Absorption of ipratropium and L-carnitine into the pulmonary circulation of the ex-vivo rat lung is driven by passive processes rather than active uptake by OCT/OCTN transporters

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ABSTRACT

The organic cation transporters OCT and OCTN have been reported to play a significant role in the cellular uptake of substrates within *in vitro* lung cells. However, no studies to date have investigated the effect of these transporters upon transepithelial absorption of substrates into the pulmonary circulation. We investigated the contribution of OCT and OCTN transporters to total pulmonary absorption of L-carnitine and the anti-muscarinic drug, ipratropium, across an intact isolated perfused rat lung (IPRL). The results obtained from the IPRL were contrasted with active transport *in vitro* using three human pulmonary cell lines and primary rat alveolar epithelial cells. *Ex-vivo* studies showed that OCT/OCTN transporters do not play a role in the overall pulmonary absorption of L-carnitine or ipratropium, as evidenced by the effect of chemical inhibition of these transporters upon pulmonary absorption. In contrast, *in-vitro* studies showed that OCT/OCTN transporters play a significant role in cellular accumulation of substrates with preferential uptake of ipratropium by OCTs, and of L-carnitine uptake by OCTNs. The results show that *in-vitro* uptake studies cannot be predictive of airway to blood absorption *in-vivo*. Nevertheless, localised submucosal pulmonary concentrations of inhaled drugs and their pulmonary pharmacodynamic profiles may be influenced by OCT/OCTN transport activity.

1. Introduction

A number of drug candidates for inhaled therapy are cationic and are therefore potential substrates for the *SLC22* superfamily of ATP-independent polyspecific cation transporters at the plasma membrane [1]. These transporters include the bidirectional organic cation transporters OCT 1 (*SLC22A1),* OCT2 (*SLC22A2), OCT3 (SLC22A3)* and the sodium-dependent carnitine/cation transporter proteins OCTN1 (*SLC22A4)* and OCTN2 (*SLC22A5).* The majority of inhaled drugs must cross the rate-limiting pulmonary epithelial barrier to access their pharmacological targets [2], e.g. smooth muscle cells. As such the interaction of cationic drugs with transporter pathways expressed in pulmonary epithelium may be important for drug access to underlying pharmacological targets. Indeed this has been the premise of research by a number of groups (reviewed in [3-5]) exploring the expression of OCTs/OCTNs within lung epithelium.

The localisation of OCT/OCTN transporters within intact lung has been reported using protein immunohistochemistry in both humans [6-9] and rodents [6, 8, 10, 11]. Evidence for expression of OCT and OCTN family members in intact human and rat lung tissue also exists at the mRNA level [6, 8, 11-13]. Nakanishi et al. [14] showed OCT/OCTN driven accumulation of ipratropium in lung epithelial tissue, namely tracheal epithelium, following drug deposition in the tracheal lumen of the mouse. A number of *in vitro* cell culture studies have reported substrate uptake via OCTs and OCTNs in lung epithelial cells including the active uptake of the model OCT/OCTN cationic substrate (4-(4-dimethylaminostyryl)-N-methylpyridinium; ASP^+) in normal human bronchial cells [7] and in a range of human bronchial epithelial cell lines [15-17]. The inhaled anti-muscarinic drug ipratropium has been implicated as a substrate of both OCTN and OCT transporters depending upon the *in vitro* model adopted [18, 19]. One report exists of the facilitative role of organic cation transporters upon the absorptive and secretory transport of ASP^+ across a cell monolayer [15] with the results showing modest effects upon the overall absorptive transport, despite a significant extent of cellular uptake.

Here we hypothesized that in a fully intact lung the OCT/OCTN transporters play little or no role in the transepithelial transport of substrates into the pulmonary vasculature. To test the hypothesis we examined in an intact perfused rat lung (IPRL) the role of OCT/OCTN transport in pulmonary transepithelial permeability of the zwitterionic substrate, L-carnitine, and the inhaled therapeutic cationic drug, ipratropium; archetype substrates for the pathways under study. We found the overall solute transport into the IPRL vasculature was predominantly driven by non-competitive passive processes that eclipse the net effect of any OCT/OCTN-mediated transport.

2. Materials and Methods

2.1 Materials

[^{3}H]-ipratropium bromide (70 Ci/mmol) was provided by GlaxoSmithKline (Ware, UK) and [^{3}H]-L-carnitine hydrochloride (70 Ci/mmol) was from American Radiochemicals Inc. (St. Louis, MO). Unlabelled ipratropium, L-carnitine, tetraethylammonium bromide (TEA) and 1-methyl-4-phenylpyridinium iodide (MPP^+) were purchased from Sigma-Aldrich (Poole, UK). Cell culture media and supplements were from Invitrogen (Paisley, UK) with cell culture plastics from Corning Costar (Hemel Hempstead, UK). All other reagents and solvents were from Fisher Scientific (Loughborough, UK) or Sigma-Aldrich. PCR primers were designed in house and supplied by Invitrogen (Paisley, UK).

2.2 Methods

2.2.1 IPRL

All animal experiments adhered to the UK Animal (Scientific Procedures) Act 1986. Rats used for all the experiments in this report weighed 250-350g. Animals were normally housed with a 12 hour day/night cycle and fed *ad libitum* until the time of surgery.

To examine the transport of ipratropium and L-carnitine across an intact pulmonary barrier an IPRL preparation was employed as previously described [20, 21]. This model includes an intra-tracheal airway dosing technique that utilises a pressurized metered dose inhaler (pMDI) reproducibly delivering a high extent (>95%) of deposited solute liquid aerosol dose into the lung periphery [22]. Using the pMDI methodology the IPRL was dosed with either vehicle control (100 μL saline) or a competitive inhibitor (in 100 μL saline), i.e. either unlabeled solute (125 nmol unlabeled L-carnitine or ipratropium) or the selective OCT inhibitor (MPP^+). Twenty minutes later the lungs were similarly dosed with the radiolabeled substrate (3 μCi of [^{3}H]-L-carnitine or [^{3}H]-ipratropium in 100μL saline). At discrete timepoints after lung dosing. 200μL samples were collected from the circulating perfusate and transferred to scintillation vials for radiochemical analysis.

2.2.2 Mathematical Modelling

Pulmonary pharmacokinetic absorption parameters were calculated by fitting Equation 1 to the individual airway to perfusate absorption data using nonlinear regression analysis (Micromath Scientist 3.0, Missouri, USA).

$$\%\ of\ deposited\ dose\ absorbed\ (t) = 100 \cdot f \cdot (1 - e^{-k.t}) \qquad \textit{Equation 1}$$

Where, f represents the available fraction to be transported, k is the absorption rate constant (min^{-1}) and t is time in minutes. Modelling of the absorption data was not improved by the use of more complex models and Equation 1 was deemed the minimum satisfactory model to provide precise parameter estimates for k and f.

2.2.3 *In-vitro* uptake studies

The *in vitro* active accumulation of substrates of either OCT and/or OCTN transporters, was studied in a range of continuous lung epithelial cell lines and primary cultures of rat lung epithelium. The human pulmonary adenocarcinoma cell line, A549 [23], and the human bronchial epithelial cell line, BEAS-2B, were obtained from ATCC (American Type Culture Collection; Manassas, VA). 16HBE14o$^-$ cells, generated by transformation of normal bronchial epithelial cells, were from Dr D. C. Gruenert (University of California San Francisco, San Francisco). Culture of these cells was performed as previously described [24], and isolation and primary culture of rat alveolar cells to a type I pneumocyte-like phenotype was undertaken as detailed in previous work [25].

Solute uptake studies described hereafter were conducted at incubation temperatures of both 37 ºC and 4 $^{\circ}$C. Radiolabelled solute was added to each well (24-well format) containing confluent cell monolayers. The dosings were 1 μCi (15 pmol) [^{3}H]-ipratropium or 1 μCi (15 pmol) [^{3}H]-L-carnitine giving a final radiolabel probe concentration in each well of 50 nM

(300 μL volume per well). Time- and temperature-dependent solute uptake was quantified at discrete timepoints over a 60 min incubation.

Radiolabelled solute uptake studies were also undertaken in the presence of unlabelled OCT/OCTN competitive inhibitors applied to the cells for a 30 min pre-incubation period prior to addition of the radiolabel probes. The unlabelled inhibitors were used to achieve concentrations of 500μM ipratropium, 100μM L-carnitine, 500μM MPP^+, 5mM TEA; a no-treatment control comprised radiolabeled solute alone in serum-free DMEM. Following the pre-incubation period, either [^{3}H]-ipratropium or [^{3}H]-L-carnitine was added to each well and the uptake of radiolabel allowed to proceed over a 60 min incubation period. The solute uptake experiments were terminated by two washes of the cell monolayers with ice-cold PBS followed by the addition of ice-cold trypsin-EDTA for 5 min, after which the cells were harvested and suspended in ice-cold DMEM and centrifuged (4 °C, 200 *g*) for 10 min after which the supernatant was discarded and the cell pellet collected. This cell washing procedure was performed three times in total. The resulting cell pellets were transferred to scintillation vials and mixed with 3mL of BioSafe 3 scintillation fluid and the cell-associated radioactivity quantified by liquid scintillation counting. The sodium-dependent nature of solute uptake for both [^{3}H]-ipratropium and [^{3}H]-L-carnitine was similarly conducted but using an incubation buffer where Na^+ was isotonically replaced with N-methyl-glucamine as previously described [7].

2.2.4 Real-time Quantitative Polymerase Chain Reaction (qPCR)

Human OCT (*SLC22A1, SLC22A3, SLC22A3),* human OCTN (*SLC22A4, SLC22A5),* rat OCT (*Slc22a1, Slc22a2, Slc22a3*) and rat OCTN (*Slc22a4, Slc22a5*) mRNA sequences were aligned using BLAST2 and the NCBI nucleotide gene search used to identify single exon regions; Oligocalc was used to validate all used transcription variants. Table 1 shows the primer sequences used in qPCR experiments. Total RNA was isolated (RNeasy, Qiagen, UK) from pulmonary epithelial cells (A549, BEAS-2B, 16HBE14o$^-$ or primary rat alveolar epithelial cells) grown under the same conditions as used in the solute uptake experiments. Total RNA was also isolated from whole rat lung and liver tissue, with the liver serving as a positive control that is known to express OCT/OCTN, albeit at different relative amounts. cDNA synthesis was performed via reverse transcription using M-MLV reverse transcriptase (Invitrogen, UK) using generic random oligonucleotide (pdN_6) primers and quantified spectrophotometrically at 260 and 280 nm by GeneQuant. The cDNA was amplified as described elsewhere [26]. qPCR was performed by SYBR® Green chemistry using a DNA Engine Opticon 2 Real-Time Cycler (BioRad, UK), previously described elsewhere [27]. The increase in fluorescence emission associated with DNA amplification was calculated throughout the run of 40 cycles and the cycle number at which fluorescence emission first reaches exponential phase was recorded.

3. RESULTS

3.1 Pulmonary transepithelial transport of [^{3}H]-ipratropium and [^{3}H]-L-carnitine

We explored if OCT/OCTN-mediated transport was a significant feature in the transepithelial absorption (airway to pulmonary vascular space) of [^{3}H]-ipratropium and [^{3}H]-L-carnitine within an intact rat lung. Over the 60 min IPRL experiment approximately 20% of the lung deposited dose of [^{3}H]-ipratropium was absorbed to the recirculating vascular perfusate (Figure 1A, Table 2) with approximately 3% of [^{3}H]-L-carnitine absorbed (Figure 1B, Table 2). Pre-administration into the airways (20 min prior to that of radiolabelled substrate) of 125

nmol of the respective unlabelled solute (either unlabelled ipratropium or L-carnitine) failed to significantly alter the extent or rate of absorption of the corresponding radiolabelled species. This despite the unlabeled solute dosed at a 3000-fold excess to that of the radiolabelled substrate. Similarly, pre-administration of 125 nmol of MPP^+ failed to alter [^{3}H]-ipratroprium absorption profile and kinetics. None of the above treatments resulted in perturbation of pulmonary barrier integrity or permeability as evidenced by the pulmonary absorption of the co-administered hydrophilic paracellular probe [^{14}C] mannitol remaining unaffected ($p>0.05$) by any treatment (Table 2).

3.2 OCT/OCTN-mediated accumulation of [^{3}H]-ipratropium and [^{3}H]-L-carnitine by primary rat pulmonary epithelial cells

Real-time qPCR confirmed the presence of *Slc22a1-Slc22a5* transcripts in both rat whole lung and in primary cultures of rat alveolar epithelial cells (Figure 1C), with the primary cultures relatively enriched, with respect to lung tissue, in *Slc22a3* and *Slc22a4*. As expected, we found the uptake of [^{3}H]-ipratropium and [^{3}H]-L-carnitine by the primary alveolar epithelial cells to display temperature dependency (data not shown). Furthermore, the accumulation of both solutes was significantly decreased ($P < 0.05$) by co-incubation with their respective unlabelled solute, or in the case of [^{3}H]-ipratropium by the OCT inhibitor MPP^+ (Figure 1D); the effect of MPP^+ upon L-carnitine uptake was not examined.

3.3 OCT/OCTN-mediated accumulation of [^{3}H]-ipratropium and [^{3}H]-L-carnitine by human pulmonary cell lines

We investigated the kinetics and transporter selectivity in the accumulation of [^{3}H]-ipratropium and [^{3}H]-L-carnitine using human lung epithelial cell lines (A549, BEAS-2B, 16HBE14o$^-$) constitutively expressing OCT and OCTN transporter proteins. The accumulation of both [^{3}H]-ipratropium and [^{3}H]-L-carnitine (applied at 50 nM) was linear over a 60 min period (Supplementary Figure 1) with parallel experiments undertaken at 4 °C showing negligible cell-associated radioactivity (< 250 DPM/10^6 cells at all timepoints; data not shown). The cell lines demonstrated differing capacities to actively accumulate [^{3}H]-ipratropium and [^{3}H]-L-carnitine (Table 3) with all three cell types showing higher ($P < 0.05$) levels of accumulation (per 10^6 cells) for L-carnitine compared to ipratropium. Of particular note was the considerable accumulation ($P < 0.05$) of [^{3}H]-L-carnitine by BEAS-2B cells, to the extent that 25% of the total applied L-carnitine radiolabel was cell-associated at 60 min

Using qPCR we evaluated solute accumulation by the various lung epithelial cell lines in the context of the absolute levels of *SLC22A1-SLC22A5* mRNA transcripts in the cells. Figure 2A shows the results of the qPCR where the mass of cDNA (femtograms) for each respective mRNA transcript is expressed relative to a 2 ng mass of total cDNA. Consistent with our observation of greater cellular accumulation of [^{3}H]-L-carnitine in all three cell lines, the combined cDNA for *SLC22A4* and *SLC22A5* was more abundant in each of the three cell lines than the respective combined cDNA for *SLC22A1*, *SLC22A2* and *SLC22A3*. The BEAS-2B cell line showed the highest total *SLC22A4* and *SLC22A5* levels of all which was mirrored by a greater uptake displayed by BEAS-2B for L-carnitine.

To further explore solute uptake and the interplay between constitutively expressed OCT and OCTN transporter proteins we examined the active accumulation of [^{3}H]-ipratropium and [^{3}H]-L-carnitine under challenge by various competitive inhibitors or co-factors (Figures 2B

and 2C respectively). Not surprisingly, in all lung epithelial cell lines the accumulation of both radiolabelled ipratropium and L-carnitine was significantly ($P < 0.05$) decreased following co-incubation with their respective unlabelled solute. Challenge with unlabelled ipratropium resulted in a significant ($P < 0.05$) reduction of [^{3}H]-L-carnitine accumulation in all three cell lines (Figure 2C). However, the effect of unlabelled L-carnitine upon [^{3}H]-ipratropium accumulation (Figure 2B) resulted in a far more restricted response, with a reduction ($P < 0.05$) in [^{3}H]-ipratropium accumulation observed only in the BEAS-2B cells, and then only at a marginal level. Co-incubation of the highly selective OCT competitive inhibitor, MPP^+, resulted in a significant ($P < 0.05$) inhibition of [^{3}H]-ipratropium accumulation in all three cell lines with inhibitory effects most pronounced in A549 cells and with the least affected being the BEAS-2B cells (Figure 2B). Notably MPP^+ had no significant ($P > 0.05$) effect upon the accumulation of the OCTN substrate [^{3}H]-L-carnitine (Figure 2C). Challenge with TEA, a mixed OCT/OCTN inhibitor, resulted in decreases in the accumulation of both [^{3}H]-ipratropium (Figure 2B) and [^{3}H]-L-carnitine (Figure 2C) in all three cell lines. OCTN1 and OCTN2 display Na^+-dependent transport, and in all three lung epithelial cell lines the active accumulation of the OCTN substrate [^{3}H]-L-carnitine was reduced by >90% ($P < 0.05$) in the absence of Na^+ (Figure 2C). In contrast, the accumulation of [^{3}H]-ipratropium was significantly less dependent on Na^+ (Figure 2B). Specifically, in the absence of Na^+ no alteration in ipratropium accumulation was observed in A549 cells. In BEAS-2B and 16HBE14o$^-$ cell lines, Na^+ depletion had a greater effect on ipratropium uptake (25-40% inhibition; $P < 0.05$), that L-carnitine accumulation. An MTT assay substantiated that none of the competitive inhibitors had any detrimental effect upon *in vitro* cell viability over the time course of experimentation (data not shown).

4. Discussion

The objective of this work was to examine the hypothesis that OCT and OCTN transporters play little or no role in the absorption of respective archetype substrates into the pulmonary circulation of a fully intact lung model. This set of experiments compliments a number of in-vitro lung epithelial cell-based studies which infer that these transporters may fulfill a role in substrate access to sub-mucosal smooth muscle targets.

Here we used an IPRL system to study transepithelial absorption from the airways, a fully intact whole lung model retaining the relevant *in-vivo* biological architecture and the various sequential and parallel, e.g. paracellular, active/facilitative and passive transcellular routes that may contribute to various extents a substrate's overall transepithelial penetration. We have previously demonstrated that the IPRL can serve as a reliable and robust model that is capable of discriminating active transport pathways for substrate movement across lung epithelia. For example, we have shown in the IPRL that P-glycoprotein (P-gp) drug efflux reduces the pulmonary absorption of certain P-gp substrates [28], an efflux mechanism which can be inhibited in the IPRL by co-administration of the chemical inhibitors such as elacridar (GF-120918). The IPRL displays similar airway perfusion characteristics to the fully intact *in-vivo* rat lung [29-31]. Indeed, in the IPRL the entire lung parenchyma from the secondary bronchi (second airway bifurcation) to the deep alveolar regions is perfused by the pulmonary circulation [30], as it is *in vivo*.

The IPRL experiments (Figs 1A,B) demonstrate a non-saturable transport pathway for ipratropium and carnitine, as well as the absence of any demonstrable inhibition of ipratropium transport by MPP^+. This demonstrates that the OCT and OCTN transporters played no significant quantitative role in the overall transport of the substrates ipratropium and L-carnitine across the pulmonary barrier into the pulmonary circulation. This particular

question has not previously been addressed. Although published studies have investigated the *in-vitro* cell uptake of OCT/OCTN substrates by pulmonary epithelial cells *per se*.

In IRPL experiments radiolabelled [^{3}H]-ipratropium and [^{3}H]-L-carnitine were administered to the airways to achieve intraluminal concentrations at least 10-fold lower than their respective K_m values (>1 μM) for OCT/OCTN [32]. These intra-luminal concentrations were determined to be close to those used in our parallel *in-vitro* uptake studies. Our calculations were based on a rat lung epithelial lining fluid (ELF) volume of 250 - 350 μL [21]. We found pre-administration of a 3000-fold excess of the respective unlabelled substrate did not alter the pulmonary absorption of either [^{3}H]-ipratropium or [^{3}H]-L-carnitine. Additionally, intra-luminal dosing of high concentration of the OCT inhibitor MPP^+ concentrations (mimicking those used in *in-vitro* studies) did not influence [^{3}H]-ipratropium absorption. These findings contrast with those of the in-vitro uptake studies which unequivocally showed saturable uptake processes for these substrates into lung epithelial cells. Nakanishi and co-workers reported that the tracheal accumulation of ipratropium in mice over a 90 second period was inhibited by both 0.2 and 1 mM carnitine and 0.1mM MPP^+ [14]. Backstrom et al [33] exploited a lung slice model to investigate the tissue binding of a number of inhaled drug molecules. For ipratropium they reported a bound drug partition coefficient of the cell i.e. the ratio of intracellular to extracellular unbound drug concentration of 7.1 and for its derivative, tiotropium, a value of 1.1. The authors concluded that OCT/OCTN transporter activity was responsible for this active accumulation of ipratropium but not for tiotropium, in spite of evidence from an analogous kidney slice model that tiotropium is an OCT substrate [19]. The tissue slice models used by Backstrom and others allow for a rapid and reliable investigation of bound:unbound drug levels in tissue homogenates. However, the model does not offer opportunity to investigate specific transport processes active at the luminal epithelial barrier such as airway to blood absorption. The epithelial and multiple sub-mucosal cell types distributed across the tissue slice are simultaneously exposed to equal concentrations of drug. This does not mimic the lung microenvironment in vivo following drug inhalation and therefore does not permit the differentiation of distinct tissue binding sites in the tissue slice.

Of note, the extent of pulmonary absorption of ipratropium (bioavailability of 20% of lung dose absorbed) was significantly greater than the zwitterion, L-carnitine (bioavailability of 3% of lung deposited dose absorbed) indicating a discrimination in the way the lung handles these charged molecules. Differences in tissue/protein binding at the epithelial surface within the lung, as dictated by physicochemical parameters such as logP, could serve to limit the fraction available for absorption into the pulmonary circulation. This whole lung absorption data nonetheless matches the low bioavailability of inhaled ipratropium in man after slow inhalation [34] and implies that the majority of the deposited dose remains either in the airway lumen or is localised to the lung submucosal tissue wherein lies the target smooth muscle cells.

For the purposes of rigorous investigation we also undertook parallel in-vitro pulmonary epithelial cell uptake studies. Consistent with others we found that OCT and OCTN transporter proteins play a significant role in the in-vitro epithelial cell uptake of ipratropium and L-carnitine. We found that human lung epithelial cell lines displayed differing and opposing capacities for active uptake of ipratropium and L-carnitine. For example, BEAS-2B cells showing the lowest capacity for ipratropium accumulation but the highest for L-carnitine, while A549 cells showed the reverse profile suggesting these solutes utilise to some extent distinct pathways for their active cellular accumulation. These results were in agreement with qPCR studies which showed BEAS-2B cells to have the highest potential for

OCTN functionality with the combined *SLC22A4* and *SLC22A5* mRNA transcript levels approximately 8500-fold greater than the combined levels of the *SLC22A1, SLC22A2 and SLC22A3* mRNA transcripts. Although our studies did not seek to directly correlate mRNA expression levels with transporter functionality in these cells lines it was noticeable that high levels of *SLC22A4* and *SLC22A5* mRNA in BEAS-2B matched the extensive accumulation of L-carnitine. We do not exclude however the contribution of another uptake transporter in L-carnitine uptake. Similar observations have been made by Nakamura et al. [18], who reported high expression of OCTN mRNA and a lack of OCT(1-3) mRNA in BEAS-2B cells using semi-quantitative PCR. In contrast, Ingoglia et al [35] recently indicated that OCT3 and OCT1 could play a role in the uptake of MPP^+ in BEAS-2B cells; a cell line which, in our hands, demonstrated negligible OCT3 expression by qPCR.

In vitro studies using overexpression kidney cell systems have shown ipratropium to variously be a substrate for either OCT [19] or OCTN transporters [18]. Consistent with these reports we show in the human respiratory cell lines, under conditions of constitutive expression and allowing for interplay between the transporter subfamilies, that ipratropium serves as a transport substrate for both OCT and OCTN but with preference for the former. This is exemplified by Na^+-free incubations, probing OCTN transporter function in particular, that revealed dramatic reductions in the uptake of the OCTN substrate L-carnitine in all the tested cell lines. Under the same conditions an appreciably smaller effect upon the uptake of ipratropium was observed, with uptake in the A549 cell line (displaying the highest relative OCT transcript levels) remaining unaffected by the absence of Na^+. Consistent with the above the OCT selective inhibitor, MPP^+, had no effect on L-carnitine uptake but resulted in significant MPP^+-mediated inhibition of ipratropium accumulation; the less profound MPP^+-mediated inhibition of ipratropium accumulation in BEAS-2B cells reflecting the considerably higher OCTN expression level in these cells that provides for a compensatory uptake. This latter point was reinforced by experiments involving co-incubation with excess unlabelled L-carnitine which reduced [^{3}H]-ipratropium accumulation only in the 'OCTN-dominant' BEAS-2B cells; a result that is in agreement with Nakamura et al. [18].

Primary cultures of rat lung alveolar epithelial cells display the phenotypic and biochemical hallmarks of the *in-vivo* type-I pneumocyte, which presents the major constituent (95% surface area) of the lung epithelial barrier. In these cultures we found expression of OCT/OCTN family members and demonstrated *in vitro* functionality of these cells in the saturable accumulation of both [^{3}H]-ipratropium and [^{3}H]-L-carnitine; accumulation which could be inhibited by co-exposure to their respective unlabelled species, and in the case of ipratropium, by the OCT inhibitor, MPP^+. Other studies in primary rat alveolar epithelial cells have reported mRNA for OCT1 and OCT3 [36] by semi-quantitative PCR, while Miakotina et al. [37] observed expression of OCT1 protein in primary rat and mouse alveolar epithelial cells.

It is not currently understood why OCT substrates, such as ipratropium, demonstrate low extents of absorption into the pulmonary vasculature in spite of extensive in vitro evidence of transporter-facilitated transport into the mucosal epithelia. Unwalla et al [38] reported that the transepithelial transport of salbutamol is increased through relaxation of epithelia tight junctional complexes that results from the β_2-receptor mediated rises in intracellular cAMP levels. This mechanism is not expected for ipratropium which acts via the M3 muscarinic receptor. More likely is a complex and dynamic interplay between membrane transporters in the epithelial and submucosal lung tissue that serve in concert to limit the access of select solutes to the pulmonary vascular bed.

5 Conclusions

In conclusion we find no evidence for a role of OCT/OCTN in the absorption of L-carnitine or ipratropium across an intact lung epithelial barrier into the pulmonary circulation. This is despite evidence in cell lines, including primary cells from the same group of rats, that the transporters play a significant role in the uptake of the substrates in to the epithelial cells. It follows that the rate of delivery of such molecules from airspace to lung sub-mucosal tissue in the intact organ will predominately be driven by passive processes. However, it is not possible to conclude that OCT and/or OCTN transporters lack influence upon localised submucosal pulmonary concentrations, and consequently upon the pharmacodynamic (PD) profiles for airway-administered cationic drugs. Indeed, while OCT and/or OCTN transporters may lack significant impact upon the aggregate systemic levels of inhaled cationic drug, these transporters may still affect localised drug concentrations (e.g. recycling) in the pulmonary PD compartment(s) which is an issue that necessitates further PK/PD investigations in the future.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Sequences of forward and reverse primers used in qPCR analysis of mRNA transcript levels in human pulmonary cell lines and rat cells and tissue.

Human			
Protein name	**Gene name**	**Primer Sequence**	
OCT1 (SLC22A1)	*SLC22A1* Forward	5'- GCTCTACTACTGGTGTGTGTGCCGGA	-'3
	SLC22A1 Reverse	5'- CTTGCCAGACCTCCCTCAGCCT	-'3
OCT2 (SLC22A2)	*SLC22A2* Forward	5'- CGCATCGGACGCCGTTACCC	-'3
	SLC22A2 Reverse	5'- CCAGCCAAGCACGCCGAAAAA	-'3
OCT3 (SLC22A3)	*SLC22A3* Forward	5'- GGCACGCAGCCCGACCACTA	-'3
	SLC22A3 Reverse	5'- CACTGCGCTTGTGAACCAAGCAAAC	-'3
OCTN1 (SLC22A4)	*SLC22A4* Forward	5'- GCCTGTCCCCCGGGAACGTT	-'3
	SLC22A4 Reverse	5'- AGATTCCACTCGGTCACGACGG	-'3
OCTN2 (SLC22A5)	*SLC22A5* Forward	5'- GCCCTATGTAAGGCCAGCCGC	-'3
	SLC22A5 Reverse	5'- CTCACACACCAGGTTCCACTCGG	-'3
Rat			
OCT1 (SLC22A1)	*Slc22a1* Forward	5'- GCCTGGCTAAACTGGTGAGGGC	-'3
	Slc22a1 Reverse	5'- CGGCCAAACCTGTCTGCAATGTA	-'3
OCT2 (SLC22A2)	*Slc22a2* Forward	5'- AGGGSCCATGTCGACCGTGGA	-'3
	Slc22a2 Reverse	5'- TTCCGGCCAAACCTGTCCGCTAG	-'3
OCT3 (SLC22A3)	*Slc22a3* Forward	5'- AGCGGACAGATACGGCAGGCT	-'3
	Slc22a3 Reverse	5'- CGGCAAAGGGAAGGCGTCGT	-'3
OCTN1 (SLC22A4)	*Slc22a4* Forward	5'- CGCCGGACCCCTTTCTCCCAA	-'3

	Slc22a4 Reverse	5'-	CAACGATGCTCCGGGGTCCC	-'3
OCTN2 (SLC22A5)	*Slc22a5* Forward	5'-	GGACGGCATGCGGGACTACG	-'3
	Slc22a5 Reverse	5'-	GGATGAACCAGAGAGCCCCA	-'3

Table 2. Pharmacokinetic parameters, rate constant (*k*) and fraction absorbed (*f*) generated from modelling IPRL lung transport data of [^{3}H]-ipratropium and [^{3}H]-L-carnitine. The extent of absorption of the control permeability probe ^{14}C-mannitol at 60 minutes is shown for comparison. Data are mean ± SD. The number of experimental replicates (n) is shown.

Substrate/ Treatment	n	*k* (min^{-1})	*f* (max =1)	% [^{14}C]-mannitol absorbed at 60 min
[^{3}H] ipratropium	6	0.042 ± 0.028	0.23 ± 0.095	28.5 ± 10.2
[^{3}H] ipratropium + 125 nmol ipratropium	6	0.043 ± 0.017	0.21 ± 0.070	26.6 ±11.5
[^{3}H] ipratropium + 125 nmol MPP^{+}	4	0.047 ± 0.022	0.17 ± 0.027	22.8 ±2.3
[^{3}H] L-carnitine	4	0.054 ± 0.020	0.030 ± 0.023	17.1 ±2.3
[^{3}H] L-carnitine + 125 nmol L-carnitine	4	0.057 ± 0.030	0.032 ± 0.005	17.3 ± 6.0

Table 3 Percentage uptake of [^{3}H]-ipratropium and [^{3}H]-L-carnitine uptake in the three human cell lines A549, BEAS-2B and 16HBE14o^{-}.

Cell Line	**A549**	**BEAS-2B**	**16HBE14o^{-}**
% Ipratropium uptake / 10^{6} cells / hr	1.6 ± 0.13	0.23 ± 0.03	0.60 ± 0.04
% L-carnitine uptake / 10^{6} cells / hr	3.4 ± 0.13	25.3 ± 2.0	4.0 ± 0.3

Figure 1. (1A) and (1B): Cumulative airway to perfusate transport (expressed as % of total lung deposited dose) following instillation into the IPRL airways of 45 pmol [^{3}H]-ipratropium or [^{3}H]-L-carnitine, respectively. Treatments included: (1A) airway co-administration of unlabelled (cold) ipratropium (125 nmol) or MPP^+ (125 nmol) or (1B) airway co-administration of unlabelled (cold) L-carnitine (125 nmol). Lines represent the predicted transport of each substrate based on mathematical modelling. (1C): Quantitative PCR results showing relative levels of cDNA transcript corresponding to *Slc22a1-5* transporters in whole rat lung tissue, primary rat lung epithelial cells and rat liver (% signal expressed to 2 ng total cDNA). (1D): Uptake (expressed as % of control) at 60 min (37°C) of 50 nM [^{3}H] ipratropium and 50 nM [^{3}H] L-carnitine in primary culture rat epithelial cells when challenged by pre-incubation with either 500 µM unlabelled ipratropium, 100 µM unlabelled L-carnitine, MPP+ (500µM). * Represents statistical significance compared to respective control at $P<0.05$ by one-way ANOVA and Dunnett's post-hoc test.

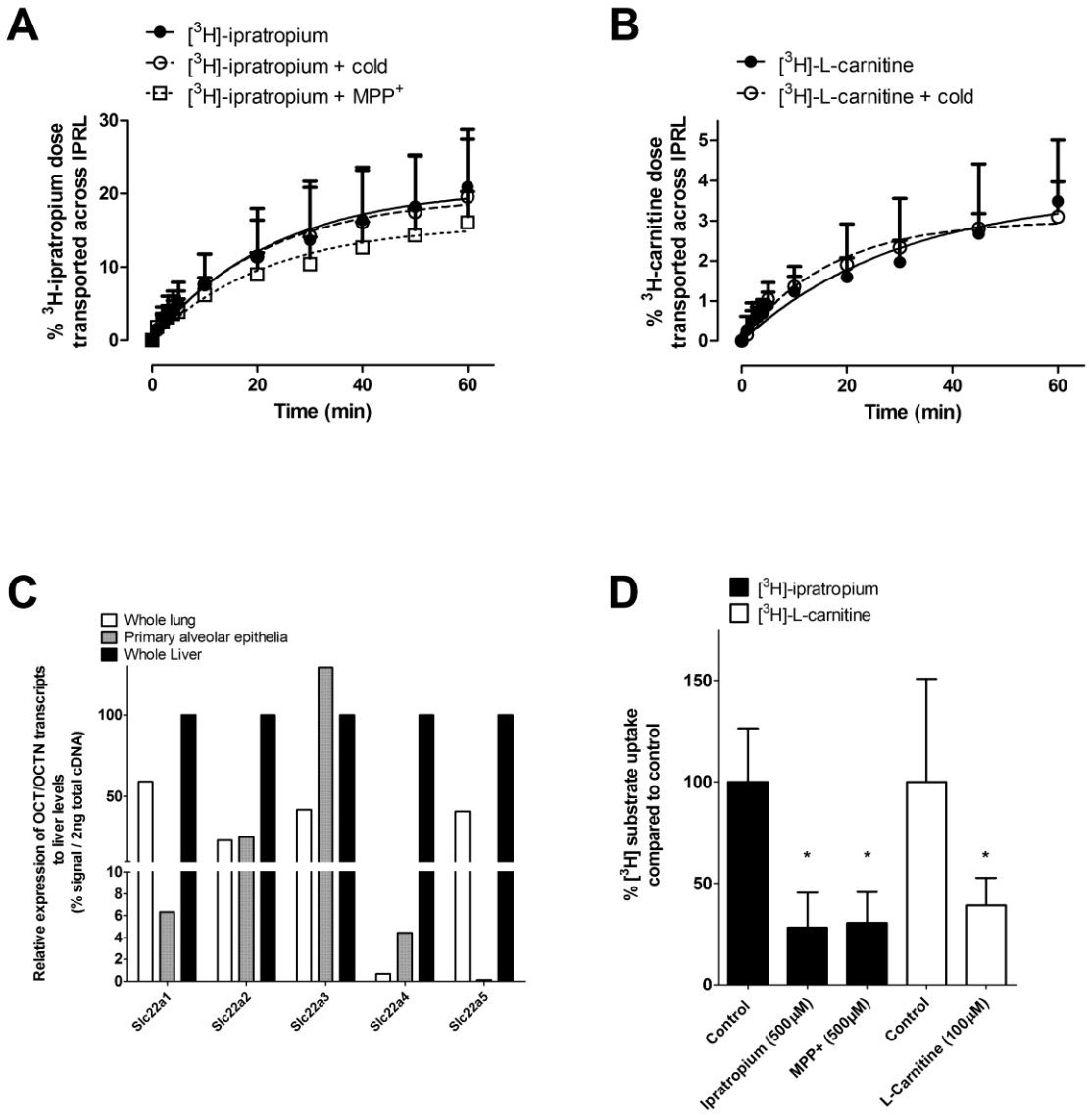
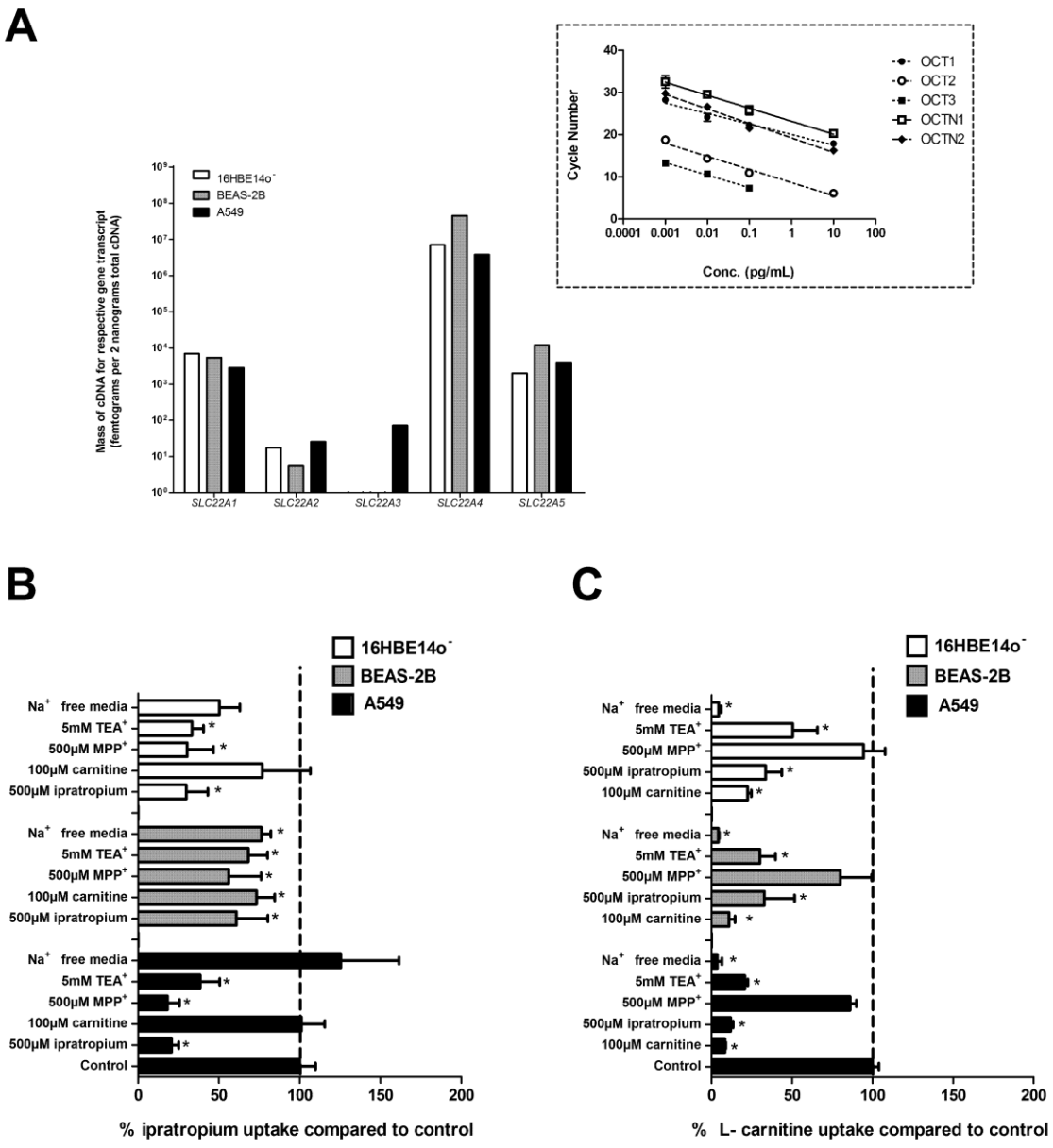


Figure 2. (2A): Real-time quantitative PCR results showing amounts of cDNA for OCT1, OCT2, OCT3, OCTN1 and OCTN2 mRNA transcripts in the three pulmonary cell lines expressed as femtograms / 2ng of total cDNA. The insert shows the standard curves for gene transcripts. (2B) and (2C): % uptake of [^{3}H]-ipratropium and [^{3}H] L-carnitine in cell lines A549, BEAS-2B and 16HBE14o$^-$ in the presence and absence of various inhibitors. Data shown are mean ± S.D., n=6-8. * denotes statistical significance ($P<0.05$) in comparison to control (radiolabelled substrate only) using one-way ANOVA with Tukey's post hoc test.



Supplementary Figures

Fig S1

Time-dependent accumulation of [^{3}H]-ipratropium (panel A) and [^{3}H]-L-carnitine (panel B) in three human lung epithelial cells lines. Cells were incubated at 37°C for pre-determined times and harvested for radiochemical analysis as described in the Materials and Methods section of the main text.