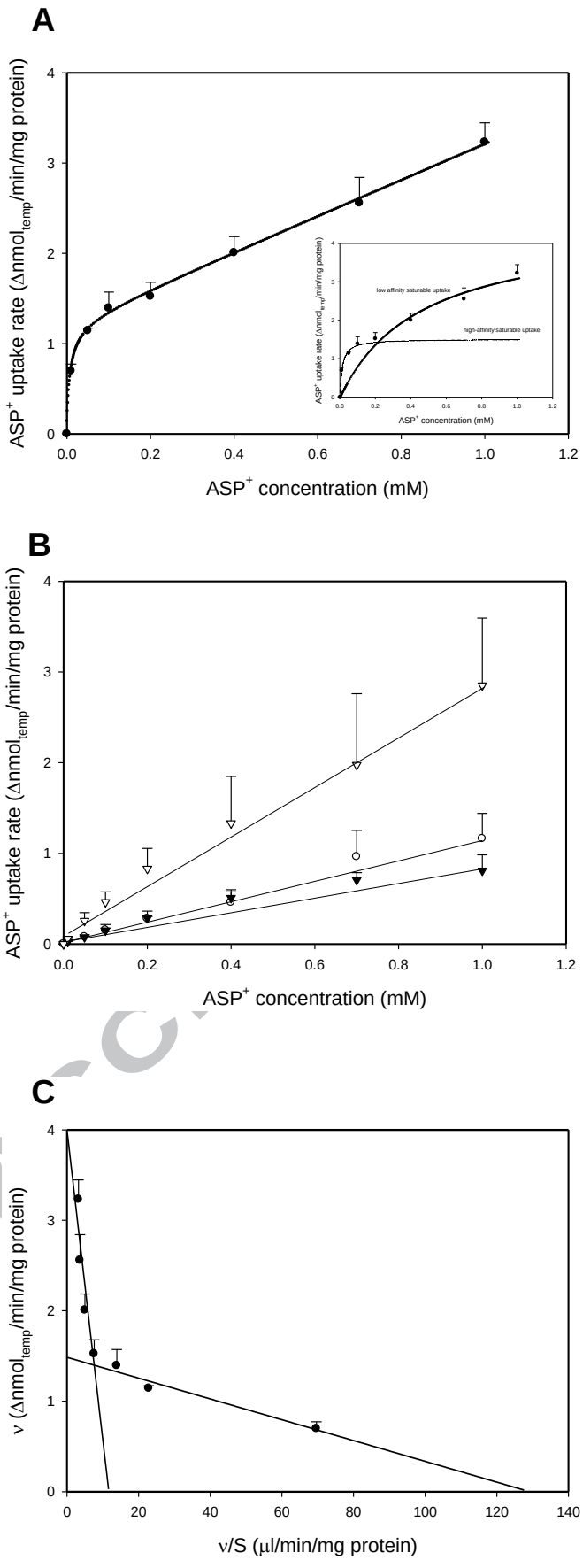

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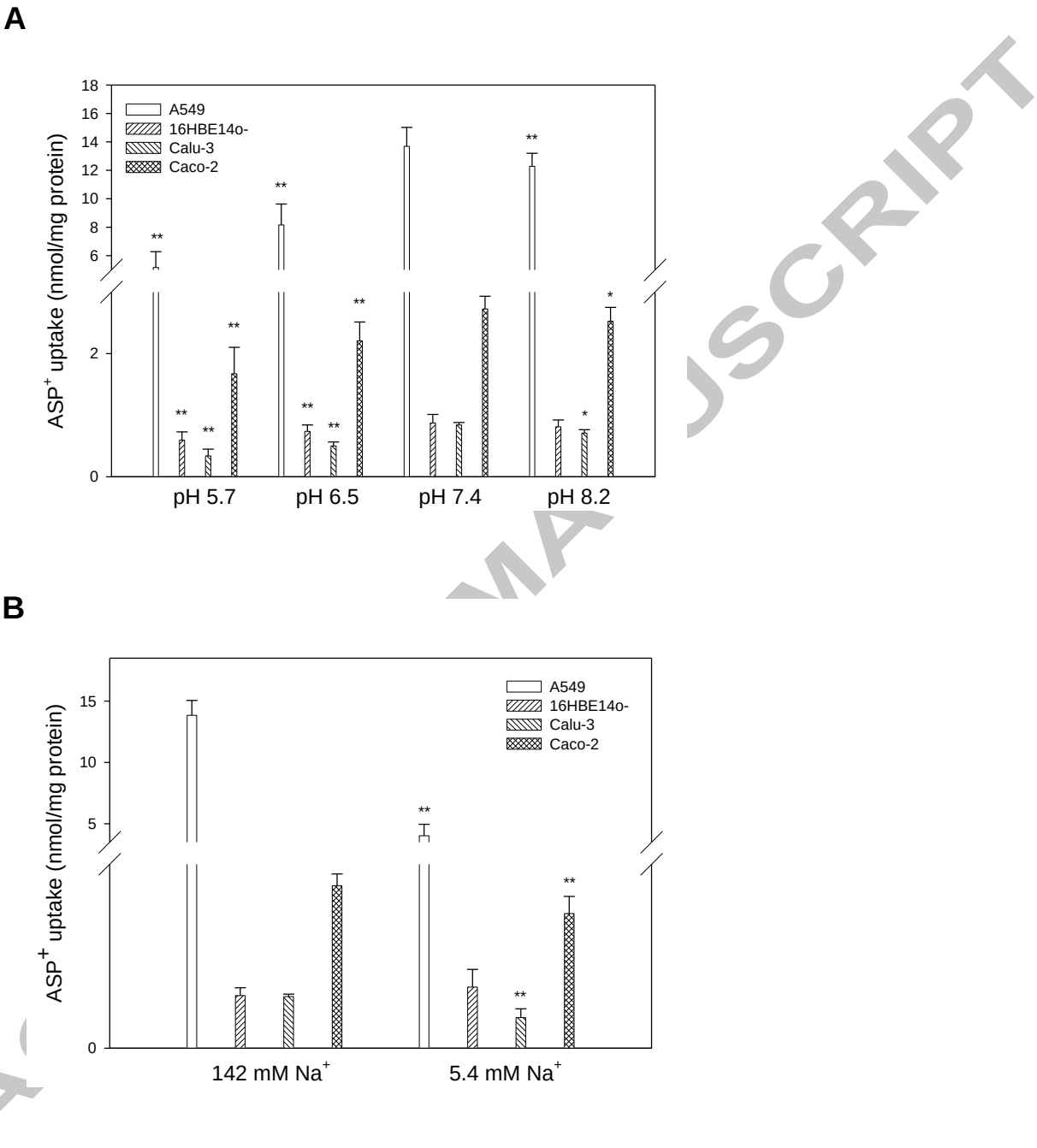
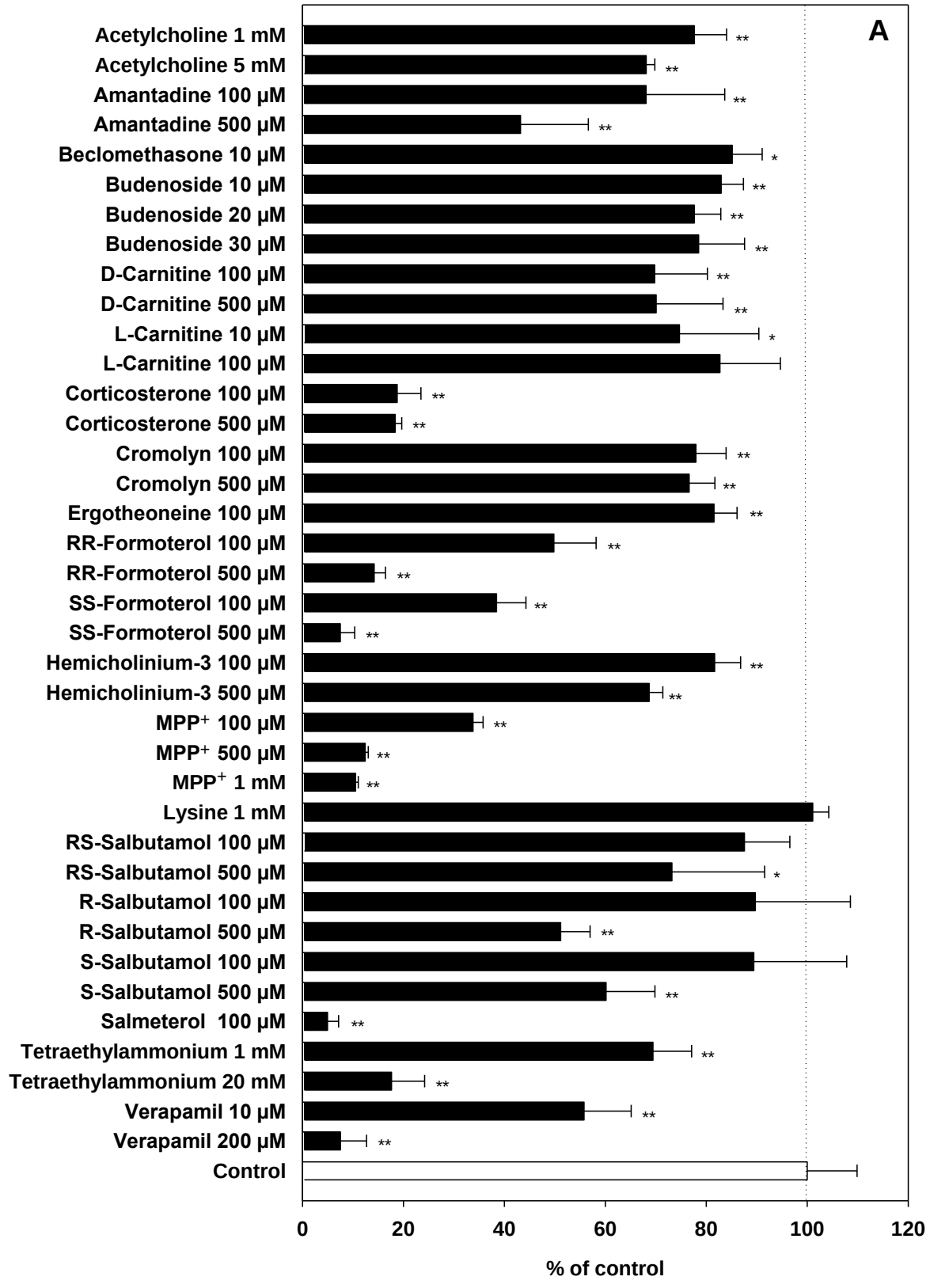
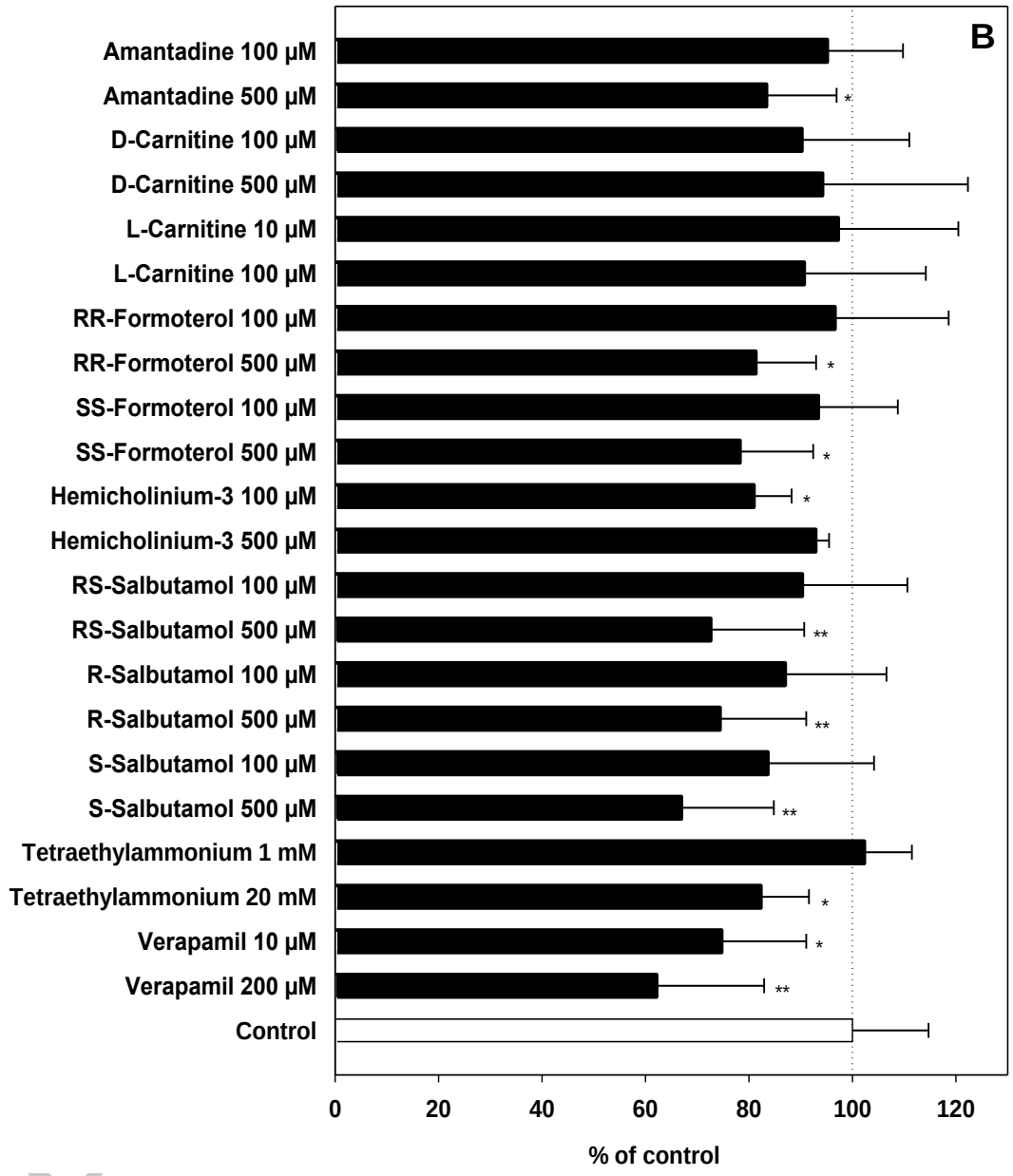
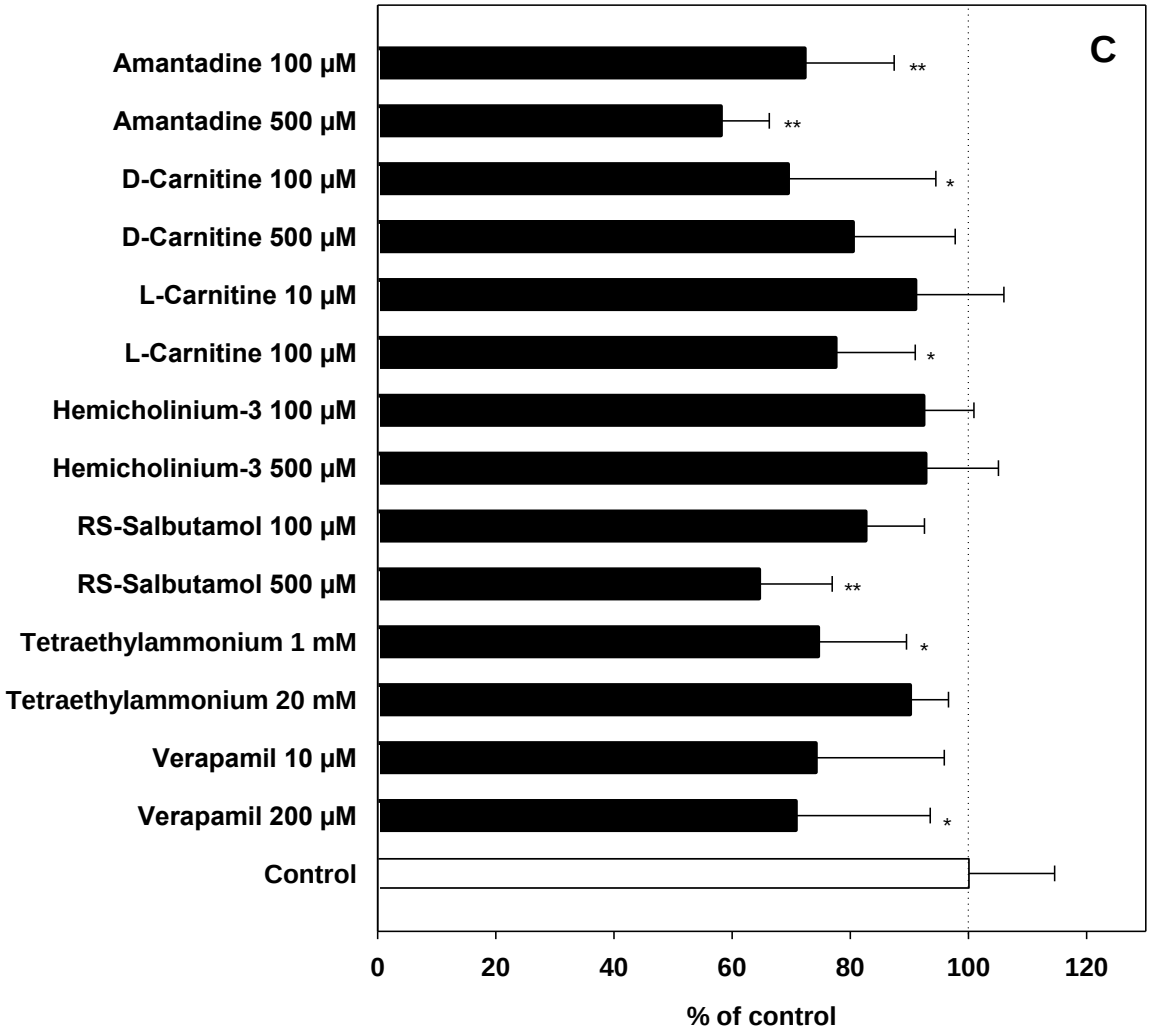


Figure 6.







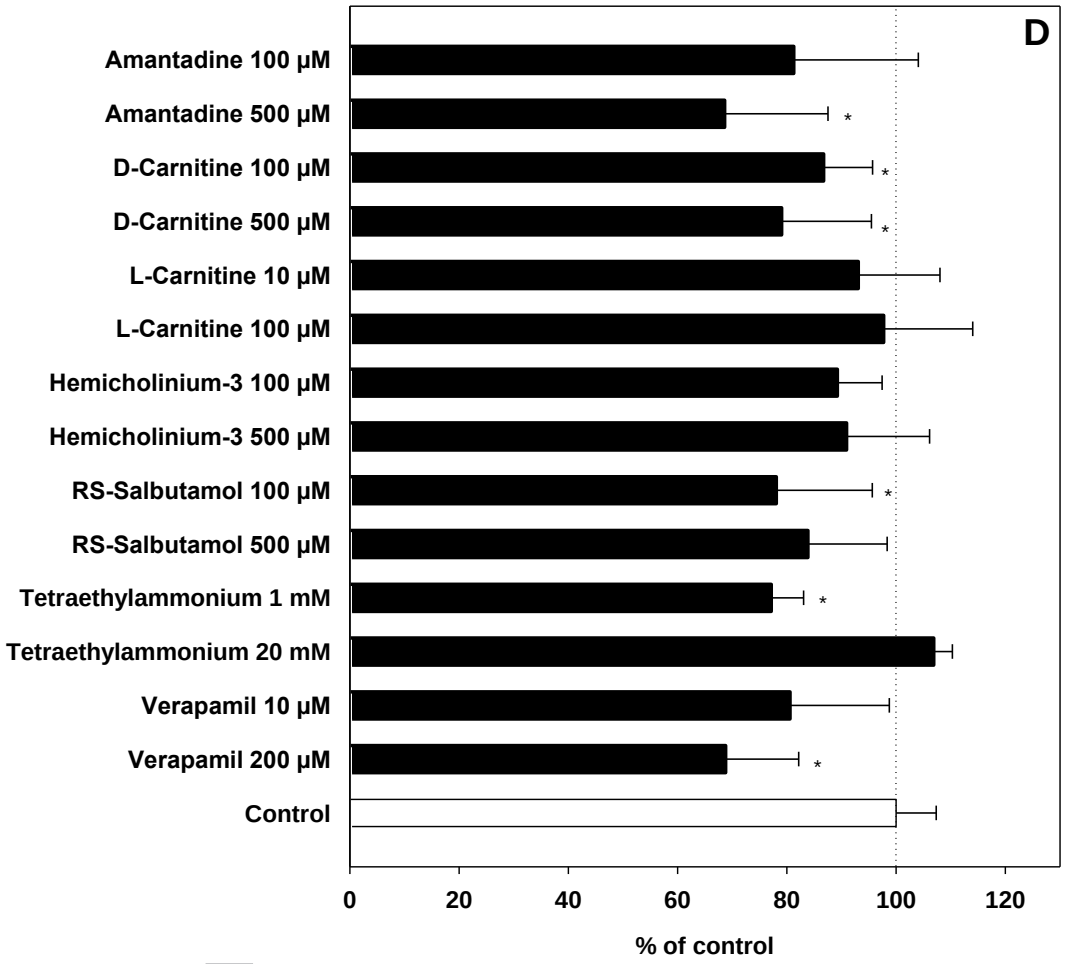


Figure 7.

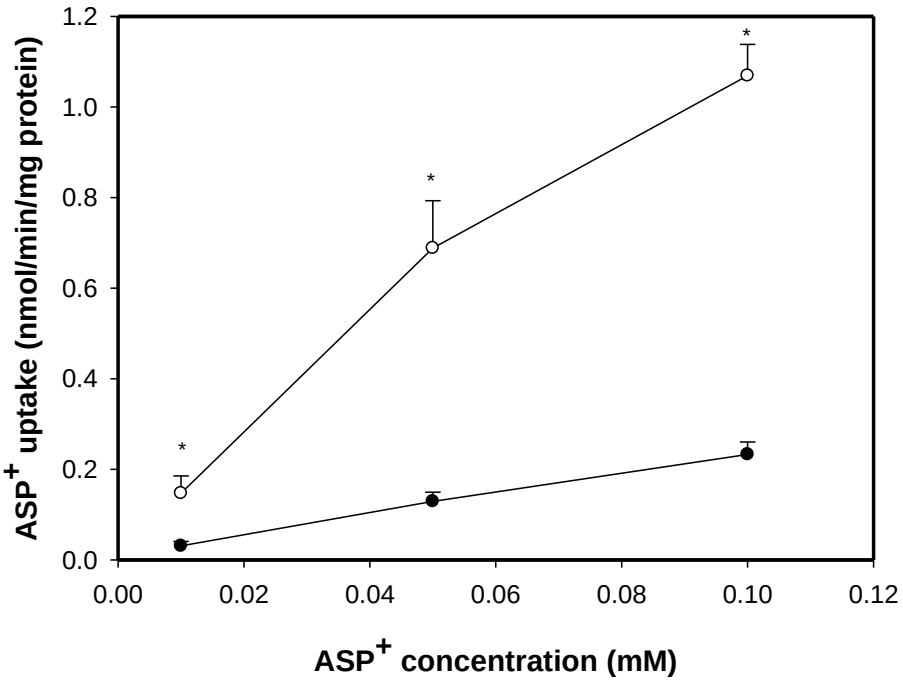
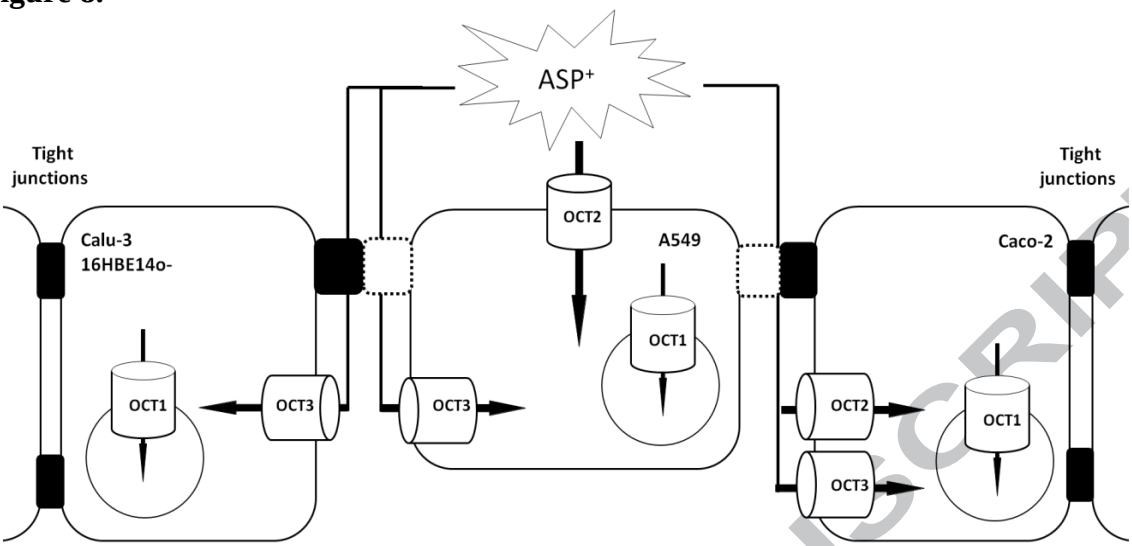
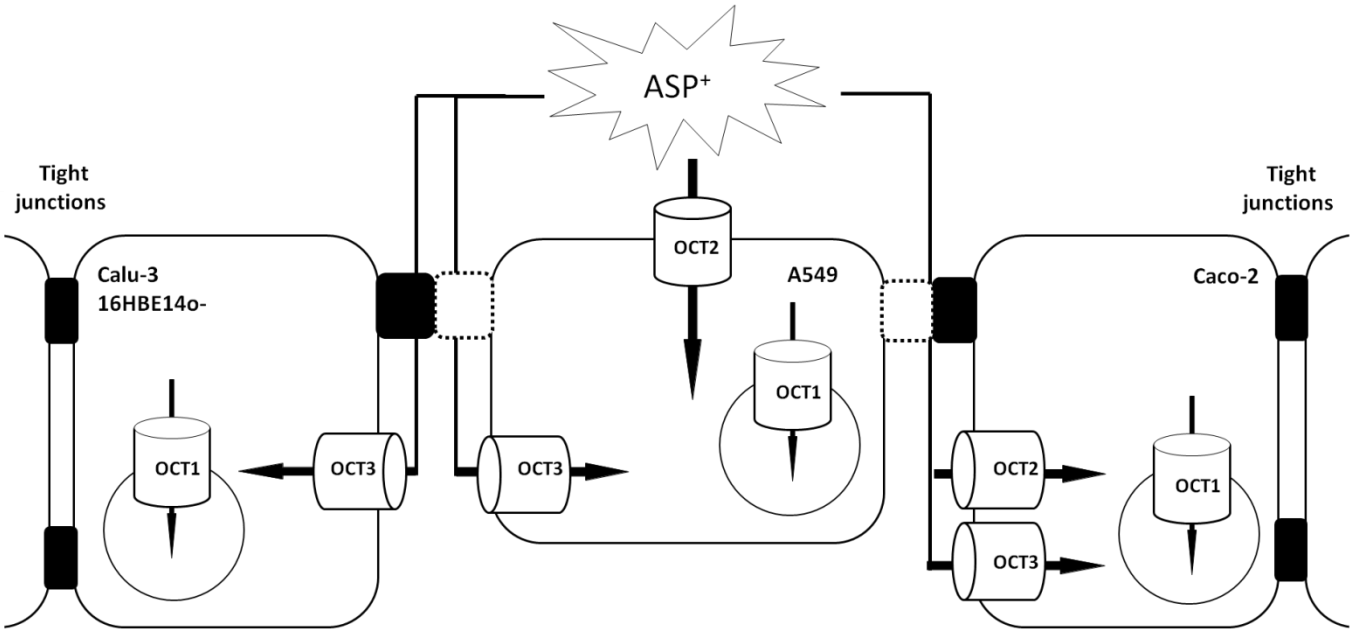


Figure 8.



*Graphical Abstract



Different absorption pathways for ASP^+ in alveolar, bronchial and intestinal epithelial cells.