Ipratropium is 'luminally recycled' by an inter-play between apical uptake and efflux transporters in Calu-3 bronchial epithelial cell layers

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ABSTRACT

The mechanism by which quaternized anticholinergic bronchodilators permeate the airway epithelium remains controversial to date. In order to elucidate the role of drug transporters, ipratropium bidirectional transport as well as accumulation and release studies were performed in layers of the broncho-epithelial cell line Calu-3 grown at an air-liquid interface, in presence or absence of a range of transporter inhibitors. Unexpectedly, a higher transepithelial permeability was observed in the secretory direction, with an apparent efflux ratio > 4. Concentration-dependent and inhibitor studies demonstrated the drug intracellular uptake was carrier-mediated. Interestingly, monitoring drug release post cell loading revealed the presence of an efficient efflux system on the apical side of the cell layers. Acting in concert, apical transporters seem to promote the 'luminal recycling' of the drug and hence, limit its transcellular transport. The data are in agreement with an apical Organic Cation Transporter (OCT) being involved in this process but also suggest the participation of unknown uptake and efflux transporters sensitive to probenecid. This study suggests the absorption of ipratropium across the pulmonary barrier is primarily governed by paracellular passive diffusion but transporters might play a significant role in controlling the drug local concentrations in the lungs.

KEYWORDS

Drug inhalation; pulmonary drug delivery; in vitro models; drug transporters; carrier-mediated transport; muscarinic M3 receptor antagonists

ABBREVIATIONS

a-*b*: apical to basolateral; ALI: air-liquid interface; *b*-*a*: basolateral to apical; FITC: fluorescein isothiocyanate; HBSS: Hank's Balanced Salt Solution; IPRL: isolated perfused rat lungs; MATE: multidrug and toxin extrusion transporters; MRP: Multidrug Resistance Protein; OAT: Organic Anion Transporters; OCT: Organic Cation Transporters; P_{app}: Coefficient of apparent permeability; TEA: tetraethylammonium; TEER: trans-epithelial electrical resistance.

1. INTRODUCTION

Anticholinergic bronchodilators are the first line therapeutic agents in the pharmacological management of Chronic Obstructive Pulmonary Disease (COPD)[1]. In order to maximise local drug concentrations in the lung with reduced systemic exposure, these drugs are administered by inhalation. Although their quaternary ammonium structure renders these molecules too polar to cross biological barriers, their engagement with the drug target on airway smooth muscles and their rapid absorption[2,3,4] (T_{max} ~ 5 minutes) following inhalation demonstrate their ability to cross the lung epithelial barrier. However, the absorption mechanism, i.e., drug transporter-mediated vs passive diffusion is not entirely clear.

The lungs express a range of transporters belonging to both the ATP-binding cassette (ABC) and solute carrier (SLC) families[5,6]. It has been hypothesised that polyspecific organic cation transporters (OCT/Ns) belonging to the SLC22 superfamily of drug carriers, may play a role in the lung disposition of the anticholinergic bronchodilators[7]. The short-acting ipratropium and the long-acting tiotropium are indeed recognised substrates for OCT1, OCT2 and OCTN2 while OCT3 only transports ipratropium and the OCTN1 subtype has a low affinity for both compounds[8,9,10]. Interactions between the more recently approved long-acting glycopyrronium and OCTs have not been systematically explored to date. Nevertheless, OCT1 and OCT2 are known to transport the drug[8].

In agreement with uptake experiments in OCT/N transfected cells, it has been shown that both ipratropium and tiotropium are internalised by the human bronchial epithelial cell line BEAS-2B via an OCTN2-mediated mechanism[11]. In addition, the transporter was also reported to be involved in the accumulation of the short-acting bronchodilator in the tracheal epithelium of mice in vivo[9]. More recently, a study in various lung epithelial cell lines highlighted the role of the OCT/N

carriers in the intracellular accumulation of ipratropium, with different subtypes playing a prominent role depending on the cell line[12].

However, in contradiction with drug uptake data, the absorption of ipratropium in isolated perfused rat lungs (IPRL) following intra-tracheal delivery was unaffected by a pre-administration of a high concentration of the drug or of the OCT1-3 inhibitor MPP^+, suggesting it is primarily mediated by passive diffusion[12]. Drug uptake studies in undifferentiated lung cells and absorption measurement in intact lungs therefore led to contradictory conclusions regarding the role of drug transporters in the trans-epithelial permeability of anticholinergic bronchodilators.

Due to their low intrinsic permeability across cell membranes, charged molecules may exploit uptake and efflux transporters to enter or exit the cells, respectively[13]. To unravel the role of transporters in the permeability of drug molecules across biological barriers, epithelial monolayer systems are considered invaluable. For instance, the potential impact of carrier-mediated transport on the oral bioavailability of various cationic molecules was demonstrated in intestinal Caco-2 monolayers[14,15]. However, to date, no such systematic investigation has been undertaken with inhaled cationic anticholinergic bronchodilators in a permeability model that anatomically represents the lung epithelium.

Amongst available human airway in vitro models, the bronchial epithelial cell line Calu-3 is the most extensively used for investigating drug transport characteristics[16,17]. When cultured at an air-liquid interface (ALI) on permeable supports, Calu-3 cells form tight layers that resemble the native bronchial epithelium[18,19] and are able to predict drug absorption in rat lungs[20]. Importantly, Calu-3 layers express the range of drug transporters found in normal human bronchial epithelial cell layers grown in similar conditions if maintained for 21 days at the ALI[21]. More specifically, we have shown that the same OCT subtypes were present in both models, i.e., OCT1, OCT3,

OCTN1 and OCTN2 with an OCT activity detected on the apical side of the Calu-3 layers[22,23]. Furthermore, the functionality of OCT1, OCT3 and OCTN2 has also been confirmed in undifferentiated Calu-3 cells[24,25].

In this study, we hypothesized that investigating the permeability characteristics of the anticholinergic bronchodilators in differentiated ALI Calu-3 layers would clarify the role of transporters in the disposition of the drugs across the lung epithelial barrier. The trans-epithelial transport of ipratropium, tiotropium and glycopyronnium was evaluated in the cell layers in both the absorptive and secretory directions. As data were similar for the three molecules, permeability measurements in presence of increasing drug concentrations or a range of transporter inhibitors were only performed with ipratropium. Bi-directional transport experiments were complemented with intracellular accumulation and release studies in order to gain a deeper understanding of the mechanisms involved in ipratropium trafficking across Calu-3 layers.

2. EXPERIMENTAL SECTION

2.1. Materials

Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and used between passage 30 and 40. Twelve well polycarbonate Transwells® with 0.4 µm pore size and a surface area of 1.12 cm^2) were purchased from Corning Costar (Kennebunk, US).
All the cell culture reagents and chemicals were procured from Sigma-Aldrich (Poole, UK). HPLC grade solvents for LC-MS/MS analysis were from Fisher Scientific UK. (Loughborough, UK).

2.2. Cell culture

Calu-3 cells were cultured in Dulbecco's modified Eagle's medium / Ham's F12 nutrient mixture (DMEM: F12) 1:1 supplemented with 10% v/v of foetal bovine serum (FBS, non USA origin and sterile filtered), 1% v/v of penicillin-streptomycin antibiotic solution, 1% v/v of 2 mM L-glutamine and 1% v/v of non-essential amino acids. They were maintained at 37°C in a humidified CO_2 atmosphere with medium changed every other day. Upon passaging, cells were seeded at a density of 1 x 10^5 cells/cm^2 on Transwells®. After 24 h of incubation, they were raised at an ALI by aspirating the medium from both the apical and basolateral chambers and adding 500 µL of medium in the basolateral chamber only. Thereafter, the cell culture medium was replaced every other day until day 21 post seeding when the differentiated cell layers were used for experimentation. The layer integrity was verified prior to and post experiments by measuring the trans-epithelial electrical resistance (TEER), using an EVOM meter with chopstick electrodes (World Precision Instruments, Stevenage, UK) after a 30 min incubation in Hank's Balanced Salt Solution (HBSS). Cell layers exhibiting TEER values greater than 500 ohm.cm^2 were selected for experimentation and only data obtained in layers which had maintained a TEER above this threshold at the end of the study were considered for analysis.

2.3. Transepithelial transport studies

The transport of ipratropium (10, 30, 100 and 300 µM), tiotropium (10 µM) and glycopyrronium (10 µM) across Calu-3 cell layers was measured in both apical to basolateral (*a*-*b*) and basolateral to apical (*b*-*a*) directions. The permeability of the passive diffusion marker metoprolol (10 µM) was assessed following the same protocol.

For *a*-*b* experiments, 0.55 mL of HBSS containing the test compound was added into the apical (donor) chamber of the Transwells® and the study was initiated by adding 1.5 mL of blank HBSS to the basolateral (receiver) chamber. For *b*-*a* experiments, 1.55 mL of HBSS with the test compound was placed in the basolateral (donor) compartment and the study was initiated by adding 0.5 mL of blank HBSS on the apical (receiver) side of the layers.

A 0.05 mL sample was collected from the donor compartments for determination of the initial concentration and the Transwell® plate was placed on an orbital shaker (60 rpm) inside the incubator (5% CO_2, 37^{o}C). Appearance of drug in the receiver compartments was monitored by collecting 0.3 mL or 0.1 mL samples for *a*-*b* and *b*-*a* experiments, respectively, after 0.5, 1, 2 and 4 h. At each time point, samples were replaced with an equal volume of blank HBSS. At the end of the experiment, 0.05 mL was collected from the donor compartments and all the study samples were stored at -20^{o}C until analysis by LC-MS/MS.

In order to further investigate the role of drug transporters in ipratropium permeability across Calu-3 layers, these were pre-incubated with HBSS containing one of the following inhibitors: 1-methyl-4-phenylpyridinium (MPP^{+}) - 500 µM, tetraethylammonium (TEA) - 5 mM, L-carnitine - 1 mM, probenecid-100 µM or verapamil-100 µM, for 30 minutes. Each experiment was initiated by co-incubating the donor side of the cell layers with ipratropium and one test inhibitor in HBSS and adding HBSS containing the inhibitor in the receiver compartment. After each sample

collection, the volume collected was replaced with HBSS containing the inhibitor in order to maintain a constant concentration throughout the experiment.

The barrier properties of the Calu-3 layers were verified by measuring the permeability of the paracellular marker, fluorescein isothiocyanate–dextran (FITC-dextran, average molecular weight 3,000-5,000 Da) at a concentration of 0.5 mg/mL following the experimental protocol described above. The fluorescent dye was also used to investigate the effect of organic cations on the paracellular space. In that case, its permeability was studied in the *a-b* direction, alone or during co-incubation with ipratropium (300 µM), MPP^+ (500 µM) or TEA (5 mM). The sample fluorescence was measured using a multimode microplate reader Spark® 10M (Tecan) at an excitation and emission wavelengths of 485 and 535nm, respectively and converted into FITC-dextran concentrations using a standard curve.

Permeability data were obtained from at least three layers and the apparent permeability coefficient (P_{app}) was calculated using the equation below.

$$P_{app} = \frac{J}{A \times C_0}$$

J = flux (moles/sec)

A= surface area of the cell layers (1.12 cm^2)

C_0 = initial concentration in the donor chamber (moles/mL)

2.4. Drug uptake studies

The role of drug transporters on ipratropium cell uptake was investigated after drug exposure from both the apical and basolateral sides of the ALI Calu-3 cell layers. The incubation and experimental conditions were similar to those described above except that the drug solution was withdrawn from

the donor compartment after 5 min incubation and the cell layers were quickly washed with cold PBS (4ºC), three times. The layers were excised from the Transwells® and collected into 1.5 mL tubes. Cells were lysed with the addition of chilled methanol containing glycopyrronium (5 nM) as an internal standard. The cell lysates were vortexed, centrifuged at 10,000 rpm for 10 minutes at 4ºC and the supernatant collected were stored at -20ºC until analysis.

For inhibitory experiments, the layers were subjected to a 30 min pre-incubation with MPP^+, TEA, L-carnitine or probenecid at the same concentration as above, then exposed to ipratropium in presence of the test inhibitor in the donor compartment while HBSS containing the inhibitor was placed in the receiver compartment. Cell uptake in presence of the inhibitors is expressed as a percentage of drug accumulation in control conditions.

2.5. Drug release studies

For initial drug release studies, cell layers were pre-loaded with ipratropium (10 µM) from their apical side. After 45 minutes of incubation, the drug solution was aspirated from the donor compartment and the cell layers were given a quick wash with cold PBS, three times. They were then exposed to 500 µL HBSS from both the apical and basolateral sides. The release of the accumulated drug was studied by collecting 50 µL from both compartments over 2 h. Each sample was replaced with 50 µL blank HBSS.

Ipratropium release was then monitored in the presence of various inhibitors. The experimental design was similar to that mentioned above, except that cell layers were pre-loaded with ipratropium (10 µM) for only 5 minutes. After a quick wash with cold PBS, the cell layers were incubated with 500 µL HBSS with or without inhibitors on both their apical and basolateral sides. Samples were taken from the apical compartment and replaced with 50 µL of either blank or

inhibitor containing HBSS. The effect of inhibitors on drug release was presented as % of drug release in the control group.

2.6. Bioanalysis

The study samples (50 µL) were processed with the addition of 150 µL of chilled methanol containing 5 nM of the internal standard (tolbutamide for metaprolol, glycopyrronium for ipratropium; ipratropium for tiotropium and glycopyrronium). The samples were vortexed and centrifuged at 10,000 rpm for 10 minutes at 4^{o}C. The resulting supernatant was mixed with 0.1% v/v formic acid (1:1 v/v) and 10 µL of this was injected into the Quattro Ultima triple-quadrupole mass spectrometer (Micromass, UK) interfaced via an electrospray ionization probe with Agilent (1100 Series, Agilent Technologies) HPLC system . Working stocks were prepared in methanol and a 12 point calibration curve (CC) was prepared in HBSS ranging from 0.24 to 500 nM utilized for each analyte. Sample quantitation was achieved by fitting curves to a weighted linear regression (1/concentration^{2}). Quality control (QC) samples prepared in blank HBSS were interspersed between study samples to monitor batch performance. Batch acceptance was set at within ±20 % of the nominal concentration for each standard and QC sample.

Chromatographic separation was achieved by an ACE Excel 2 C18-AR (50 x 2.1mm) column with a mobile phase consisting of methanol: water with 0.1% v/v formic acid. A gradient chromatographic method was used, where the % of methanol was increased from 45 to 90 within 2 minutes and maintained for 1 minute, before returning to the initial level of 45% within another 3.5 minutes. The flow rate was set at 0.3 mL/min. Sample temperature was kept at 4^{o}C, and column temperature was set at 40^{o}C.

The retention time was determined as 1.1, 1.37, 2.4, 3.8 and 3.9 min for ipratropium, metaprolol, tiotropium, tolbutamide and glycopyrronium, respectively. Analytes were detected in the positive

ionization mode and data acquisition was carried out by using multiple reaction monitoring (MRM) as follows: ipratropium (332.16 > 165.89), metaprolol (268.5>115.9), tiotropium (392.06 > 151.87), (tolbutamide: 271.2>172) and glycopyrronium (318.09 > 115.84). Instrument control and data acquisition were performed by Masslynx software packages (version 4.1). Data processing and analysis were performed using the QuanLynx software.

2.7. Statistical analysis

The results are presented as mean ± standard deviation (n = 3-4 layers). GraphPad Prism version 6 was used for statistical analysis. The normal distribution of initial transport and uptake data consisting of $n \geq 5$ was confirmed and thereafter, assumed for all data sets. Unpaired t-test was used to compare two groups and ANOVA with Dunnett's multiple comparison test was used for more than 2 groups. A p value < 0.05 was considered as indicative of statistical significance.

3. RESULTS

3.1. Transepithelial transport

The bi-directional permeability of the three anticholinergic bronchodilators ipratropium, tiotropium and glycopyrronium was measured in ALI Calu-3 layers. These were deemed to exhibit appropriate barrier properties since the P_{app} of the paracellular marker FITC-dextran was measured as 0.14 and 0.19 $\times 10^{-7}$ cm/s in the *ab* or *ba* direction, respectively, i.e., was in the expected range[18] (Figure 1).

A similar asymmetrical transport was observed for all drugs with, surprisingly, the P_{app} ($\times 10^{-6}$ cm/s) in the *b-a* direction found to be significantly higher than in the opposite direction: 1.27 ± 0.1 vs. 0.16 ± 0.02 for ipratropium; 1.19 ± 0.18 vs.0.22 ± 0.07 for tiotropium and 1.09 ± 0.1 vs 0.23 ± 0.06 for glycopyrronium, giving efflux ratios of 8.1, 5.5 and 4.8 respectively (Figure 2). In contrast, the transport of the transcellular passive diffusion marker metoprolol was one order of magnitude higher and similar in both directions, with an efflux ratio of 0.9 (Figure 1). Transport data were very reproducible as ipratropium P_{app} values were not statistically different when measured over three different passage numbers (Supplementary info 1).

As a common mechanism of transepithelial transport was suspected for the three bronchodilators, the study was pursued with only ipratropium. Concentration dependent permeability measurements showed a trend towards a decrease in *a-b* transport with an increase in the apical donor concentration, the P_{app} value being significantly lower at 100 and 300 µM than at 10 µM (Figure 3). In contrast, increasing ipratropium basolateral concentration up to 300 µM had no effect on its secretory P_{app} (Figure 3).

Ipratropium transport across Calu-3 ALI layers was subsequently studied in presence of a range of drug transporter inhibitors. MPP^+ is known to inhibit OCT1-3 without affecting the OCTNs while

TEA interacts with all the OCT/N family members[7]. L-carnitine is a recognised inhibitor of the OCTN2 subtype and to a lesser extent of OCTN1[7]. Due to the apparent efflux observed, inhibitors of the ATP-binding cassette (ABC) family of transporters were also tested, although, to our knowledge, there is no indication in the literature that ipratropium might be one of their substrates. Verapamil is commonly used as a P-glycoprotein (P-gp) inhibitor[26] but has also been shown to interfere with the activity of a range of drug transporters, including some of the Multidrug Resistance Proteins (MRPs)[27], multidrug and toxin extrusion (MATE) transporters[28] and all the OCTs[7]. Probenecid is known to inhibit the MRPs without affecting P-gp[27] and although it also interacts with the Organic Anion Transporters (OATs)[29], we have shown previously that these proteins are not expressed in Calu-3 ALI layers[21].

MPP^+ significantly reduced ipratropium P_{app} in both the absorptive and secretory directions. Interestingly, the extent of the reduction in permeability was similar in both directions; i.e., ~30% of the control group (Figure 4). Co-incubation of ipratropium with TEA also caused a significant decrease in both P_{app}, supporting a possible role of OCTs in its broncho-epithelial permeability. However, in contrast to MPP^+, the decrease in permeability in the *b-a* direction was more pronounced than in the *a-b* direction with the P_{app} dropping to 23 or 45% of the control value, respectively (Figure 4). L-carnitine did not alter ipratropium transepithelial transport, ruling out an involvement of OCTN2 in the drug trafficking across the Calu-3 layers (Figure 4). Finally, when the permeability of ipratropium was assessed alongside verapamil or probenecid, both agents caused a significant decrease in its secretory transport without affecting the P_{app} in the *a-b* direction, thus abolishing the asymmetric transport observed in absence of inhibitors (Figure 4).

3.2. Uptake studies

In order to gain a better understanding of the role of transporters in ipratropium trafficking across ALI Calu-3 layers, drug uptake measurements were undertaken in presence or absence of transporter inhibitors. As verapamil is known to interact with multiple transporter families, it was not tested as an inhibitor in those studies.

Ipratropium cell-associated concentration was first quantified after a 5 min apical or basolateral exposure. That was significantly higher when the drug had been added in the apical chamber (Figure 5), likely as a consequence of carrier-mediated internalisation at the air interface.

Both the apical and basolateral uptake of ipratropium by Calu-3 layers was significantly inhibited to ~50% of the control in presence of MPP^+ (Figure 6). On the other hand, TEA caused a dramatic reduction (~14 % of control) in ipratropium uptake from the apical side without affecting the uptake from the opposite side (Figure 6). Probenecid impact on ipratropium internalisation was intriguingly similar to that of TEA. Indeed, it largely inhibited the apical uptake (~10% of control) but had no effect on the internalisation from the opposite side (Figure 6). This is in contradiction with its alleged interactions with an MRP transporter and indicates it interferes with the activity of an uptake transporter in Calu-3 layers.

3.3. Drug release studies

The ability of ipratropium to be secreted across either membrane of Calu-3 cell layers was assessed following a 45 minute pre-loading from the apical side. Indeed, the drug concentrations in the basolateral compartment were below the quantification limit of the LC-MS/MS method after a 5 minute pre-exposure.

Ipratropium showed a preferential efflux into the apical compartment, the drug apical concentrations being ~ 13 - 60 times higher than in the basolateral chamber throughout the course

of the study (Figure 7), which confirmed the presence of a secretory mechanism across the apical membrane of Calu-3 layers, as suggested by the bi-directional transport studies.

The mechanism behind the efficient secretion of ipratropium at the air-epithelium interface was further explored by monitoring its release into the apical compartment following a 5 minute apical pre-incubation in the presence of inhibitors. Interestingly, both MPP^+ and TEA significantly reduced the release of intracellular ipratropium into the apical compartment to ~20 % of the control while probenecid caused a ~50% decrease (Figure 7). This showed that all three inhibitors impacted on transporters involved in both the uptake and release of ipratropium at the luminal side of the Calu-3 cell layers.

3.4. Effect of organic cations on FITC-dextran permeability

Since it has been suggested organic cations might interact with negatively charged sites within the tight junctions, potentially affecting paracellular diffusion[30], the permeability of FITC-dextran across Calu-3 layers was measured with or without ipratropium, TEA or MPP^+ in the test medium. FITC-dextran was selected as the paracellular marker due to its relatively high molecular weight. Any alteration of the tight junctions is likely to have a more significant impact on its trans-epithelial permeability than on that of a small molecule like mannitol or fluorescein.

The dye P_{app} was unchanged in the presence of ipratropium or TEA but was significantly reduced by MPP^+ (Figure 8). This indicates the latest compound might partly obstruct the paracellular space upon binding to anionic components of the tight junctions. The impact of MPP^+ on ipratropium permeability in Calu-3 layers must therefore be interpreted with caution. In contrast, TEA and concentration dependent effects on the drug transport can be confidently ascribed to transporter inhibition.

4. DISCUSSION

The permeation pathway of anticholinergic bronchodilators across the absorption barrier in the lung remains controversial to date. Based on in vitro drug uptake studies in undifferentiated cells, it has been proposed OCT/Ns facilitate their transport across the airway epithelium[11]. On the other hand, absorption studies in IPL failed to demonstrate an involvement of the transporters, suggesting it is primarily driven by passive diffusion[12]. Given the importance of anticholinergic bronchodilators in the management of COPD, gaining further insight into the mechanisms by which they are processed by lung cells could help optimising their therapeutic benefits.

In this study, we used ALI human broncho-epithelial Calu-3 layers, a physiologically relevant in vitro model[18,19] of intermediate complexity between undifferentiated lung epithelial cells and intact lungs, with the aim to elucidate the role of drug transporters in the pulmonary disposition of inhaled anticholinergics. A combination of transepithelial permeability and drug uptake/release measurements were performed in the layers, in presence or absence of drug transporter inhibitors. Our data highlights the existence of different drug transport mechanisms on the apical and basolateral surfaces of Calu-3 cells. It also reveals the absorption of anticholinergic bronchodilators across the bronchial epithelium is likely governed by a complex process involving an inter-play between paracellular passive diffusion and transporter mediated uptake/efflux across the apical cell membrane.

The three anticholinergic bronchodilators tested, i.e., ipratropium, tiotropium and glycopyrronium, exhibited a polarised transport across Calu-3 cell layers, with, unexpectedly, a higher permeability in the *b-a* direction (Figure 2). This implies the presence of a secretion mechanism across the layers that is more efficient than absorption processes facilitated by uptake transporters such as OCT/Ns. Permeability values were similar for the short acting bronchodilator, ipratropium and the long

acting bronchodilators tiotropium and glycopyrronium, reflecting their similar pulmonary absorption profile in vivo[3] and alluding to common disposition pathways across the epithelial barrier in the lung. Interestingly, a similar net secretion has been reported for the prototypical organic cation MPP^+ across Caco-2 layers[31], which suggests different epithelia might handle quaternised molecules in a similar manner.

Increasing ipratropium donor concentrations caused a significant reduction in its permeability in the *a-b* direction while *b-a* transport was unaffected over the range investigated (Figure 3). Furthermore, the OCT inhibitors TEA and MPP^+ decreased the drug absorptive transport while all inhibitors tested but L-carnitine limited its secretory permeability (Figure 4). It is noteworthy that the effect of MPP^+ on trans-epithelial transport must be interpreted with caution due to its impact on FITC-dextran permeability (Figure 8). These observations nevertheless demonstrated drug transporters play a significant role in ipratropium asymmetric permeability across Calu-3 layers, although drug uptake and release studies were required to fully understand their contribution.

To account for a suspected high unspecific binding of the drug to cell membranes, ipratropium uptake in the layers was primarily evaluated in presence of inhibitors. However, the higher cell-associated concentrations measured after 5 min luminal exposure of the cells to the drug alone as compared to those after a basolateral exposure pointed towards a more efficient transporter-mediated drug uptake from the apical side (Figure 5). This assumption was confirmed by the reduction in uptake observed in presence of TEA, MPP^+ and probenecid (Figure 6). Interestingly, an extensive release of ipratropium in the apical chamber was measured post luminal loading of the cells with the drug which was, in addition, reduced by co-incubation with TEA, MPP^+ and probenecid (Figure 7). This efficient apical efflux mechanism can explain why, despite a transporter facilitated uptake from the apical side, the *a-b* permeability of ipratropium across Calu-

3 layers remains in the same range as that of low molecular weight paracellular markers such as mannitol or fluorescein[16].

A plausible hypothesis arising from our data is that, in the absorptive direction, ipratropium is unable to significantly permeate Calu-3 layers by the transcellular route due to its incapacity to freely diffuse across biological membranes and the absence of an efficient efflux transporter on the basolateral side of the cells. Following transporter mediated uptake, the drug seemed to be shuttled back into the apical compartment, suggesting paracellular diffusion is the predominant process by which it permeates the epithelium. Interestingly, a similar 'luminal recycling' has been observed in the intestinal Caco-2 model with the antidiabetic drug metformin which, like ipratropium, is a hydrophilic cation with a low membrane permeability[13]. This phenomenon has been proposed to contribute to metformin relatively high oral bioavailability by creating a sustained concentration gradient across the intestinal absorption barrier that enhances its diffusion by the paracellular route. A similar modulation of ipratropium concentration gradients across both Calu-3 cell membranes by apical transporters might account for the reduction of its absorptive transport in the cell layers at high concentrations as well as by TEA and MPP^+ (Figures 3 & 4), whereas, overall, our data suggests it is primarily governed by paracellular passive diffusion. In contrast, ipratropium absorption in the IPRL was not influenced by a pre-administration of high concentrations of the drug or the OCT inhibitor MPP^+ twenty minutes before dosing[12]. This could be due to the small volume of lung fluid which, in the IPRL, maintains a high concentration gradient across the airway epithelium or to a rapid disappearance of the drug or inhibitor from the epithelium surface in the *ex vivo* model.

Importantly, Calu-3 layers provided a unique insight into the mechanism of drug transport from the blood circulation into the lung tissue, which is extremely challenging to investigate in a whole

lung model. Ipratropium basolateral uptake was reduced by MPP^+ (Figure 6), while its *b-a* transport was independent of the concentration over the range studied (Figure 3), which suggests a low affinity transporter might facilitate its entry into the cells from the basolateral side. One or several efflux transporters apically expressed could then recognise the drug, allowing its translocation across the luminal cell membrane. Our data indicate that, in the secretory direction, the transcellular transport route is rendered possible for ipratropium thanks to the collaboration of drug transporters expressed on both sides of the cell layers. Due to their high OCT-mediated renal clearance[2,8,32], it is unlikely this process redistributes inhaled anticholinergics back into the lungs following their absorption into the bloodstream. However, such an inter-play between basolateral uptake transporters and apical efflux transporters might play a role in maintaining the pulmonary concentrations of other drug classes. It could also potentially be targeted to promote the accumulation of drugs in the lungs following their systemic administration.

Due to the complexity of the mechanisms controlling the disposition of ipratropium in Calu-3 cell layers and the non-specificity of the inhibitors, identifying the uptake and efflux transporters involved is extremely challenging. Nevertheless, studies with TEA, MPP^+ and L-carnitine support a role for an apically expressed OCT in the 'luminal recycling' of the drug. OCTs are indeed known to transport their substrates in both directions across the plasma membrane according to the concentration gradient[7]. The most probable candidate would be OCT1. Although ipratropium is a substrate for all three OCTs[8-10], OCT2 is not expressed in ALI Calu-3 layers[22] and the drug concentration tested (10 µM) is above its recently reported inhibitory concentration against OCT3[33]. In contrast, OCTN2 was reported to be the main transporter responsible for the intracellular accumulation of the drugs in the BEAS-2B cell line and mice tracheal epithelial cells in vivo, likely because its expression is high in those cells in comparison to that of the OCTs[9,11].

Moreover, although OCTN2 was shown to be functional in undifferentiated Calu-3 cells[25], it is unclear whether its activity is maintained when cells are cultured at an ALI.

The bidirectional ipratropium permeability data with/without inhibitors can nevertheless not be fully interpreted considering the sole activity of OCT1 on the luminal side of the cells. Inhibition studies with probenecid suggest the presence of an unknown uptake transporter together with an efflux transporter sensitive to the drug at the air-epithelium interface. Intriguingly, although no data were presented, Koepsel et al noted in their 2007 review that probenecid inhibits the OCTs without being transported[7]. In light of our study, the interactions of probenecid with transporters of organic cations warrant further investigation. It can nevertheless be speculated that the unidentified apical transporters might be members of the OCT or MATE families. MATE transporters have been reported to efflux cationic drugs across the apical membrane of hepatocytes and tubular renal cells[34]. Ipratropium has very recently been recognised as a MATE substrate[33] and verapamil, which significantly decreased ipratropium transport in the *b-a* direction (Figure 4) is an inhibitor[28]. Furthermore, interactions between anionic drugs and the transporters have been reported[35]. It is however currently unknown whether MATE transporters are expressed in ALI Calu-3 layers or even, in the bronchial epithelium. Similarly, the MPP^+ sensitive basolateral uptake transporter remains to be unravelled. This is unlikely to be an OCT member since its activity is unaffected by TEA (Figure 6).

The clinical relevance of our findings in differentiated Calu-3 layers remains to be determined. However, if the carrier-mediated 'luminal recycling' of ipratropium observed *in vitro* also occurs *in vivo* in epithelial cells, it may participate in the control of the local drug concentrations in the lung tissue by creating a 'drug depot' in the airway epithelium. This could influence exposure of the smooth muscles to ipratropium and therefore, the drug therapeutic efficacy. Furthermore, the

probable involvement of different drug transporters in the disposition processes of anticholinergic bronchodilators in the lung raises questions around the potential impact of genetic mutations on patients' response to the drugs.

5. CONCLUSION

Bidirectional transport and uptake studies in ALI Calu-3 layers were able to reconcile conflicting absorption data in undifferentiated lung epithelial cells and in intact lungs previously reported for the anticholinergic bronchodilators. The data confirm the absorption of ipratropium across the pulmonary barrier is unlikely to be significantly facilitated by drug transporters after pulmonary delivery. However, they highlight a potential role of both uptake and efflux transporters in modulating local drug concentrations in the lungs, which might have implications for the development of future inhaled drugs. In addition, this study showed that the use of simple yet anatomically relevant cell culture models of the airway epithelium is essential to gain understanding on the mechanisms controlling drug disposition in the lungs.

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FIGURE LEGENDS

Figure 1. Coefficient of apparent permeability (P_{app}) of FITC-dextran-0.5 mg/mL, (A) and metoprolol-10 µM (B), measured in 21 day old Calu-3 cell layers cultured under air-liquid interface (ALI) conditions in the apical to basolateral (ab) and basolateral to apical (ba) direction. Data are presented as the mean ± SD (n= 3-4 layers).

Figure 2. Coefficient of apparent permeability (P_{app}) of ipratropium, glycopyrrolate and tiotropium measured in 21 day old air-interfaced Calu-3 cell layers, in the apical to basolateral (ab) and basolateral to apical (ba) direction at a concentration of 10 µM. Data are presented as the mean ± SD (n=4 layers). * indicates a statistically significant higher permeability in the ba than ab direction ($p<0.05$)

Figure 3. Coefficient of apparent permeability (P_{app}) of ipratropium measured over a range of concentrations (10 - 300µM) in 21 day old air-liquid interfaced Calu-3 layers in the apical to basolateral direction (A) and in the basolateral to apical direction (B). Data are presented as mean ± SD (n=4 layers). * indicates a statistically significant lower permeability than at a drug concentration of 10 µM ($p<0.05$).

Figure 4. Coefficient of apparent permeability (P_{app}) of ipratropium measured at a concentration of 10 µM in the apical to basolateral (A) or the basolateral to apical (B) direction in 21 day old air-interfaced Calu-3 layers in the presence of transporter inhibitors (LC: L-carnitine, VP: verapamil, PB: probenecid). Data are presented as mean ± SD (n= 3 layers).* indicates a statistically significant lower permeability than in the control groups ($p<0.05$).

Figure 5. Cell associated concentrations (nmol/mg of protein) of ipratropium following incubation for 5 minutes in 21 day old air-interfaced Calu-3 cell layers, in the apical to basolateral (ab) and

basolateral to apical (ba) direction at a concentration of 10 µM. Data are presented as the mean ± SD (n=4 layers). * indicates a statistically significant lower cell associated concentration in the ba than ab direction ($p<0.05$)

Figure 6. Ipratropium uptake from the apical (A) or basolateral (B) side of 21 day old air-interfaced Calu-3 layers in the presence of transporter inhibitors. Data are presented as mean ± SD of 3 or 4 layers.* indicates a statistically significant lower uptake as compared to the control groups ($p<0.05$).

Figure 7. Release of ipratropium from 21 day old air-interfaced Calu-3 layers into the Transwell® chambers following an apical pre-loading at a concentration of 10 µM. (A) drug concentrations measured in the apical or basolateral chamber over time following 45 min of pre-loading; (B) effect of transporter inhibitors on the apical release following 5 min of pre-loading. Data presented as mean ± SD (n=4 layers). * indicates a statistically significant lower release as compared to the control ($p<0.05$)

Figure 8. Coefficient of apparent permeability (P_{app}, as % of control) of FITC-dextran measured in the apical to basolateral direction at a concentration of 0.5 mg/mL in 21 day old air-interfaced Calu-3 layers in presence of the organic cations ipratropium (300 µM), TEA (5 mM) and MPP^{+} (500 µM). Data presented as mean ± SD (n=3 or 4 layers). * indicates a statistically significant lower permeability as compared to the control ($p < 0.05$)

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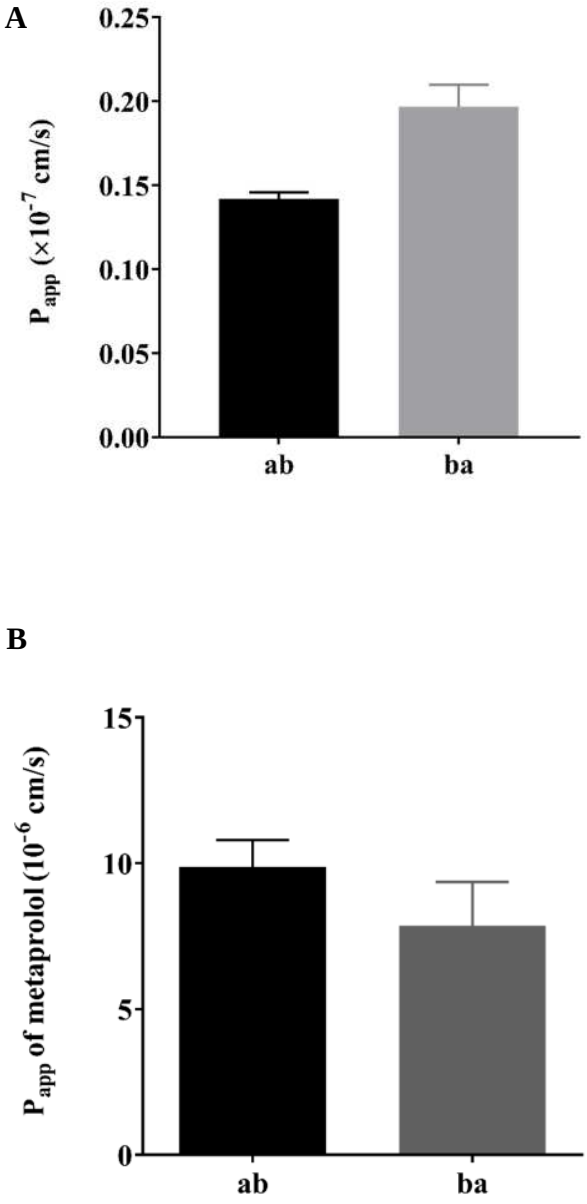

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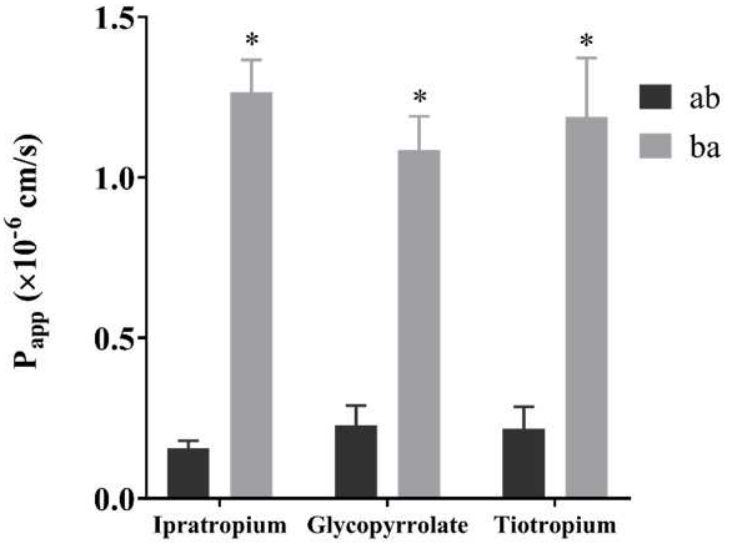

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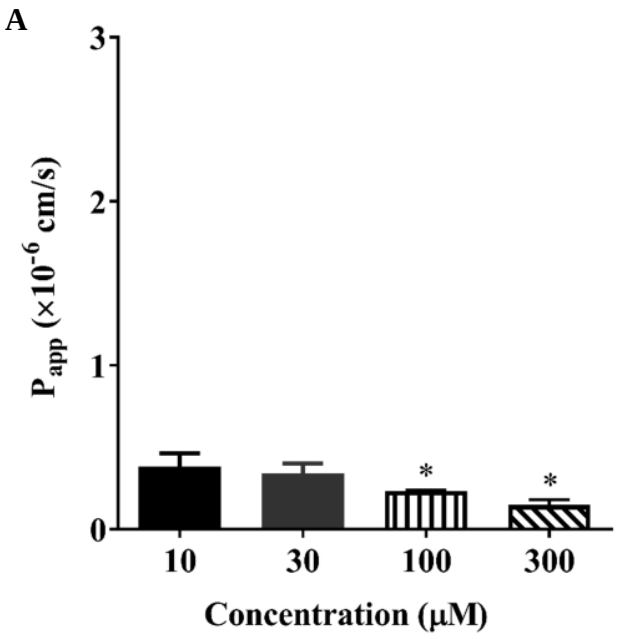

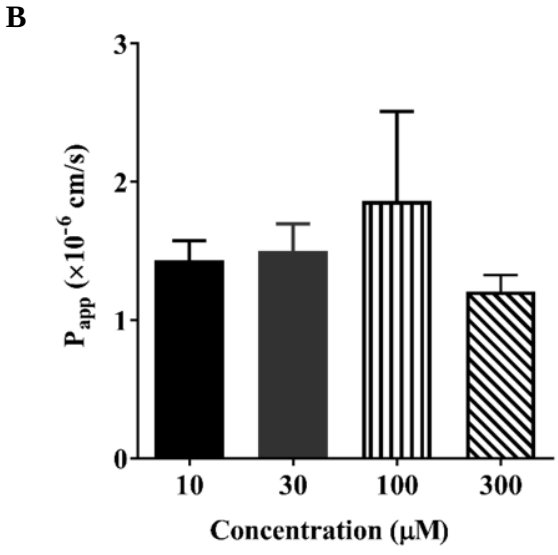

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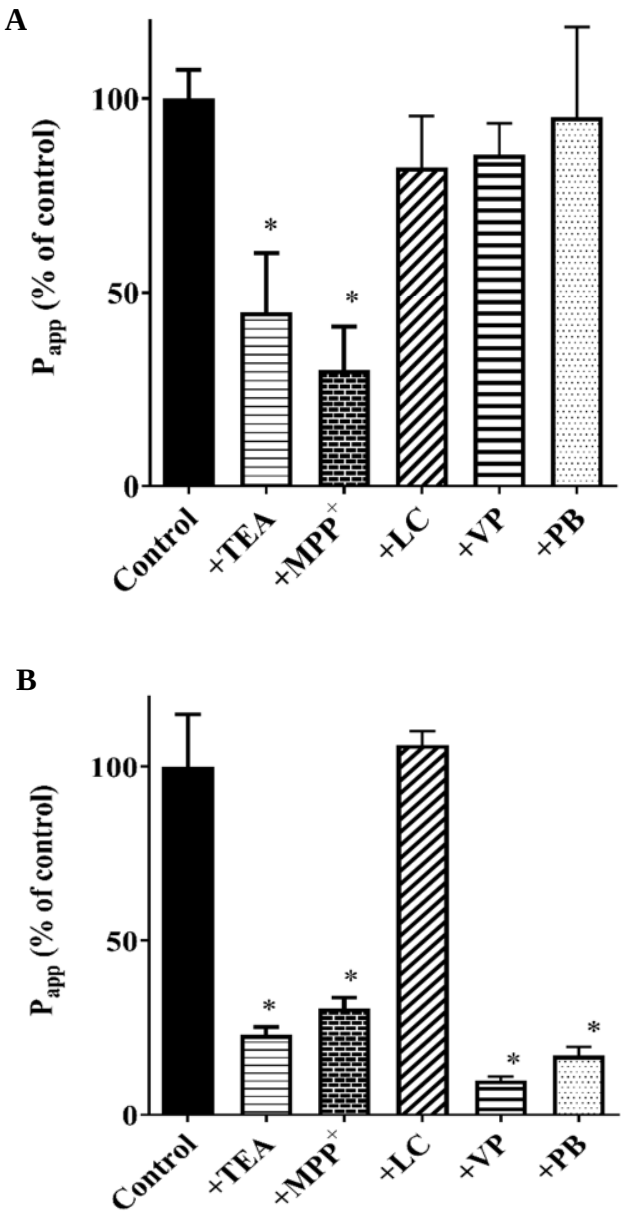

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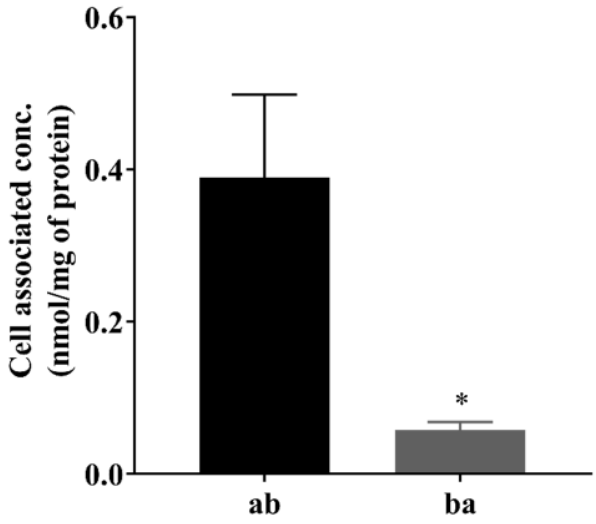

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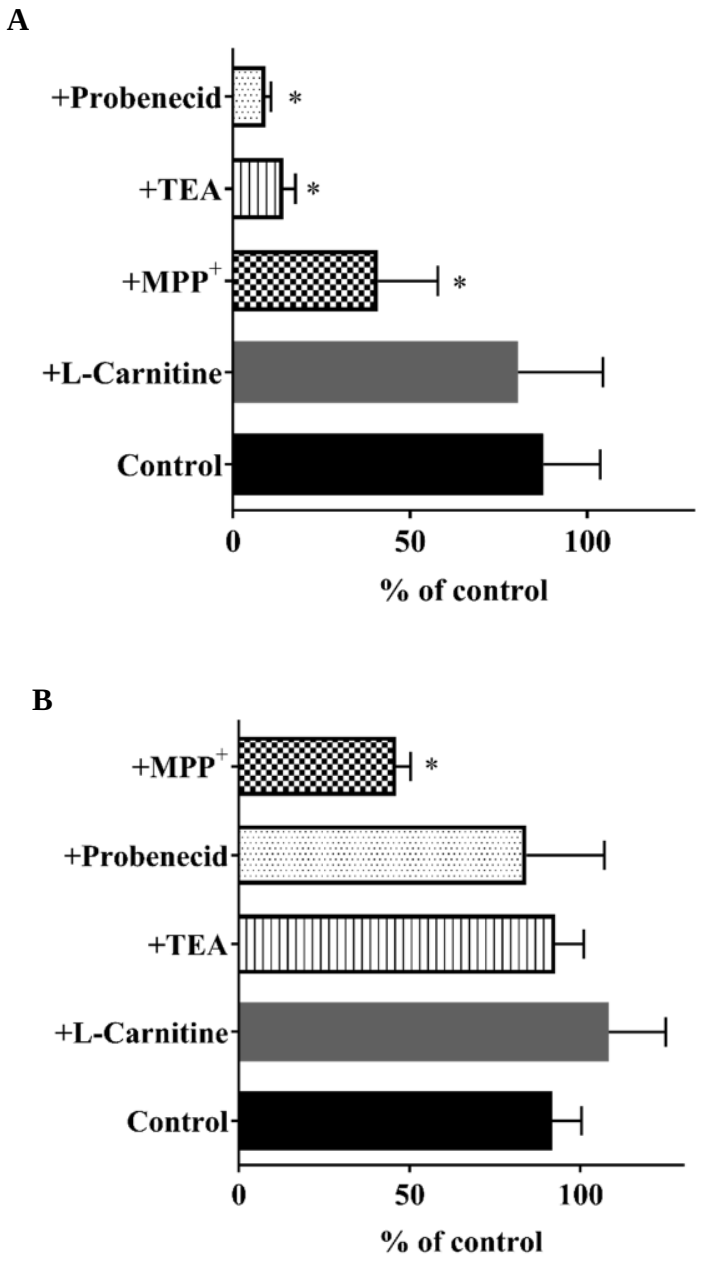

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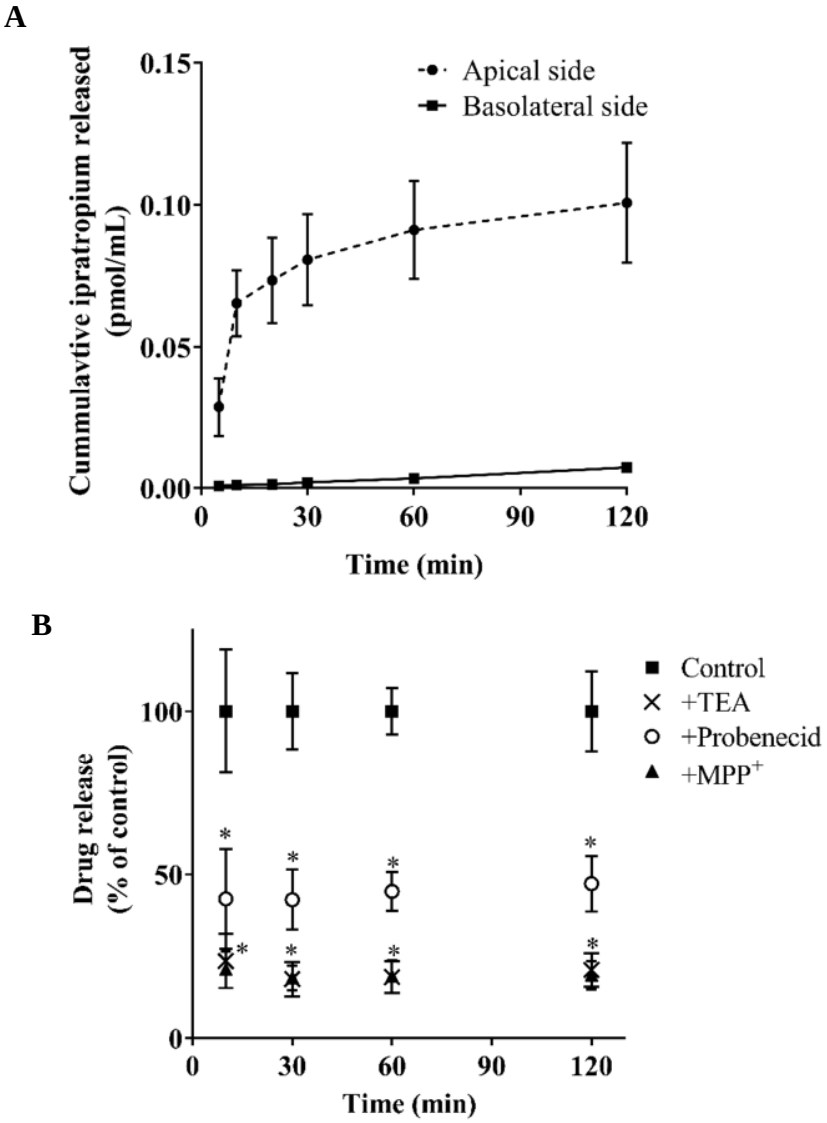

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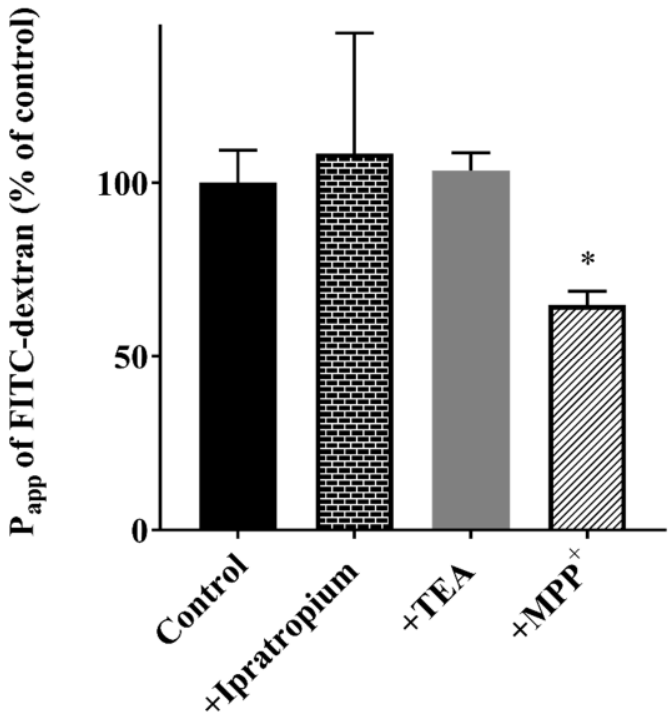

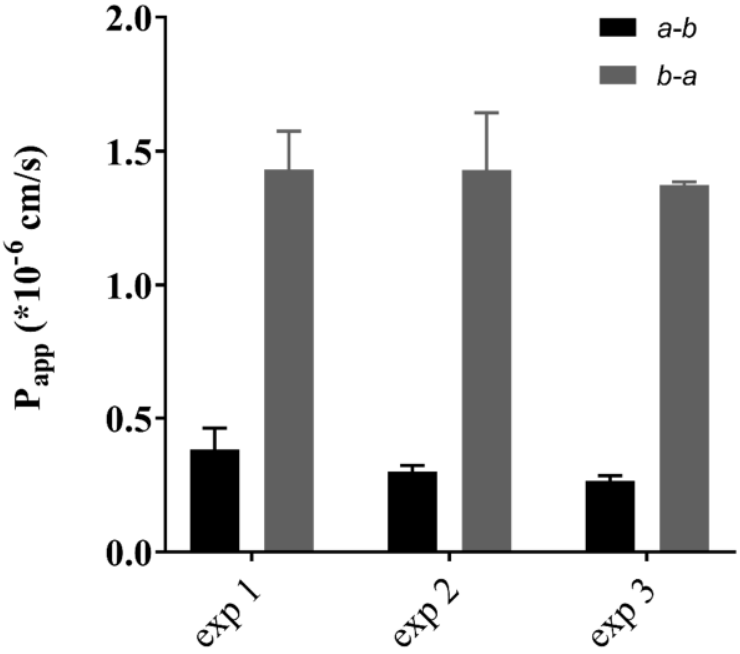

Figure SM1. Coefficient of apparent permeability (P_{app}) of ipratropium measured in 21 day old air-interfaced Calu-3 cell layers, in the apical to basolateral (ab) and basolateral to apical (ba) direction at a concentration of 10 µM. Data were collected in three independent experiments in layers at three different passage numbers and presented as the mean ± SD (n=3-4 layers).