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RESEARCH ARTICLE

Characterization of Calu-3 cell monolayers as a model of bronchial epithelial transport: organic cation interaction studies

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Abstract

Background: To fully exploit organic cation transporters for targeted drug delivery in the lung, the use of a readily available and well-characterized tissue culture model and cheap easily detectable substrates is indispensable.

Objectives: To investigate the suitability of Calu-3 as tissue model for characterizing organic cation permeation across the bronchial cells using a fluorescent dye, 4-(4-(Dimethylamino)styryl)-N-methylpyridinium iodide (4-DI-1-ASP).

Methods: Substrate uptake, inhibition, and transport were performed to establish active transport mechanism. Organic cation transporter expression was determined with quantitative polymerase chain reaction (qPCR), immune-histochemistry, and fluorescent microscopy.

Results: 4-Di-1-ASP uptake in Calu-3 cells was concentration (K_m = 2.7 ± 0.3 mM, V_{max} = 4.6 ± 2.6 nmol/µg protein/30 min), temperature (uptake at 37°C>>4°C), and pH dependent (higher uptake at pH ≥ 7). L-carnitine, verapamil, and corticosterone significantly inhibited its uptake with IC_{50} of 28.2, 0.81, and 0.12 mM, respectively. Transport of the dye across the cells was polarized (AP→BL transport was 2.5-fold > BL→AP), saturable (Km = 43.9 ± 3.2) (µM; Vmax =0.0228± nmol/cm²/sec) and reduced 3-fold by metabolic inhibition. The expression pattern of the organic cation transporters (OCT) and carnitine/organic cation transporter (OCTN) isoforms was: OCT1<<OCT3 <OCTN1<OCTN2; OCT2 was not detected.

Conclusions: Based on qPCR, immunohistochemistry, uptake and transport data, the Calu-3 cells can be used as a model for not only studying strategies for optimizing the effect of inhaled organic cations, but also for cross-validating newly-developed respiratory cell lines.

Keywords: Pulmonary epithelium, organic cation transporters (OCT1-3), carnitine/organic cation transporter (OCTN1, OCTN2)

Introduction

Drug "developability" assessment has become an increasingly important addition to traditional drug efficacy and toxicity evaluations, as pharmaceutical scientists strive to accelerate drug discovery and development processes in a time- and cost-effective manner (Sun et al., 2004). The use of *in vitro* methods for drug absorption screening is a useful strategy in drug development facilitation (Reichl et al., 2011; Haycock, 2011; Angelis & Turco, 2011). *In vitro* investigations often employ appropriate epithelial cells of mucosal tissue of interest (Vllasaliu et al., 2011) and a wealth of knowledge, especially mechanistic information, has been gained from these studies.

The airway epithelium represents a barrier through which inhaled bronchodilators must pass to reach targeted receptors in the underlying airway smooth muscle. Therefore, a better understanding of pulmonary absorption mechanisms should provide information that can be used to develop more effective inhaled drugs for treatment of COPD and asthma (Nakamura et al., 2010).

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An evaluation of the impact of active transport systems on drug absorption from the lung may help in the interpretation and optimization of pharmacokinetics-pharmacodynamics (PK-PD) parameters after drug inhalation (Bosquillon, 2010). This type of information may be easily obtained using tissue culture models – particularly Calu-3 cells (Fernandes & Vanbever, 2009). The cell line has been extensively characterized for drug metabolism, toxicity, and absorption studies (Florea et al., 2003; Steimer et al., 2005; Sporty et al., 2008). Although other cell lines such as 16HBE14o, BEAS-2B, and A549 have been investigated as models for screening pulmonary drug administration, the Calu-3 cell line is the most commonly used based on the following advantages: (a) It is relatively well-differentiated, characterized, and originated from bronchial submucosal glands, (b) The cells can be grown at an air-liquid interface easily to form polarized cells. (c) The Calu-3 cells have been used for pulmonary drug delivery and deposition studies more than other respiratory cell lines, hence more published data exist about the cell line compared to others (Zhu et al., 2010).

Many drug transporters that are expressed in the intestine, liver, kidney and brain are also present in the lung. Most inhaled β_2-agonists are cations with a positive net charge at physiologic pH and thus are likely substrates for organic cation transporters (OCTs and OCTNs) expressed in the airway cells (Horvath et al., 2007a). In a recent study, Gnat et al linked significant delay of pulmonary absorption of inhaled salbutamol and GW597901 in human lung perfusion model to competitive absorption inhibition of OCT2 transporters by carnitine (Gnadt et al., 2012). In order to fully exploit these transporters for targeted drug delivery in the lung (e.g. reduced systemic absorption/increased residence time by competitive OCT/OCTN inhibition), the use of a readily available and well-characterized tissue culture model, as well as cheap and easily detectable substrates is indispensable. There is an immense need for reliable, physiologically relevant *in vitro* models of the bronchial and alveolar epithelium (following well established *in vitro* models of the liver, kidney, and intestine), which will allow correlation of *in vitro* results with *in vivo* data (Salomon & Ehrhardt, 2012). Although the Calu-3 cell line has been broadly characterized as a biopharmaceutical tool, information on the characterization of the cell line as a potential research tool for investigating carrier-mediated pulmonary absorption of organic cations is limited to two recent publications (Salomon et al., 2012; Mukherjee et al., 2012). Salomon et al., (2012) compared the uptake of 4-Di-1-ASP in human alveolar (A549), bronchial (16HBE14o- and Calu-3), and intestinal (Caco-2) epithelia. In a related study, Mukherjee et al., (2012) quantified the cellular uptake of the OCT substrates in the presence of inhibitors. Their studies suggested that several OCTs were functionally active on the apical side of the cell layers. Studies by both groups confirmed that 4-Di-1-ASP was actively taken up in the Calu-3 cell line. However, as active solute uptake does not necessarily imply active transport, there is still a need to characterize the transport characteristics of 4-Di-1-ASP across the Calu-3 cells. The focus of our study was to explore other important aspects of organic cation transport that were not covered by other published papers. The information provided in this paper, together with other published work on organic cation transporters in Calu-3 cells provides strong evidence on the suitability of the Calu-3 as tissue culture model for characterizing organic cation permeation across the trachea-bronchial cells.

Materials and methods

Chemicals

4-(4-(Dimethylamino)styryl)-N-methylpyridinium iodide (4-Di-1-ASP), triton X-100®, tetraethyl ammonium (TEA), choline, L-carnitine, D-carnitine, verapamil, bovine serum albumin (BSA), pronase, isopropanol, Hanks' balanced salt (HBSS), and penicillin/streptomycin were supplied by Sigma (St. Louis, MO, USA). DMEM-F12 1/1, Oligo dT primer, M-MVL reverse transcriptase, cDNA buffer, and dNTPs were purchased from Invitrogen (Burlington, ON, Canada). SYBR green mix was from Qiagen (Mississuaga, ON, Canada). Taq polymerase and gene ladder (100 bp GeneRuler) were from Fermentas (Burlington, ON, Canada). Rabbit polyclonal antibodies (OCT1, OCT2, OCT3, OCTN1, and OCTN2) and goat-anti-rabbit IgG conjugated with Alexa 488 were from Santa Cruz Biotech (CA, USA) and Jackson ImmunoResearch Lab Inc. (West Grove, PA, USA), respectively. Fluorescence mounting medium and Bicinchoninic acid (BCA) protein assay kit were from Dako (Carpinteria, CA) and Millipore (Billerica, MA, USA), respectively. Human bronchial/sub-bronchial gland cell line (Calu-3) was purchased from American Type Culture Collection (Manassas, VA, USA).

Cell culture

The Calu-3 cells were grown according to a standard protocol. The cells were cultured in 1:1 D-MEM/F-12 supplemented with 10% FBS, 1% Glutamax, 100U/ml penicillin, and 100 mg/ml streptomycin. Initially they were grown submerged in the culture medium for 3 days. After this period, they were maintained at an air–liquid interface by not including culture medium on the donor compartment of the inserts (cells for transport studies). Except for cells that were used for RNA extraction, immunehistochemistry and uptake studies all cell batches were grown on Costar® Transwell® clear polyester membrane (Corning Inc., MA) with a diameter of 12 mm and 0.4 μm pore-size. The cells were fed every other day with a DMEM-F12 containing 10% FBS, 1% glutamax, 1% 10,000 units/mL penicillin and 1%, 100 mg/ml streptomycin. The cells were maintained at 95% O_2 and 5% CO_2 environment. Cells within passage 8–15 were used for the studies.

Drug uptake studies

Cells that were grown either in 12- or 24-well plates were used for uptake experiments. On day 21 of the culture, the cells were washed twice with transport medium and were allowed to equilibrate for 30 min. Subsequently, test solutions of 4-Di-1-ASP were prepared either in transport medium or in a 1 mM inhibitor solution depending on the specific study. Solubility of 4-Di-1-ASP was enhanced by sonication for 15–30 min. At the beginning of an uptake study 200 µl (24-well plate) or 400 µl (12-well plate) of appropriate concentrations of 4-Di-1-ASP test solutions were added to properly labeled wells on either the 12- or 24-well plate. The plates were then incubated at the desired temperature (4 or 37°C) for 30 min. Depending on the experimental condition (4 or 37°C or in the presence of inhibitors), the cells were pre-incubated at 4 or 37°C or in the presence of the inhibitors for 15 min prior to uptake studies. The experiment was brought to an end by removing the test solutions and immediately washing the cells three times with ice-cold transport medium. The cells were then lysed with 500 µl (24-well plate) or 1000 µl (12-well plate) of 1% Triton X-100 in 0.1N NaOH on a shaker for 2–3 hours at 10°C.

Transport studies

4-Di-1-ASP was used as a model compound for organic cation transport studies. The effect of concentration (0.25–1 mM), metabolic inhibition, and polarity on the transport of the compound were investigated. Prior to the permeation experiments, the cells were washed twice and pre-incubated with transport medium (Hanks' balanced salt supplemented with 10 mM HEPES buffer and 25 mM glucose, pH 7.4) for 30 min at 37°C. Transport studies were initiated by adding 250 µl of test solutions to the donor compartment. At stipulated time points (0–120 min), 100 µl aliquots were sampled from the acceptor compartment (750 µl) and were replaced immediately with an equal volume of TM. Cells on each insert were checked for epithelial integrity before and after permeation experiments by transepithelial electrical resistance (TEER) measurement and sodium fluorescein permeation assay. Inserts with TEER values below 250 $\Omega.cm^2$ before experiments were not used for transport studies. Similarly, data from inserts with TEER readings below this value and sodium fluorescein permeation above 1.0% after 1 h incubation following permeation experiments were discarded.

RNA isolation and RT-PCR

Total RNA was isolated from Calu-3 cells after 21 days in culture. The cells were suspended, homogenized, and RNA extracted with 1ml TriZol® according to the manufacturer's instructions. The extracted RNA pellet was dissolved in 20 µl of diethylpyrocarbonate (DEPC) water by pipetting and was subsequently stored at –80 degrees.

Reverse transcriptase was performed with 0.5 µg of isolated RNA from the Calu-3 cells using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) in a 14 µl reaction volume for 30 min at 42°C. The reverse transcriptase enzyme was then inactivated by heating to 95°C for 3 min and the resulting cDNA was used in the PCR study. To assess organic cation mRNA expression levels, polymerase chain reaction was used with custom made gene-specific primers (Invitrogen, Carlsbad CA). Real-time PCR amplification reactions were performed with Quantitect SYBR® Green PCR kit. The following cycling conditions that consisted of 10 min polymerase activation at 95°C, and 60 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s were used. The genes were amplified with a Hybaid PxE Thermal Cycler PCR machine (Thermo Scientific, Waltham, MA). PCR products were separated on a 2% agarose gel and stained with ethidium bromide (0.5 µg/ml). Primers used are summarized in Table 1.

Immuno-histochemistry and fluorescent microscopy

Prior to staining with antibodies, the cells were fixed with 4% paraformaldehyde for 10 min and subsequently blocked with horse serum for 15 min. Rabbit polyclonal antibodies, OCT1, OCT2, OCT3, OCTN1, and OCTN2 were incubated with the cells at 1:40 dilution for 1 h. The cells were subsequently incubated with propidium iodide (1 µg/mL) and goat-anti-rabbit IgG conjugated with Alexa 488 at a dilution of 1:1000. After each step the cells were washed three times with Tris buffer that contained

Table 1. Primers used for PCR.

Name	Gene symbol	Sequence	Position	Bp
Forward primer				
hOCT1	SLC22A1	GATTTAAAGATGCTTTCCCTCG	1050-1077	141
hOCT2	SLC22A2	GATGTACAACTGGTTCACGA	1222-1242	100
hOCT3	SLC22A3	CTCTGATCATCTTTGGTATCCTG	1522-1545	103
hOCTN1	SLC22A4	CTACATCGTCATGGGTAGTC	1631-1651	125
hOCTN2	SLC22A5	TCTCCCTACTTCGTTTACCT	1649-1669	169
Reverse primer				
hOCT1	SLC22A1	CCTGATAGAGCACAGAGTCC	1191-1171	141
hOCT2	SLC22A2	GGGCAGAGTAGAAGAAATCC	1322-1302	100
hOCT3	SLC22A3	TTCTACATCATCCACTGTCTC	1625-1604	103
hOCTN1	SLC22A4	CCAGATCTGAACCATTTCAC	1756-1736	125
hOCTN2	SLC22A5	CTGTGTTTCATTCCTTTGACTC	1818-1796	169

0.5% Triton-X100. The cover slips were mounted on glass slides with fluorescence mounting medium and left to dry overnight. Epifluorescence microscopy was performed using Zeiss Axiovert 10 fluorescent microscope (emission = 488 nm, excitation = 520 nm) with oil immersion objectives (Carl Zeiss Ltd).

Sample analyses

Sodium fluorescein samples were assayed with Cary 50 UV-Vis spectrophotometer (Varian Inc, CA, USA) at 490 nm wavelength. Fluorescence intensity of 4-Di-1-ASP samples was monitored with Modulus single tube multimode florescence reader (Flurometer model 9200 from Turner Bio systems) at an excitation of 477 nm and emission of 557 nm wavelengths.

Protein content of cell lysates was quantified using bovine serum albumin as standards according to BCA protein assay protocol.

Data analyses

Apparent permeability coefficients [P_{app} (cm/s)] and 4-Di-1-ASP flux were calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

Where, $\frac{dQ}{dt}$ = Time-dependent 4-Di-1-ASP flux, C_0 = 4-Di-1-ASP initial concentration in the apical compartment, A = Costar insert surface area (1.12 cm^2).

Kinetic parameters (K_m, V_{max}) for uptake and transport and IC_{50} for 4-Di-1-ASP inhibition studies were determined by non-linear regression using Prism® 5.0 (GraphPad, San Diego, CA, USA). Passive diffusion was accounted for by subtracting the rate constant for 4-Di-1-ASP passive diffusion at 4°C. Unless stated otherwise, all experiments were performed in triplicates and data presented as mean ± SD. Where appropriate, statistical significance of the results were determined using students *t*-test (polarity and sodium azide transport studies) and ANOVA (uptake inhibition studies) using InStat® 3.0 (GraphPad, San Diego, CA, USA). $p < 0.05$ was considered significant.

Results

Uptake studies

Figure 1 shows the effect of concentration on 4-Di-1-ASP uptake in Calu-3 cells. There was a limit to the maximum concentration of the compound that we could investigate due to limited aqueous solubility. Within the tested concentration range of 0–2000 μM, the uptake of the compound was concentration-dependent (K_m = 2.7 ± 0.3 mM, V_{max} = 4.6 ± 2.6 pmol/μg protein/30 min). The non-linear regression coefficient (r^2) for the kinetic parameters determination was 0.98. Statistically significant difference was observed between total uptake at 37°C and 4°C ($p < 0.05$). Higher dye accumulation at 37°C may be attributed to active transport, possibly by the organic cation transporters that were less active at a cold temperature of 4°C.

To further investigate possible involvement of organic cation transporters, we investigated the effect of some organic cation substrates and inhibitors on 4-Di-1-ASP uptake (choline chloride, TEA, DL-carnitine, L-carnitine, quinine, 1-methyl-4-phenylpyridinium (MPP^+), and verapamil). At 1 mM concentration, MPP^+ and verapamil significantly reduced 4-Di-1-ASP uptake, with the latter inhibiting the uptake by more than 50%. Similarly, at the same concentration, L-carnitine and quinine inhibited intracellular accumulation of the compound by 24 ± 2% and 34 ± 4%, respectively. Choline chloride, TEA, and DL-carnitine had minimal effect ($p > 0.05$). Further inhibition studies were conducted to explore concentration-dependent reduction of 4-Di-1-ASP uptake by competing molecules. In these studies, we selected six compounds reported to have significantly inhibited the various five OCTs isoforms (OCT1-3, OCTN1, and OCTN2). The results of this study are shown in Figure 2. Clear concentration-dependent 4-Di-1-ASP uptake inhibition was observed for L-carnitine (IC_{50} = 28.2 mM), verapamil (IC_{50} = 0.81 mM), and corticosterone (IC_{50} = 0.12 mM). D-carnitine, quinine, and N-methylnicotinamide inconsistently reduced 4-Di-1-ASP uptake. For the three compounds, 3–5 mM did not result in up to 50% inhibition of the substrate uptake.

The activity of some organic cation transporters is affected by pH. The effect of pH on 4-Di-1-ASP uptake is shown in Figure 3. As demonstrated in the figure, the dye accumulated in the cells in a pH-dependent manner. Highest uptake was observed at an alkaline pH of 8.5. The quantity of dye taken up at this pH was about 20% higher than the amount taken up at physiological pH of 7.4. Acidic pH of 4 showed significantly lower uptake that decreased by 26.1% ± 5.4% relative to the physiological pH of 7.4.

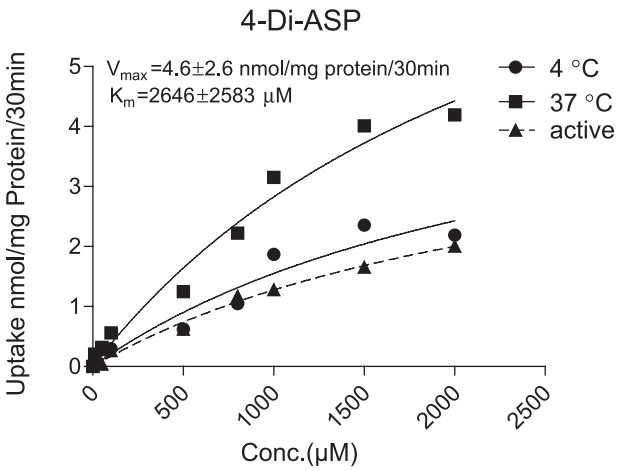

Figure 1. Effect of concentration on 4-Di-1-ASP (10μM) uptake in Calu-3 cells. Cells were incubated with 4-Di-1-ASP for 30 min 37°C followed by ice-cold PBS wash and subsequent extraction with Triton X-100/0.2N NaOH. Dotted line represents the difference between uptake at 37 and 4°C (active transport). Data represent mean ± SD, n = 3.

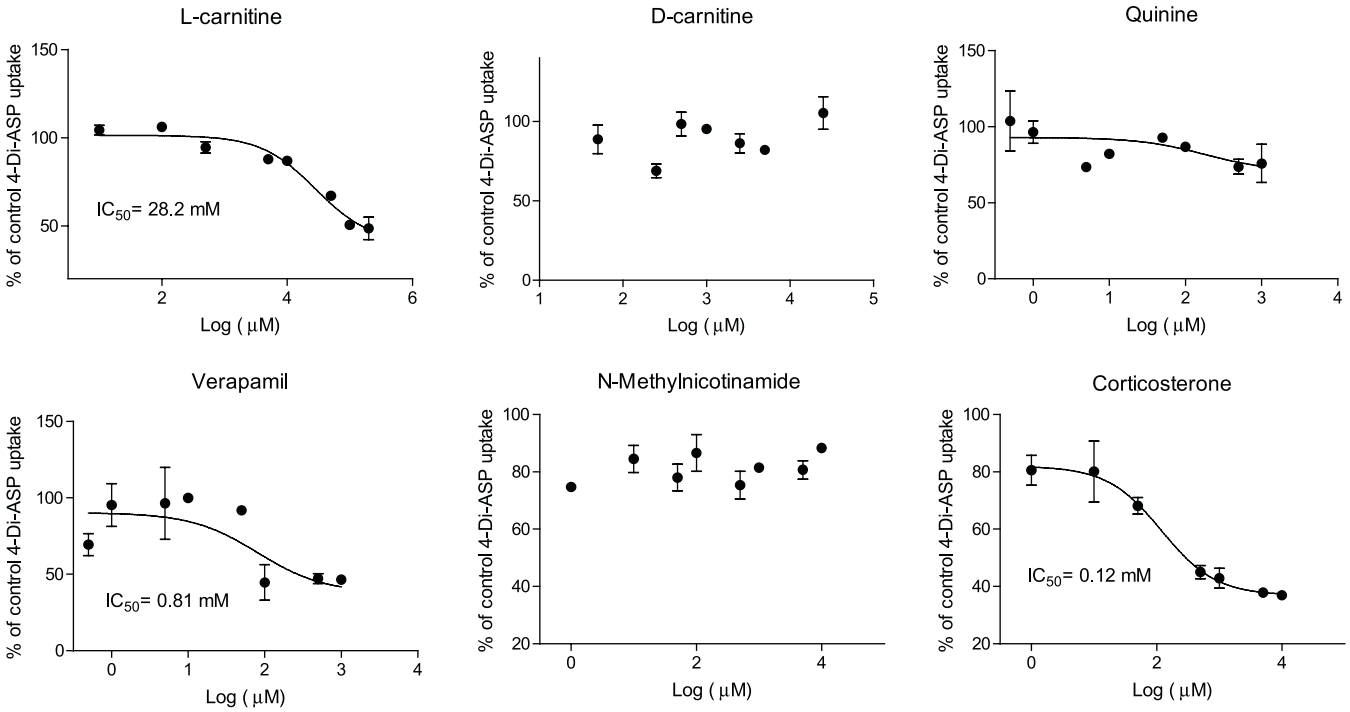

Figure 2. Effect of OCTs and OCTNs substrates and inhibitors on 4-Di-1-ASP (10 μM) uptake in Calu-3 cells. Cells were pre-incubated with inhibitors/substrates for 15 min, followed by incubation of the inhibitors in combination with 4-Di-1-ASP for an additional 30 min at 37°C. The cells were subsequently washed with ice-cold PBS and extracted with Triton X-100/0.2 N NaOH. Data represent mean ± SD, n = 3.

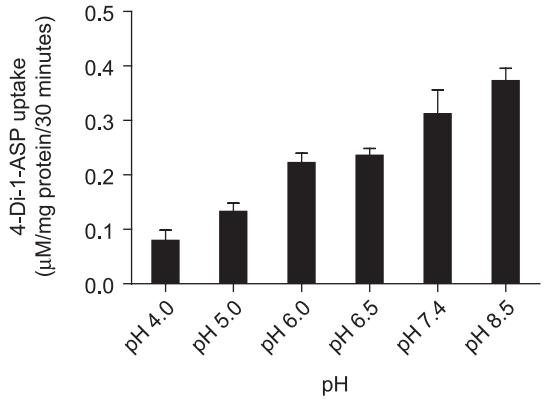

Figure 3. Effect of pH on 4-Di-1-ASP uptake in Calu-3 cells. Cells were incubated with 4-Di-1-ASP for 30 min 37°C followed by ice-cold PBS wash and subsequent extraction with Triton X-100/0.2 N NaOH. Data represent mean ± SD, n = 3.

Transport studies

Figure 4 shows the transport data for 4-Di-1-ASP (0.025 - 1 mM) permeation across the Calu-3 cells. The permeation of the compound was clearly concentration-dependent with saturation occurring between 0.2 and 1 mM. Its flux (μM/s/cm^2) was linear up to 0.2 mM (Figure 4B). An inverse relationship was observed between the permeability coefficient of the dye and its concentration (Figure 4C). As concentration increased, permeability coefficient decreased, suggesting transporter saturation at higher substrate concentrations – a hallmark of carrier-mediated active transport mechanism. Figure 5A and 5B demonstrates polarized transport of 4-Di-1-ASP across the Calu-3 cells. The cumulative amount of the dye transported in 2 h from AP → BL (37.7 ± 0.8 nM) was 2.5 times higher than its BL →AP (14.5 ± 1.5 nM) transport. Similarly, the mean AP → BL flux of the compound was significantly higher than BL →AP flux. These data suggest that the OCTs/OCTNs responsible for 4-Di-1-ASP transport in Calu-3 cells are possibly located on the apical side of the cells. If indeed the transport of the dye was carrier-mediated, one would expect inhibition of the transport proteins responsible for the transport (OCTs/OCTNs) by metabolic inhibitors to result in reduced transport. Figure 6 shows the effect of sodium azide (metabolic inhibitor) on 4-Di-1-ASP transport across the Calu-3 cells. The inhibitor reduced both the cumulative transport and flux of the compound by approximately three times. Over the 2 h transport period, the cumulative amount of the dye transported in the absence and presence of the inhibitor were 37.1 ± 0.8 and 12.3 ± 2.0 nM, respectively. Flux (μM/s/cm^2) of the compound was 0.015 ± 0 (without azide) and 0.0040 ± 0.002 (with azide). The studies described in this section functionally confirmed that 4-Di-1-ASP is not only taken up, but actively transported from the apical to the basolateral side of the cells in Calu-3 cells.

Molecular biology studies

Although functional studies proved that OCTs transporters were involved in 4-Di-1-ASP transport in the Calu-3, it was important to confirm this observation using molecular biology methods. The results of gene expression studies are shown in Figure 7A and 7B. Among the expected OCTs genes, three showed strong bands (OCT3, OCTN1,

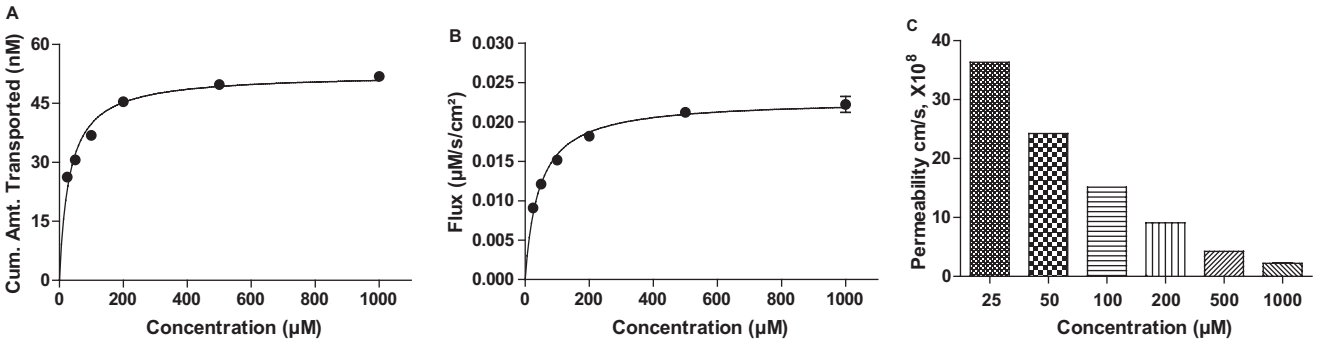

Figure 4. Effect of concentration on the cumulative amount of 4-Di-1-ASP transported (A), Flux of the dye (B) and permeability coefficient (C) in Calu-3 cells. Experiments were conducted by measuring the permeation of 0.025–1 mM 4-Di-1-ASP over a 2 h period. Data represent mean ± SD, n = 3. The kinetic parameters for the permeation of the compound were: Km = 43.9 ± 3.2 μM, Vmax = 0.02280 ± 0.0004 nmol/cm²/s.

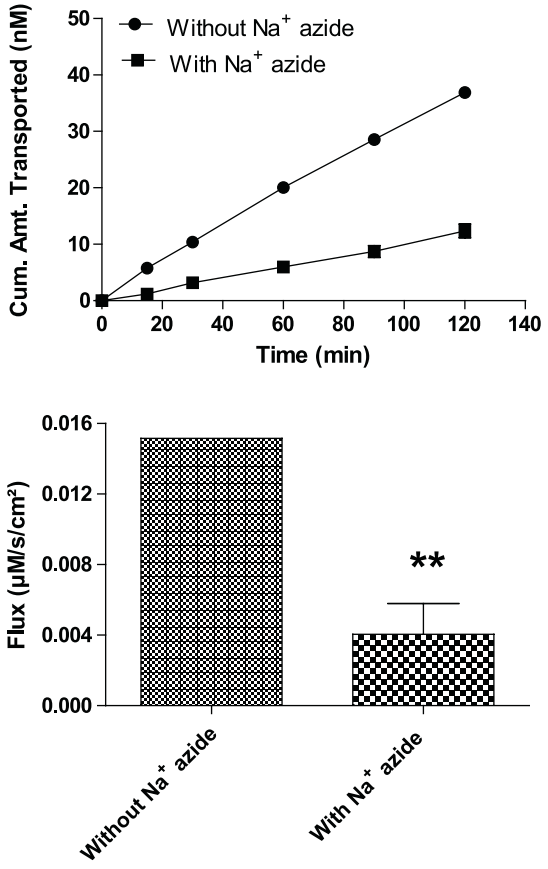

Figure 5. Effect of sodium azide (metabolic inhibitor and energy uncoupler) of cumulative amount of 4-Di-1-ASP transported and flux of the compound in energy in Calu-3 cells. Experiments were conducted by measuring the permeation of 100 μM 4-Di-1-ASP from AP→BL side of the cells in the absence and presence of sodium azide, respectively. Data represent mean ± SD, n = 3.

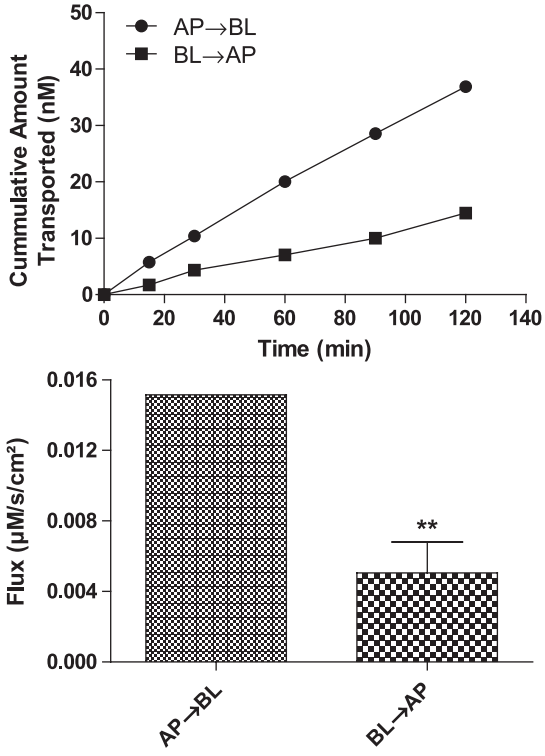

Figure 6. Effect of direction of transport (polarity) on cumulative amount of 4-Di-1-ASP transported in Calu-3 cells (A) and flux of the compound (B). Experiments were conducted by measuring the permeation of 100 μM 4-Di-1-ASP from AP→BL and BL→AP sides of the cells, respectively over a 2 h period. Data represent mean ± SD, n = 3.

and OCTN2). OCT1 showed a faint band, while no band was seen for OCT2. The corresponding gene transcripts for OCT1, OCT3, OCTN1, and OCTN2 were 141, 103, 125, and 169, respectively. Quantitative PCR was used to investigate the relative expression levels of the transporters in the cells. The results are shown in Figure 7B. In agreement with non-quantitative PCR data, the expression levels were as follows: OCT1<< OCT3<OCTN1<OCTN2. OCT2 could not be detected quantitatively.

Further confirmation of the OCTs gene expression was done by immuno-histochemistry and fluorescent microscopy. Figure 8 highlights the results of the studies. Panels A, B, C, D, E, and F, represent cells stained with propidium iodide only (control), OCT1, OCT2, OCT3, OCTN1, and OCTN2, respectively. The protein expression pattern was in agreement with the PCR results. For instance, no fluorescent OCT staining (green) was observed in the control and OCT2 panels. Although only a faint signal was observed in OCT1 panel, strong fluorescent signals were seen in OCT3, OCTN1, and OCTN2 panels, respectively.

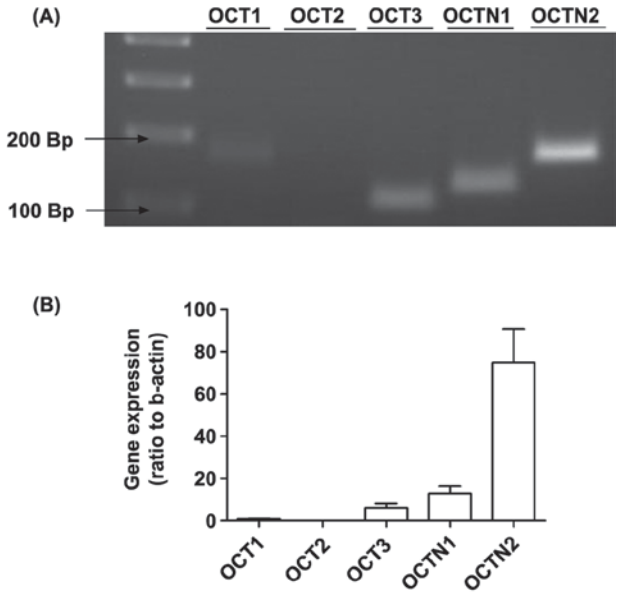

Figure 7. Non-quantitative (A) and quantitative OCTs and OCTNs gene expression in Calu-3 cells. RNA was extracted from cells grown in Costar® 6-well plates for 14 days.

Discussion

Based on availability, level of differentiation, ease of culture, robustness, reproducibility, development of measurable TEER and possibility for high-throughput screening of a large number of drug candidates, the Calu-3 cell culture model appears to be the cell line of choice for screening drug absorption mechanisms in the lung (Hamilton et al., 2001; Florea et al., 2001; Paturi et al., 2010; Ehrhardt et al., 2005). Our study was designed to validate the use of the Calu-3 cell line in conjunction with 4-Di-1-ASP a non-radioactive fluorescent tracer as model for characterizing pulmonary drug absorption by organic cation transporters. During the preparation of this manuscript, we identified two papers that recently investigated the permeation of 4-Di-1-ASP in Calu-3 cells (Salomon et al., 2012; Mukherjee et al., 2012). Although both published papers addressed the same issue as our studies (recognition of the relevance of Calu-3 as organic cation transporter model in the respiratory system), the three studies approached the issue somewhat differently. Importantly, a comparison of the data from three independent studies that addressed the same issue in different laboratories will give some insight on inter-laboratory variation in organic cation transporter expression and function in Calu-3 cells.

In a study to explore the basis for putative participation of OCTs in non-neuronal cholinergic signalling by airway epithelial cells, Lips et al. (2005) showed that OCT3 was expressed in luminal membrane of ciliated epithelial cells in human tracheal and bronchial cells. In a related study, low expression levels of OCT1 and OCT3, but nearly undetectable levels of OCT2 were reported in cells isolated from the human lung (Horvath et al., 2007a). In contrast to the low expression levels of the membrane potential-sensitive transporters (OCT 1-3), the authors reported high expression levels of the pH-sensitive OCTNs (OCTN1 and OCTN2). Based on our data [OCT2 = 0 (undetectable), OCT1< OCT3<OCTN1<OCTN2], it can be inferred that the OCTs and OCTNs expression in Calu-3 cells were considerably similar to the data from the human lung primary cells. The OCTs and OCTNs expression pattern in our study were also comparable to the data reported in other respiratory cell lines. Salomon et al., (2012) confirmed using immunoblot that OCT1-3, OCTN1, and OCTN2 are expressed in the alveolar epithelial cell line (A549). The authors also reported that with the exception of OCT2, the other isoforms (OCT1, OCT3, OCTN1, and OCTN2) were expressed in bronchial cell lines (16HBE14o- and Calu-3 cells). In a similar study, Mukherjee et al., (2012) showed that the Calu-3 cells expressed OCT1, OCT3, OCTN1, and OCTN2; but not OCT2. Based on our data and other published studies, it can be concluded that the Calu-3 does not express OCT2, but OCT1, OCT3, OCTN1, and OCTN2 to a variable extent. Substrates with higher affinity for OCT3, OCTN1, and OCTN2 may be better for lung targeting. Similarity in the organic cation expression pattern of the Calu-3 cells from three different laboratories highlights the potential usefulness of the cell line as a model of organic cation transport in the bronchial region of the lung. As the presence of the OCT members and their relative expression levels in the human bronchial epithelium remain unclear to date (Mukherjee et al., 2012), our quantitative PCR studies provided a quantitative estimate of the expression levels of the isoforms that may be used for future reference.

In agreement with literature information, the uptake of 4Di-1-ASP in the Calu-3 cells was carried-mediated. This was not only evident in the concentration- and temperature-dependent uptake of the compound, but also in its inhibition by classical organic cation inhibitors. Corticosterone, L-carnitine, and verapamil inhibited the compound uptake with IC_{50s} of 28.2, 0.81, and 0.12 mM, respectively. Corticosterone is a known inhibitor of OCT1, OCT2, and OCT3 (Inazu et al., 2005). Therefore, the observed 4-Di-1-ASP inhibition may be attributed to OCT3 inhibition because of low or no OCT1 and OCT2 expression, respectively. L-carnitine inhibition suggested the involvement of OCTN2. The data were in agreement with the suggestion that organic cation transport is largely mediated by OCTN2 in human airway epithelia (Horvath et al., 2007a). However, Grigat et al., (2007, 2009) showed that 4-Di-1-ASP is not an OCTN2 substrate. Therefore, the relatively remarkable inhibition of 4-Di-1-ASP uptake by verapamil, an inhibitor of both OCTN1 and OCTN2 transporters implies that OCTN1 may be more important than OCTN2 for 4-Di-1-ASP uptake in Calu-3 Cells. It is important to emphasize that verapamil is a non-specific inhibitor that also inhibits other transporters capable of transporting organic cations (Martel et al., 2001). The observed pH effect also solidified the argument for the involvement of pH-sensitive organic cation transporters

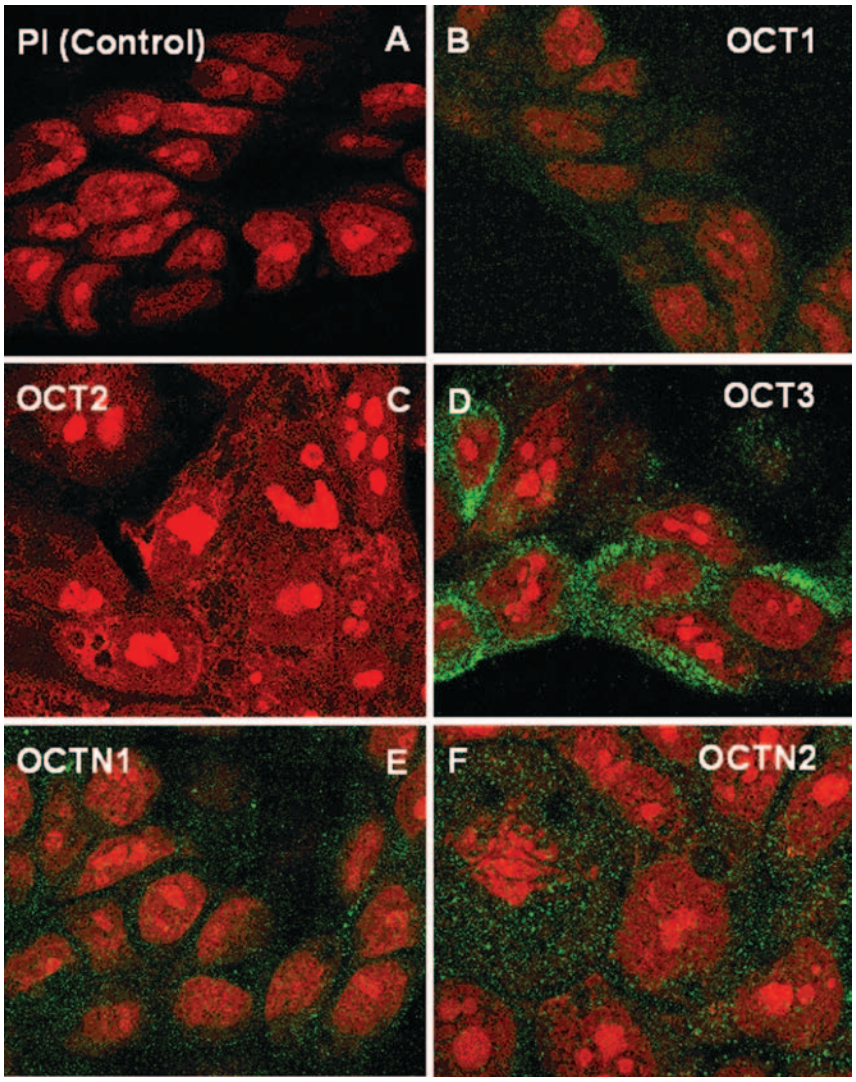

Figure 8. Expression of organic cation transporter isoforms in Calu-3 cells. Cells stained with propidium iodide (PI) only (A), OCT1 (B), OCT2 (C), OCT3 (D), OCTN1 (E), and OCTN2 (F). The nuclei were stained red with PI. The green fluorescence represents OCTs and OCTNs. Magnifications: ×630.

(Schild et al., 2007). TEA has similar, but higher affinities for OCT1 and OCT2 in comparison to OCT3. Its low-inhibitory effect on 4-Di-1-ASP uptake may be explained, by the low or no expression of OCT1 and OCT2, and its low affinity for OCT3. Similarly, quinine (OCT1) and N-methylnicotinamide (OCT2) inhibitors had little or no effect on 4-Di-1-ASP uptake. The effect of chirality on the dye uptake was investigated with L-and D-isomers of carnitine. Significantly higher uptake inhibition by the L-isomer relative to the D-isomer showed the importance of stereo-selectivity in the uptake process. L-carnitine showed higher inhibitory effect to 4-Di-1-ASP uptake than D-carnitine. This effect may be related to the observation that D-carnitine and L-carnitine show different substrate and inhibitor function on OCTN1 and OCTN2 transporter systems. According to Grigat et al., (2009), D-carnitine is an inhibitor of OCTN2 but not OCTN1. Nevertheless, it has also been reported that D-carnitine is a good OCTN2 substrate (Ohashi et al., 1999). Based on our data and other published studies involving Calu-3 cells, no one knows exactly which transporter isoform that is responsible for 4-Di-1-ASP uptake in the cell line. Therefore, it is hard to ascribe poor D-carnitine inhibitory effect to its lack of effect on OCTN1 as OCNT1 is a multi-specific, bidirectional, and pH-dependent organic cation transporter that is energized by proton antiport mechanism (Yabuuchi et al., 1999). Furthermore, OCTN1 transports zwitterions (e.g. L-carnitine) and organic cations (e.g. TEA, quinidine, pyrilamine, and verapamil) and can also be inhibited by some of these same substrates (e.g. L-carnitine, D-carnitine, quinine, quinidine, tetrabutylammonium, tetrapentylammonium, nicotine, and verapamil) (Koepsell et al., 2007). In summary, the functional uptake studies suggested that 4-Di-1-ASP uptake in the Calu-3 cells is possibly mediated by low-affinity organic cation transporters. However, according to Martel et al., (2001), there is substantial evidence for the involvement of several distinct transport mechanisms in the secretion and absorption of organic cations across the brush-border membrane of enterocytes. These transporters

include: (1) sodium-independent thiamine transporter, (2) sodium-independent, potential-dependent transporter that mediates absorption of tyramine, tryptamine, and disopyramide; (3) sodium- and electrical potential-independent transporter that mediates absorption of choline; (4) proton/cation antiporter, involved in guanidine secretion; (5) ATP-dependent, polyspecific transporter that mediates secretion of hydrophobic organic cations; and (6) a polyamine transporter, involved in the absorption of spermine, spermidine. Some of these transporters might also contribute to 4-Di-1-ASP uptake and transport in the Calu-3 cells if they are expressed in the cells.

The OCTs are either located on the apical or basolateral side of an epithelium. The location of these transporters within the cells affects their roles in solute transport. Attempts to use immuno-histochemistry coupled with confocal microscopy to pin-point the location of these transporters in cultured respiratory cells have not unequivocally defined their locations within these cells. Functional permeation studies appear to be a useful strategy for demonstrating the location of the transporters in polarized cells. As members of the SLC super-family, OCTs and OCTNs can transport solutes either from the AP→BL regions of the cells or vice versa, depending on their location and function. Despite the importance of solute transport in drug permeation process, and the relevance of 4-Di-1-ASP as a fluorescent organic cation tracer, published information on 4-Di-1-ASP transport in respiratory cells is limited compared to published uptake data. Polarity in transport is often observed with active transport because of preferential transport of substrates in one direction. Salomon et al., (2012) used an indirect method to show that 4-Di-1-ASP is transported by comparing its uptake in the presence and absence of EDTA. Their studies supported the notion that at least one of the responsible transporter sites is localized at the basolateral side of the cells (Salomon et al., 2012). Furthermore, Mukherjee et al., (2012) used the permeation of cationic bronchodilators to show carrier-mediated permeation characteristics of organic cations in Calu-3 cells. Results of our transport studies showed that 4-Di-1-ASP, is not only actively taken up, but also actively transported across the Calu-3 cells. The kinetic parameters for the permeation of the compound were: K_m = 43.9 ± 3.2 μM, V_{max} = 0.02280 ± 0.0004 nmol/cm^2/s. The fact that the dye was transported via a high affinity transporter, suggests possible involvement of other transporters. Metabolic inhibition by sodium azide resulted in reduced rate (flux) and extent (cumulative transport) of solute permeation. The polarized transport data indicated that the main transporters responsible are located on the apical side of the cell as AP→BL transport was 2.5 times higher than BL→AP transport. As suggested by Salomon et al., (2012), the involvement of basolaterally located transporters such as OCTN1 that mediates multi-specific, bidirectional, and pH-dependent transport of organic cations cannot be ruled out (Yabuuchi et al., 1999).

Functionally, the physiological roles of OCT3, OCTN1, and OCTN2 in the respiratory cells are not known. The actual role of OCT3 in the basolateral membrane of various airway epithelial cell types remains unclear as human OCT3 transcript expressed by *Xenopus oocytes* do not translocate acetylcholine (Kummer et al., 2008). However, the steroid-sensitive nature of OCT3 transporters in the respiratory cells including the Calu-3 cells (Horvath et al., 2007b) suggests some form of neuroendocrine functional capability and possible involvement in local disposal of cationic drugs in airway tissues. OCTN2 has the same functional characteristics as OCT1, but also functions as L-carnitine transporter with affinity for zwitterions, hence may play a role in endogenous organic cation transport.

Conclusions

The data reported in this work showed that the Calu-3 cells functionally express various isoforms of organic cation transporters except OCT2. This observation was confirmed by quantitative PCR and immuno-histochemistry. The expression pattern and the uptake characteristics of 4Di-1-ASP were comparable to data reported for biopsies excised from various parts of the lung and recently published data using Calu-3 cells. Additionally, our study provided new information on transport characteristics of 4-Di-1-Asp in Calu-3 cells, which was found to be concentration-dependent, polarized, and energy-dependent. Collated data from the molecular biology, uptake and transport studies showed that the Calu-3 cells can be used as a model for not only studying strategies for optimizing the effect of topically applied organic cation bronchodilators for treatment of respiratory tract diseases such as asthma and COPD, but also for cross-validation of newly-developed respiratory cell line.

Declaration of interest

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