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DRUG TRANSPORT ACROSS THE HUMAN RESPIRATORY EPITHELIAL BARRIER

by

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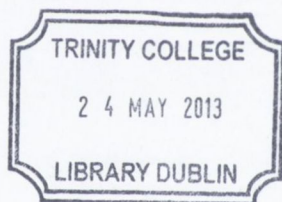
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SUMMARY

Drug transporters have only recently moved into the centre of attention of pulmonary drug delivery research, and of the more than 400 identified transporter proteins, only little information is available. In a first step, protein expression levels of several members of the ABC- and SLC-super family were determined in human lung epithelial cells and human alveolar epithelial primary cells. Protein abundance was found to be more or less equivalent for organic cation/carnitine (OCT/N, *SLC22A1-5*) and multidrug and toxin extrusion (MATE1, *SLC47A1*) transporters in human alveolar (primary culture and A549), bronchiolar (H441), bronchial (16HBE14o-, Calu-3) epithelial cells, except for OCT2 (not detected in bronchial cells), OCTN2 (higher expression levels in the bronchial cells). The expression pattern of OATPs revealed high protein levels of OATP2A1, OATP3A1 and OATP4A1 in alveolar (primary cell culture and A549) cells, whereas low levels of transport proteins were detected in 16HBE14o- and Calu-3 cells. Intestinal Caco-2 epithelial cells were used as comparison. P-glycoprotein (*ABCB1*/MDR-1) protein expression was confirmed in A549 and H441 cells. Our investigations continued by functionally characterising in particular OCT/Ns in human respiratory cell culture models by using a fluorescently-labelled cationic compound 4-(4 (dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺) as well as radiolabelled substrates, e.g., the model cation [¹⁴C]-TEA and the OCTN probe [³H]-acetylcarnitine in cell uptake studies. We showed that A549 cells exhibited the highest uptake activity of organic cations in comparison to the other cell types. In A549 cells we found evidence that the uptake of organic cations was mediated mainly by OCT1 and OCT2 at the alveolar epithelium. An OCT-driven uptake of organic cations has been shown at the bronchial and bronchiolar epithelium at lower levels with a speculative involvement of OCT1. In the case of acetylcarnitine uptake,

OCTN2 was thought to play a major role in this translocation in alveolar and bronchial epithelial cells. There is an immense need for reliable, physiologically relevant *in vitro* models of the respiratory epithelium, therefore, H441 cells were characterised as an *in vitro* model for biopharmaceutical studies of the distal lung. We confirmed the expression of a number of key drug transporters and P-gp function with parallels to A549 and human alveolar epithelial primary cells. We concluded that H441 cells are a good candidate as an *in vitro* model for transport studies of distal lung epithelial barrier. We undertook further investigations to study the susceptibility of organic cation pathways to the presence of β_2 -adrenergic drugs and to explore the uptake and transport of [^3H]-salbutamol across the lung epithelium. We provided evidence that all three β_2 -receptor agonists competitively inhibit uptake of [^{14}C]-TEA, but not of [^3H]-acetylcarnitine at the alveolar epithelium. Moreover, we demonstrated in hOCT-over-expressing HEK-293 cells that β_2 -agonists exhibited selective inhibition effects on hOCT1-mediated uptake. The uptake of salbutamol, however, was not mediated by the five cloned organic cation transporters. Besides organic cation translocation, we studied the suitability of bile acid analogues to study functional activity in Caco-2 and A549 epithelial cells. Interestingly, we found evidence for an active uptake process which was clearly dependent on chemical features such as diastereoselectivity. Lastly, we investigated the effect of latex nanoparticles to interact with drug transporter functionality in A549 epithelial cells. It was the first study comparing pharmacological P-glycoprotein inhibitors with nanoparticulates. We observed an attenuation of Rh123 release, and hence P-glycoprotein function by nanoparticles, which might further increase the interest in nanoparticulate drug delivery systems. Collectively, the results of this work uncover novel functional expression patterns of ABC and SLC drug transporters in different respiratory *in vitro* models.

To Anna-Lena

"A fool ... is a man who never tried an experiment in his life."

Erasmus Darwin (1711-1802, English physician, grandfather of C. Darwin)

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CHAPTER 1

INTRODUCTION

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1.1 THE LUNG

The lung is a highly complex organ, whose furcated structure ensures efficient oxygenation of the blood and simultaneous removal of carbon dioxide from the body. The human pulmonary system can be divided into two regions: the conducting (or proximal) airways, including bronchi and bronchioles, and the respiratory (or distal) region with terminal bronchioles, alveoli and the alveolar capillary network (Weibel, 1997). The tree-like structure of the lung enormously increases the surface area, permitting a fast diffusive gas exchange. The airways bifurcate about 16–17 times before the alveoli are encountered (Patton et al., 2007). The alveolar-capillary barrier of the lower lung is formed by a layer of pulmonary epithelial cells (140 m^2) in contact with the airspace, a thin basement membrane and sheets of microvascular endothelial cells (130 m^2) in contact with the blood (Weibel, 1997). The proximal part, on the other hand, mainly serves as a filter, heater and humidifier of incoming air. In order to fulfil the specific roles of the different lung regions, the respiratory epithelium is composed of several distinct cell types. Generally, the epithelial surface cells become flatter, the deeper they are located within the lung. Columnar ciliated epithelial cells form up to 50% of the airway epithelium which are important for the transport of mucus to the throat. Furthermore, the large (bronchial) and small (bronchiolar) airways are composed of basal cells, brush cells, Clara cells, goblet cells and pulmonary neuroendocrine cells. Under physiological conditions, two epithelial cell types are present in the alveolar region: the squamous type I (thickness of $0.1\text{--}0.2\text{ }\mu\text{m}$) cells which cover approximately 96% of the surface area and the cuboidal type II cells covering the remaining surface area. Alveolar type II (ATII) cells are responsible for the biosynthesis and secretion of surface active materials and immunoglobulins. Furthermore, they are considered to be

the progenitor cells of the alveolar epithelium due to their ability to differentiate into alveolar type I-like cells (*in vitro*). Excellent reviews have been published on lung anatomy and respiratory cell physiology by Knight *et al.*, Mason *et al.* and Crystal *et al.* (Knight *et al.*, 2003; Mason *et al.*, 1998; Crystal *et al.*, 2008).

The epithelial surface of the lung is covered by a lining fluid and surfactant layer and in addition, mucus in the upper parts, trapping inhaled particulate matter and preventing alveolar collapse (Liao *et al.*, 2003; Patton *et al.*, 2004). This fluid is assumed to have a thickness of only 5-10 μm , progressively decreasing with the airway level (Patton *et al.*, 2004; Wiedmann *et al.*, 2000). Given the fact that numerous epithelial cell types exist throughout the different regions of the lung, a divergence in tight junctional architecture of both bronchial and alveolar epithelial cells can be found (Godfrey *et al.*, 1993). Tight junctional complexes determine the cell polarity by dividing the cell membrane into apical and basolateral domains.

1.2 PULMONARY DRUG DELIVERY

Pulmonary drug delivery has been extensively reviewed by Smyth and Hickey (Smyth and Hickey (Ed): Controlled Pulmonary Drug Delivery, 2011) and Tronde *et al.* (Tronde *et al.*, 2008). Pulmonary drug therapy may be used in the treatment of local conditions of the lung such as asthma, chronic obstructive pulmonary disease (COPD) or cystic fibrosis. The lung, however, can also be used as a portal to deliver drugs to the systemic circulation. The latter is particularly attractive due to the high surface area available for absorption, avoidance of the liver first pass metabolism and a rapid onset of action (Patton *et al.*, 2010; Forbes and Ehrhardt, 2005; Ehrhardt and Kim (Ed): Drug absorption studies. In situ, in vitro and in silico models. 2008). Inhaled medicines have been used for the treatment of respiratory diseases for decades, either as liquid or dry powder formulations. Intriguingly, the therapeutic targets have not changed significantly, e.g., COPD and asthma are mainly treated using bronchodilators (β_2 -agonists and anticholinergics) and inhaled corticosteroids. While advanced inhaler devices and novel formulations have been employed to improve and control pulmonary deposition (Tayab *et al.*, 2005) the fate of inhaled therapeutics is still poorly understood (Patton and Byron, 2007; von Wichert and Seifart, 2005). In this regard, it is particularly important to improve our knowledge base on molecular processes involved in the disposition of inhaled drugs (Gumbleton *et al.*, 2001).

Figure 1 summarises different clearance pathways following oral inhalation. After the deposition of the aerosol particle, there are two main competing mechanisms determining its fate: dissolution followed by absorption of the drug or removal of the particle (Tayab *et al.*, 2005). The removal mechanisms include mucociliary clearance in the upper airways and phagocytosis by macrophages in the alveolar region. The role of

airway clearance in pulmonary drug disposition was recently reviewed by Edsbäcker *et al.* (Edsbäcker et al., 2008).

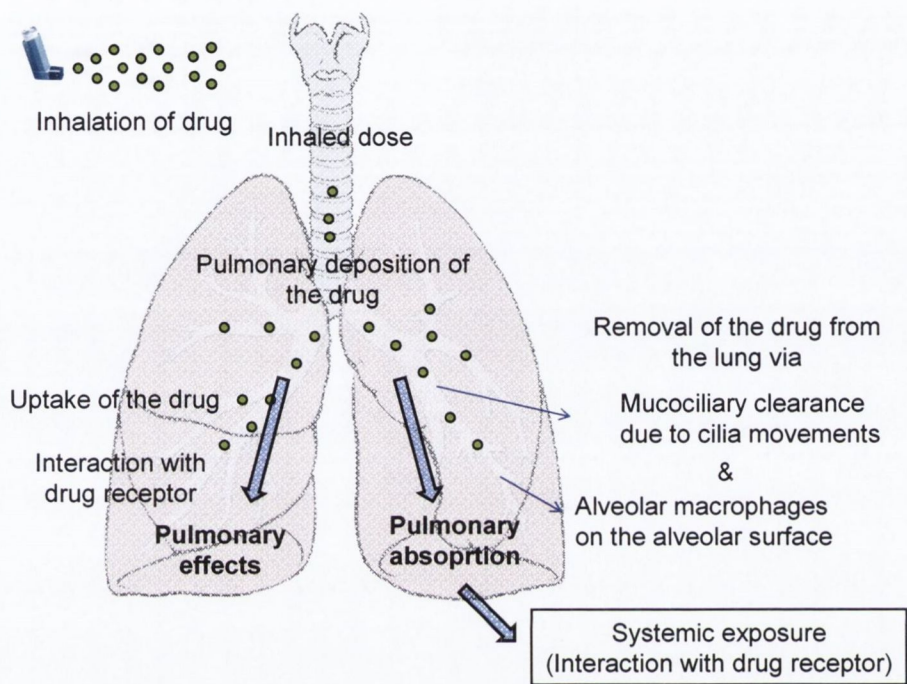


Figure 1. Schematic illustration of the fate of an inhaled drug (modified from Tronde et al., 2008).

Efficient drug deposition and distribution are the main challenges of inhalation therapy. The inhaled drug, in most cases, will have to cross the lung epithelium in order to reach its site of action. If a local effect is desired, absorption of these substances into the systemic circulation is unwanted and will worsen adverse reactions (Tronde et al., 2008; Schanker et al., 1986; Pilcer et al., 2010). Systemic delivery of drugs via the inhalation route, on the other hand, is governed by pulmonary absorption and metabolism (Patton and Byron, 2007).

The pharmacokinetic characterisation of pulmonary administered drugs comprises a variety of *in silico*, *in vitro*, *ex vivo* and *in vivo* techniques. *In silico* methods include

computational models to estimate and predict drug absorption based on drug physicochemical properties such as octanol/water partitioning coefficient. The cell phenotype heterogeneity of the lung complicates the study of drug absorption *ex vivo* or *in vivo*. Hence, numerous airway epithelial cell models have been established to study absorption processes *in vitro*. They offer a number of advantages, e.g., low costs, precise dosing, defined local drug concentration and surface area of exposure and the ability to reduce the variability in cultures. More importantly, cell culture methods are widely accepted as they offer a higher and quicker throughput possibility compared to *in vivo* animal tests (Forbes and Ehrhardt, 2005).

On the other hand, isolated perfused lung models allow lung-specific investigations on the permeability of barriers and interaction between different cell types *ex vivo* (Tronde et al., 2008). The perfusate and lavage fluids can be easily sampled and drugs can be directly administered to the airway lumen or the circulation. *In vivo* animal studies are accompanied by ethical issues and immense costs. Moreover, animal derived pharmacokinetic data has to be analysed with caution as the lung anatomy and physiology in humans show significant differences.

1.3 CELL-CULTURE MODELS OF THE PULMONARY MUCOSA

As outlined above, the respiratory epithelium is composed of a variety of different cell phenotypes, thus choosing the appropriate model to study drug transport *in vitro* is quite challenging. A number of different bronchial and alveolar epithelial cell cultures have been characterised regarding their suitability to serve as models for drug absorption and disposition (Ehrhardt et al., 2006; Elbert et al., 1999; Fiegel et al., 2003; Florea et al., 2001; Foster et al., 1998; Grainger et al., 2006; Hamilton et al., 2000; Lin et al., 2007; Zhu et al., 2010). The most commonly used human epithelial cell lines include 16HBE14o- and Calu-3 (bronchial) and A549 (alveolar) cells (Forbes et al., 2003; Ehrhardt et al., 2002; Bosquillon 2010) which were also the main cell culture models in the current work (Figure 2).

16HBE14o- is a human bronchial epithelial cell line generated by transformation of normal bronchial epithelial cells obtained from a one-year-old male heart-lung patient. Transformation was accomplished with SV40 large T antigen using the replication defective pSVori plasmid (Cozens et al., 1994). 16HBE14o- cells are non-serous, non-ciliated and smaller in size than, for example Calu-3 cells. These cells form confluent, polarised cell layers with functional tight junctional complexes exhibiting transepithelial electrical resistance (TEER) values of approximately $700 \Omega \cdot \text{cm}^2$ (Ehrhardt et al., 2002). The Calu-3 epithelial cell line is derived from lung adenocarcinoma (Fogh and Trempe, 1975) and is known to exhibit serous cell properties. This cell line also forms tight monolayers (Shen et al., 1994) and TEER values of $1000 \Omega \cdot \text{cm}^2$ can be reached when grown under liquid-covered conditions (LCC), whereas $\sim 300\text{-}600 \Omega \cdot \text{cm}^2$ are peak values in the case of air-interfaced conditions (AIC) (Grainger et al., 2006). It is the fact that A549 cells do not form electrically tight monolayers, due to their inability to

develop functional tight junctional complexes (Foster et al., 1998). Nevertheless, they are probably the most frequently used alveolar epithelial cell model. A549 cells are derived from human pulmonary adenocarcinoma exhibiting morphologic phenotypes of human pulmonary alveolar type II cells *in situ* (Lieber et al., 1976).

A similar deficiency as for A549 cells has been reported for the immortalised bronchial epithelial cell line, BEAS-2B (Eaton et al., 1996; Nakamura et al., 2010). Although functional tight junctions are of paramount importance for trans-monolayer absorption studies, cells without this feature might, nevertheless, be suitable for uptake or metabolic studies. A cell line that thus far has received little attention in biopharmaceutical research is H441. H441 cells were originally isolated from the pericardial fluid of a patient with papillary adenocarcinoma of the lung. They possess characteristics of both alveolar as well as of bronchiolar epithelial phenotype and can grow to confluent monolayers under the right culture conditions (Vuong et al., 2002; Hermanns et al., 2004).

In addition to continuously growing cell lines, primary cultures of human bronchial and alveolar epithelial cells have been reported. Whilst it is generally accepted that primary cultures most closely resemble the situation *in situ*, their use is linked to a number of limitations, e.g., restricted availability of human donor tissue, relatively high costs and a short life-time (Forbes and Ehrhardt, 2005; Kim et al., 2001). In the case of human alveolar epithelial primary cell culture of isolated alveolar type II cells, monolayers with TEER values $>1000 \Omega \cdot \text{cm}^2$ were generated (Elbert et al., 1999).

A number of review articles discuss respiratory cell-based *in vitro* models (Buckley et al., 2011; Ehrhardt et al., 2005; Forbes and Ehrhardt, 2005; Forbes, 2000; Sakagami, 2006; Shen and Lee, 2002; Sporty et al., 2008).

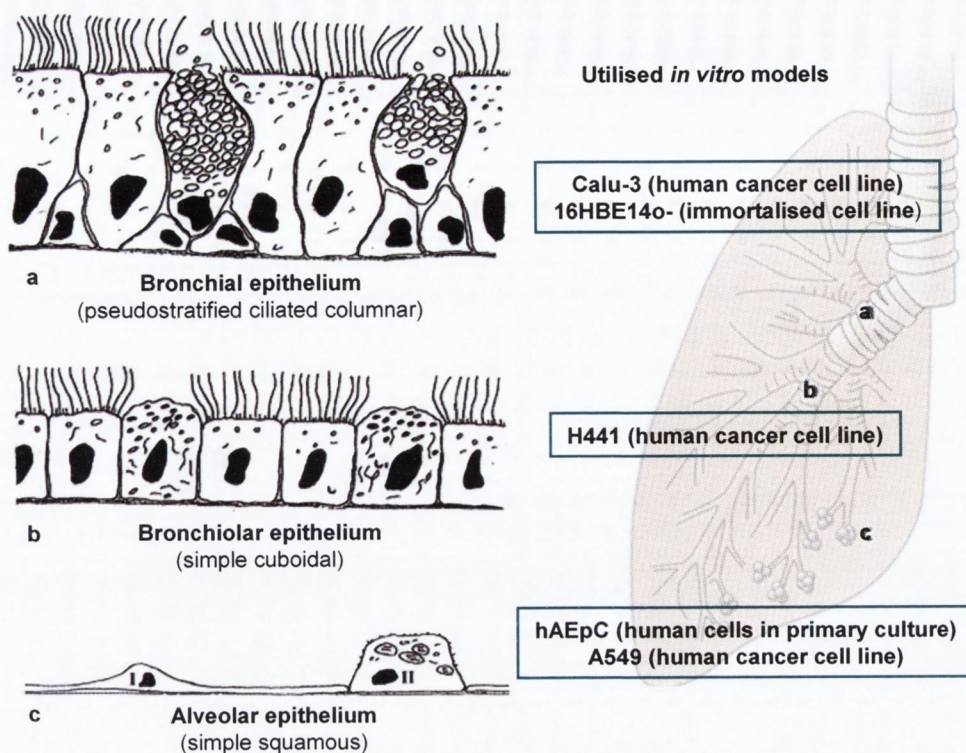


Figure 2. The lung epithelium with major cell types and major respiratory epithelial models pertaining to each area. *Left panel:* **a** The bronchial epithelium with ciliated cells, interspersed with goblet cells and basal cells. **b** The bronchiolar epithelium with ciliated cells and Clara cells. **c** The alveolar epithelium comprised of the thin alveolar type I cell (I) and the cuboidal type II cell (II). *Right panel:* **a** Calu-3 and 16HBE14o- are both bronchial epithelial cell lines. **b** H441 cells are described to have characteristics of ATII and bronchiolar (Clara) epithelial cells. **c** A549 cells are derived from pulmonary adenocarcinoma with alveolar type II characteristics. Human alveolar epithelial primary cells (hAEpC) represent most closely the characteristics of the alveolar epithelium *in situ*. All shown respiratory epithelial cell models have been used in expression and functional activity studies (modified from Forbes, 2000; Wetsel et al., 2011).

1.4 DRUG TRANSPORT ACROSS THE LUNG EPITHELIAL BARRIER

Once arrived at the lung surface, the physicochemical properties of an inhaled drug molecule govern its absorption. A freely water soluble drug can dissolve in the epithelial lining fluid and be absorbed quickly, whereas for more hydrophobic substances dissolution might become a rate limiting factor (Olsson et al., 2011).

Absorption across the lung epithelial barrier is considered to represent the limiting step for inhaled drugs to reach their pharmacological target sites (Olsson et al., 2011).

Generally, the absorption of small lipophilic substrates is believed to occur mainly by passive diffusion through the cell membrane; whilst small hydrophilic compounds are absorbed by diffusion through tight junctional pores or by specific carriers such as transporter proteins (see Figure 3) (Patton and Byrne, 2007; Tronde et al., 2008).

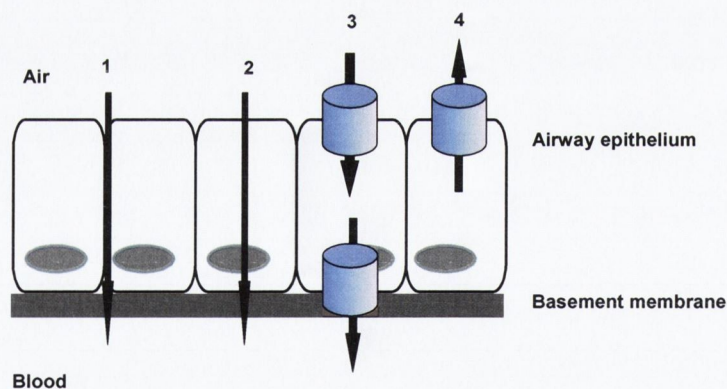


Figure 3. Simplified schematic epithelial drug transport mechanisms between the airway lumen and the blood side (processes of interest in this thesis). The lung epithelium plays a major role in drug absorption processes. Nevertheless, the basement membrane and the endothelial layer (not shown) are also contributing to pulmonary drug metabolism, clearance and absorption and followed by systemic absorption. **1** Simple paracellular diffusion **2** Simple transcellular diffusion **3** Carrier-mediated transport **4** Efflux process.

Evidence of carrier-mediated transport across the pulmonary epithelial barrier is, however, relatively limited (Gumbleton et al., 2011; Bosquillon, 2010; Patton et al.,

2004). In one study, disodium cromoglycate was observed to be absorbed from the rat lungs in part by a carrier-type transport process and in part by diffusion (Hemberger and Schenker, 1979). Moreover, transepithelial transport of dipeptides across rat alveolar epithelial cell monolayers and rabbit tracheal epithelial cells was reported in 1993 (Morimoto et al., 1993) and 1998 (Yamashita et al., 1998), respectively.

To date, more than 400 membrane transporters belonging to two major super families, ATP-binding cassette (ABC) transporters and solute carriers (SLC) have been identified. ABC transporters are generally known as efflux transporters, while SLC transporters mediate uptake of substrates, although most are capable of bidirectional transport. ABC transporters are a family of transmembrane proteins that are mostly responsible for pumping a wide variety of substrates from the intracellular to the extracellular compartment in an energy-dependent manner. There are 49 *ABC*-related functional genes in the human genome, e.g., the genes encoding the P-glycoprotein (*ABCB*) family, such as multidrug resistance protein 1 (MDR1) and the bile salt export pump (BSEP) and the multidrug resistance-associated (MRP, *ABCC*) protein family (van der Deen et al., 2005, Ho and Kim, 2005). The uptake of drugs into cells is mainly accomplished by SLC transporters. Within the 51 subgroups there are members of the organic anion transporting polypeptide (OATP, *SLCO*) family, organic cation transporter (OCT, *SLC22A*) family, organic cation/carnitine transporter (OCTN, *SLC22A*) family and multidrug and toxin extrusion (MATE, *SLC47A*) family.

The importance of genetic and molecular identification of drug transporters and their substrates has been increasingly recognised. The functional characterisation of drug transporters provides important information that allows improvement of drug delivery

and drug design. Although transporters have been characterised and localised in several tissues, most attention was paid to intestine, liver, kidneys and blood-brain-barrier, focussing on the interaction of drugs and their metabolites with transporters present in the epithelium and endothelium, respectively (Figure 4) (International Consortium, 2010).

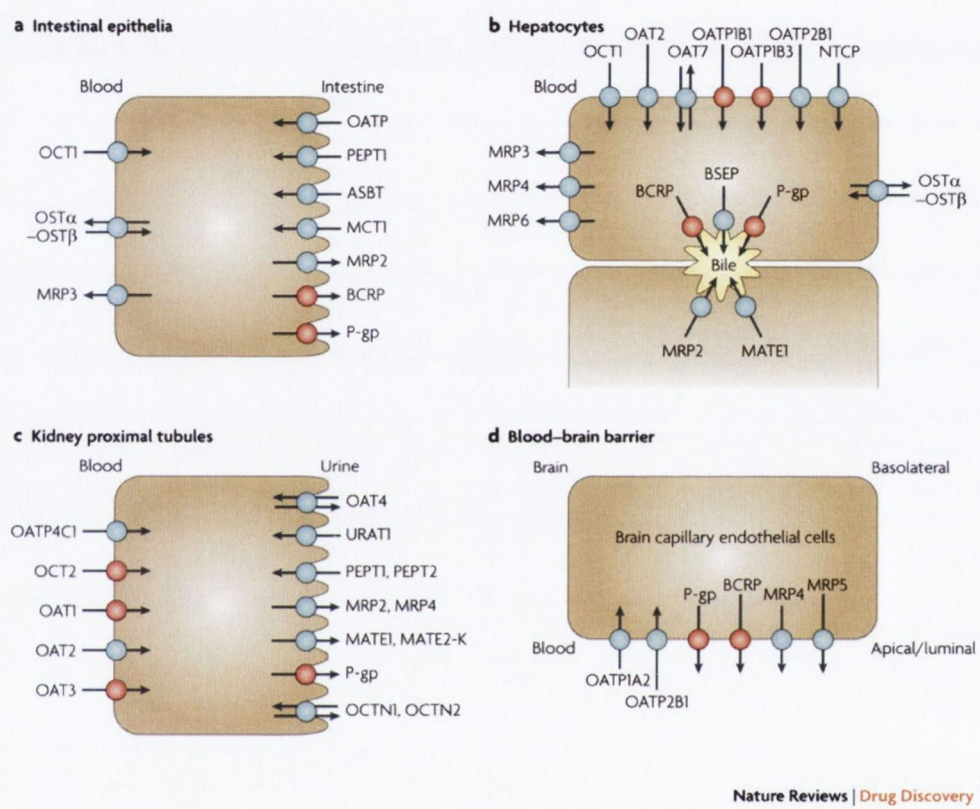


Figure 4. ABC and SLC-transporters in membranes of intestinal epithelia, hepatocytes, kidney proximal tubules and brain capillary endothelial cells are shown. The expression of transporters in several compartments is a key role for drug disposition (International Transporter Consortium, 2010).

The following chapters will focus on the functional expression of drug transporter protein at the air-blood barrier with emphasis on investigations on organic cation/ carnitine transporters (*SLC22A1-5*).

1.4.1 P-GLYCOPROTEIN

P-glycoprotein/MDR1 (P-gp) is a 170 kDa membrane glycoprotein, encoded by the *ABCB1* gene. It plays an important role as an efflux pump in drug disposition, particularly in cancer chemotherapy (Baguley, 2010). P-gp is also expressed in many tissues under physiological conditions, indicating its major role in cellular transport of a variety of endogenous substrates and therapeutic agents (Baguley, 2010). Presently, over 100 compounds have been identified to be P-gp substrates, of which the majority are basic lipophilic amines freely permeating the plasma membrane (van der Deen et al., 2005). The lung is thought to accumulate lipophilic amines and the similarity in substrate specificity of the efflux pump strongly suggests that P-gp mediated efflux plays an important role in pulmonary accumulation (Bosquillon, 2010). Functionally expressed P-gp within the alveolar epithelium may limit transport of therapeutic molecules and hence, the systemic absorption of inhaled products. It may also be able to extrude inhaled xenobiotics from the alveolar epithelium back into the alveolar airspace away from the systemic circulation, to increase the residence time of drugs if a local effect is desired (Campbell et al., 2003).

The presence of P-gp/MDR1 has been reported in the conducting airways of the lung (Lechapt-Zalcman et al., 1997) and in the alveolar epithelium (Campbell et al., 2003) *in situ*. P-gp has also been detected in human respiratory epithelial cells *in vitro*, in bronchial epithelial Calu-3 and 16HBE14o- cells (Hamilton et al., 2001; Florea et al., 2001, Ehrhardt et al., 2003). Moreover, this efflux transporter is functionally expressed in freshly isolated human alveolar epithelial cells in primary culture (hAEpC) and in the continuously grown A549 cell line (Endter et al., 2007; Salomon and Ehrhardt, 2009).

1.4.2 TRANSPORTERS FOR ORGANIC ANIONS (OATPs)

OATPs are encoded by genes of the SLCO super family. OATPs are expressed in multiple organs such as the blood-brain-barrier, choroid plexus, intestine, testis, spleen, placenta, lung, kidney, heart and liver (Hagenbuch et al., 2004). The tissue distribution and expression of OATPs has been recently reviewed (Roth et al., 2012). OATPs can be divided into six families (OATP1-6) based on their amino acid homology. To date, only a few members, e.g., OATP1A2, OATP1B1, OATP1B3 and OATP2B1 are well characterised and have shown to play an important role in drug disposition (Shitara et al., 2006). OATP1A2 and OATP2B1 are widely distributed in the body, at high levels in the brain, liver, kidney and testis (Kullak-Ublick et al., 2001). OATP1B1 and OATP1B3 are predominantly expressed in the liver and at lower levels in the brain and testis (Hagenbuch and Meier, 2004). They are able to translocate a variety of endogenous and exogenous compounds. The endogenous compounds include bile acids, thyroid hormones 3,5,3'-triiodo-L-thyronine (T3), thyroxine (T4), prostanoids (PGE₁, PGE₂ and LTE₄) and conjugated steroids (estradiol-17 β -glucuronide and estrone-3-sulphate). OATP-mediated transport is thought to be ATP- and sodium-independent, and several studies suggest electro neutrality (Roth et al., 2012). OATP2B1, OATP2A1, OATP3A1, OATP4A1 and OATP4C1 mRNA expression in the lung was reported by Bleasby *et al.* and Tamai *et al.* (Bleasby et al., 2006; Tamai et al., 2000). Whilst initial data is emerging (Seki et al., 2009), information on the functional activity and substrate specificity of OATPs in the lung is lacking. Isomeric 3-desoxy-3-NBD-amino derivatives of deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) were synthesised as probes in-house for studying the distribution of bile acids and used to characterise transport activity at the lung epithelium as part of this thesis.

1.4.3 MEMBRANE TRANSPORTERS FOR ORGANIC CATIONS AND THEIR EXPRESSION PATTERNS IN RESPIRATORY EPITHELIA

The SoLute Carrier (SLC) super family comprises 51 gene families having at least 362 putatively functional protein-coding genes (Hediger et al., 2004; Sugiura et al., 2006). Proteins encoded by the *SLC5*, *SLC6*, *SLC7*, *SLC22*, *SLC29*, *SLC44* and *SLC47* gene families are among the membrane transporters involved in the uptake and efflux of organic cations across biological membranes. Although a relevant organ of absorption, the lung remains mostly uncharted terrain in transporter research, and most data available in the public domain is limited to PCR-based expression studies (Nakamura et al, 2010; Endter et al., 2009; Ishiguro et al., 2007; Wang et al., 2007, Courcot et al., 2012). Little data is published on protein abundance, localisation or indeed transporter function. Tables 1 to 4 summarise our current understanding of the distribution pattern of organic cation transporters in the lung (Table 1) and in human *in vitro* cell culture models (Table 3). Tables 2 and 4 compile information on negative data regarding transporter expression in the lung and in relevant *in vitro* models, respectively. There is, however, considerable discord about the exact spatial expression patterns of membrane transporters. The data summarised in Tables 1-4 clearly show that quantitative information is often lacking, and that information on subcellular localisation is scarce.

The most investigated of the aforementioned proteins are the polyspecific organic cation transporters of the *SLC22* family, OCT1, -2 and -3, and the organic carnitine transporters OCTN1 and OCTN2. These OCT/Ns are able to translocate small (< 500 Da) organic cations with broad, overlapping affinities for endogenous substrates, such as choline, acetylcholine and monoamine neurotransmitters, as well as a variety of

xenobiotics (Jonker et al., 2004). OCTs transport organic cations electrogenically and sodium-independently across the cell membrane in both directions. OCTNs, on the other hand, mainly operate as carnitine transporters in a sodium- and pH-dependent manner (Koepsell, 2005; Koepsell et al., 2007). A considerable body of knowledge on structure, expression, function and regulation of OCT/Ns has been published, since the first member (OCT1) was cloned in the early 1990's (Koepsell, 2005; Bleasby et al., 2006; Ciarimboli, 2008; Nies et al., 2011; Roth et al., 2012; Gruendemann et al., 1994). Evidence is now emerging (Gumbleton et al., 2008; Bosquillon, 2010; Lips et al., 2005) that OCT/Ns also have relevant functions in the lung. In particular, OCT1 and OCT2 (but not OCT3) have been reported to mediate luminal acetylcholine release in human airways (Lips et al., 2005). Our lab very recently reported on the functional expression of OCT/Ns in human bronchial (Calu-3, 16HBE14o-) and alveolar (A549) cell lines in comparison to the intestinal Caco-2 model. We concluded that OCT/N protein levels were more or less equivalent for all transporters across the respiratory epithelial cell types. The notable exceptions were OCT2, which was only detected in A549 cells at relevant levels, and OCTN2, which was found at lower levels in A549 compared to the bronchial cell types. In comparison with signal intensities obtained from Caco-2 cells, it was noted that OCT1, OCT2, OCTN1 and OCTN2 were synthesised at lower levels in the lung-derived cell lines. OCT3 was observed at similar levels in all cell types studied (Salomon et al., 2012). The high-affinity choline transporter CHT1 (*SLC5A7*) is mainly responsible for the translocation of choline at the airway epithelium. It localises to the apical membrane of ciliated cells in rat trachea (Lips et al., 2005). In addition to CHT1, the choline-specific transporter-like protein family (*SLC44A1-4*) is of interest for the transport of organic cations. CTL1 and CTL2 were found to be expressed in human lung and trachea tissue by PCR (Ishiguro et al., 2007) as well as in Calu-3 and A549 cells

(Ishiguro et al., 2007; Wang et al., 2007). These data are consistent with Northern blot analysis, confirming CTL1, CTL2 and CTL4 in rat lung (Traiffort et al., 2005).

Little is known about the expression and function of members of the *SLC29* and *SLC47* family in the lung. The *SLC29A* family including the equilibrate nucleoside transporters (ENT1, ENT2 and ENT4) are expressed in BEAS-2B cells (Allen-Gipson et al., 2009), but no studies have yet been carried out to assess the expression of MATE-1 and MATE-2 (*SLC47A1-2*) in respiratory epithelia. Likewise, the role of *SLC6* and *SLC7* family members in cation transport is not fully investigated.

Table 1. Expression and localisation of transporter proteins involved in the translocation of organic cations in lung tissue *in situ*.

Gene name	Protein	Cellular distribution & localisation	Method	Specimen	References
<i>SLC5A7</i>	CHT1	<i>Lamina propria</i> , Bronchial epithelial cells: apical	WB IH	Lung sections	(Horiguchi et al., 2009)
<i>rSLC5A7</i>		Tracheal epithelium, ciliated cells: apical	RT-PCR, WB, IH	Tracheal epithelium	(Lips et al., 2005; Pfeil et al. 2003)
<i>hSLC6A14</i>	ATB ^{0,+}	Localisation n.s.	RT-PCR WB	Total lung tissue Airway and distal lung	(Sloan et al., 1999) (Sloan et al., 2003)
<i>mSLC6A14</i>		Epithelial cells: lining the trachea, bronchioles and alveoli, trachea: apical membrane of epithelial cells, bronchioles: apical membrane of ciliated cells, membrane of alveolar type I cells	WB, IH	Total lung tissue	(Sloan et al., 2003)
<i>hSLC7A1</i>	CAT1		RT-PCR	Alveolar macrophages	(Rotoli et al., 2007)
<i>rSLC7A1</i>			qRT-PCR	Type II cells from foetal lung	(Wang et al., 2006)
<i>mSLC7A2A</i>	CAT2		RT-PCR	Total lung tissue	(Devès et al., 1998)
<i>mSLC7A2B</i>			RT-PCR	Total lung tissue	(Devès et al., 1998)
<i>rSLC7A3</i>	CAT3		qRT-PCR	Type II cells from foetal lung	(Wang et al., 2006)

<i>hSLC7A6</i>	y ⁺ LAT2	Localisation n.s.	RT-PCR qRT-PCR	Alveolar macrophages Pulmonary parenchyma, bronchial mucosa (BM)	(Rotoli et al., 2007) (Courcot et al., 2012)
<i>hSLC7A7</i>	y ⁺ LAT1	Localisation n.s.	RT-PCR, qRT-PCR Northern blot	Alveolar macrophages, pulmonary parenchyma, BM Total lung tissue	(Rotoli et al., 2007) (Courcot et al., 2012) (Torrents et al., 1998)
<i>SLC7A9</i>	b ^{0,+} AT	Not yet investigated			
<i>hSLC22A1</i>	OCT1	Bronchial ciliated cells: mainly localised within the cells and weakly apical Airway epithelium Airway smooth muscle cells Ciliated epithelial cells: apical	RT-PCR, IH qRT-PCR RT-PCR qRT-PCR	Trachea and bronchus (AEC) Trachea Trachea, lung Pulmonary parenchyma, BM	(Lips et al., 2007) (Horvath et al., 2007) (Horvath et al., 2007) (Courcot et al., 2012)
<i>mSLC22A1</i>		Tracheal epithelium, ciliated cells: apical	RT-PCR, IH	Trachea, bronchi	(Hollenhorst et al., 2012; Kummer et al., 2006)
<i>rSLC22A1</i>		Alveolar epithelium Alveolar epithelial cells Fibroblast, alveolar macrophages	RT-PCR, IH bDNA amplification assay WB	Tracheal and bronchial epithelium (AEC) ATII cells, lung tissue Total lung tissue Primary type II cells	(Lips et al., 2007) (Ishiguro et al., 2007) (Slitt et al., 2002) (Miakotina et al., 2005)
<i>hSLC22A2</i>	OCT2	Bronchial ciliated cells: mainly apical, Basal cells: entire membrane	RT-PCR, IH	Trachea and bronchus (AEC)	(Lips et al., 2007)
<i>mSLC22A2</i>		No specific labelling	RT-PCR, IH	Trachea, bronchi	(Hollenhorst et al., 2012; Kummer et al., 2006)

<i>rSLC22A2</i>	OCT3	Fibroblast, alveolar macrophages	WB	Primary type II cells	(Miakotina et al., 2005)
		Tracheal epithelium, ciliated cells: apical Fibroblast, alveolar macrophages	RT-PCR, IH WB	Tracheal epithelium (AEC) Primary type II cells	(Lips et al., 2007) (Miakotina et al., 2005)
<i>hSLC22A3</i>		Basal cells: membrane Intermediate cells: basolateral membrane Ciliated cells: apical Pulmonary blood vessels, airway smooth muscle cells	RT-PCR, IH	Trachea and bronchus (AEC)	(Lips et al., 2007)
			RT-PCR, qRT-PCR, IH qRT-PCR	Trachea, lung Pulmonary parenchyma, BM	(Ishiguro et al., 2007; Horvath et al., 2007) (Courcot et al., 2012)
<i>mSLC22A3</i>		Bronchial smooth muscle, bronchial epithelium	RT-PCR, IH	Trachea, bronchi	(Hollenhorst et al., 2012; Kummer et al., 2006)
<i>rSLC22A3</i>		Tracheal epithelium, brush cells: apical, luminal membrane, non-polarised basal cells: the whole membrane Alveolar epithelium	RT-PCR, IH	Tracheal epithelium	(Lips et al., 2007)
			RT-PCR bDNA amplification assay	ATII cells, lung Total lung tissue	(Ishiguro et al., 2007) (Slitt et al., 2002)
<i>hSLC22A4</i>	OCTN1	Tracheal epithelium: apical, luminal side, Alveolar macrophages	RT-PCR, qRT-PCR, IH qRT-PCR	Trachea sections Pulmonary parenchyma, BM	(Horvath et al., 2007; Horvath et al., 2007a, Courcot et al., 2012)
<i>rSLC22A4</i>			bDNA amplification assay	Total lung tissue	(Slitt et al., 2002)
<i>hSLC22A5</i>	OCTN2	Airway epithelial cells: apical	RT-PCR, qRT-PCR, IH	Trachea sections	(Horvath et al., 2007; Horvath et al., 2007a)

<i>rSLC22A5</i>			qRT-PCR	Pulmonary parenchyma, BM	(Courcot et al, 2012)
			bDNA amplification assay	Total lung tissue	(Slitt et al., 2002)
<i>SLC29A1</i>	ENT1		qRT-PCR	Distal pulmonary parenchyma BM	(Courcot et al., 2012)
<i>SLC29A2</i>	ENT2		qRT-PCR	Distal pulmonary parenchyma BM	(Courcot et al., 2012)
<i>SLC29A4</i>	PMAT (ENT4)		qRT-PCR	Distal pulmonary parenchyma BM	(Courcot et al., 2012)
<i>hSLC44A1</i>	CTL1	Localisation n.s.	RT-PCR, WB	Lung, trachea	(Ishiguro et al., 2007; Michel et al., 2006)
<i>rSLC44A1</i>		Localisation n.s. Alveolar epithelium	RT-PCR RT-PCR	Lung ATII cells	(Ishiguro et al., 2007) (Ishiguro et al., 2007)
<i>SLC47A1</i>	MATE1		qRT-PCR	Distal pulmonary parenchyma BM	(Courcot et al., 2012)
<i>hSLC47A2</i>	MATE2		RT-PCR	Total RNA from tissue	(Komatsu et al., 2011)

RT-PCR: reverse-transcriptase-polymerase chain reaction; qRT-PCR: quantitative RT-PCR; WB: Western blot; IH: immunohistochemistry;
AEC: abraded epithelial cells; BM: bronchial mucosa, n.s. not specified;

Table 2. Negative data on transporter expression in lung tissues.

Gene name	Protein	Cellular distribution & localisation	Method	Specimen	References
<i>hSLC7A2A</i>	CAT2		RT-PCR	Alveolar macrophages	(Rotoli et al., 2007)
<i>hSLC7A2B</i>			RT-PCR	Alveolar macrophages	(Rotoli et al., 2007)
<i>hSLC22A1</i>	OCT1	Localisation n.s.	RT-PCR	Lung, trachea	(Ishiguro et al., 2007)
<i>rSLC22A1</i>		Localisation n.s.	RT-PCR	Lung	(Ishiguro et al., 2007)
<i>mSLC22A1</i>		Fibroblast	WB	Primary type II cells	(Miakotina et al., 2005)
<i>hSLC22A2</i>	OCT2	Localisation n.s.	RT-PCR	Lung, trachea	(Ishiguro et al., 2007)
			qRT-PCR	Pulmonary parenchyma	(Cournot et al., 2012)
<i>rSLC22A2</i>		Alveolar epithelium	RT-PCR	ATII cells, lung	(Ishiguro et al., 2007)
			bDNA amplification assay	Total lung tissue	(Slitt et al., 2002)
<i>hSLC47A2</i>			qRT-PCR	Pulmonary parenchyma	(Cournot et al., 2012)

Table 3. Expression profiles of organic cation transporters of the *SLC5*, 6, 7, 22, 29, 44 and 47 families in human respiratory epithelial cell cultures (continuously growing and primary cell culture).

Gene name	Protein	Method	Cell line	Comments	References
<i>SLC5A7</i>	CHT1	Functional studies	A549	LCC	(Kleinzeller et al., 1994)
<i>SLC6A14</i>	ATB ^{0,+}	RT-PCR	Calu-3	Culture conditions unknown	(Rotoli et al., 2005)
<i>SLC7A1</i>	CAT1	RT-PCR	Calu-3		(Rotoli et al., 2005)
<i>SLC7A2A</i>	CAT2	RT-PCR	Calu-3		(Rotoli et al., 2005)
<i>SLC7A2B</i>		RT-PCR	Calu-3		(Rotoli et al., 2005)
<i>SLC7A3</i>	CAT3	RT-PCR	Calu-3		(Rotoli et al., 2005)
<i>SLC7A6</i>	y ⁺ LAT2	RT-PCR qRT-PCR	Calu-3 A549, BEAS-2B, 16HBE14o-hBEpC		(Rotoli et al., 2005) (Courcot et al., 2012)
<i>SLC7A7</i>	y ⁺ LAT1	qRT-PCR	A549, hBEpC		(Courcot et al., 2012)
<i>SLC7A9</i>	b ^{0,+} AT	Not yet investigated			
<i>SLC22A1</i>	OCT1	RT-PCR, WB, IH RT-PCR	Calu-3, BEAS-2B,	AIC and LCC LCC	(Endter et al., 2009; Salomon et al., 2012; Mukherjee et al., 2012) (Endter et al., 2009)

<i>SLC22A2</i>	OCT2	RT-PCR, q-RT-PCR, WB	16HBE14o-	LCC	(Endter et al., 2009; Salomon et al., 2012; Cournot et al., 2012)
		RT-PCR, q-RT-PCR RT-PCR, qRT-PCR, WB	hBEpC A549	LCC LCC	(Endter et al., 2009; Cournot et al., 2012) (Endter et al., 2009; Salomon et al., 2012; Cournot et al., 2012)
		WB	A549	LCC	(Salomon et al., 2012)
<i>SLC22A3</i>	OCT3	RT-PCR, WB, IH	Calu-3,	AIC and LCC	(Endter et al., 2009; Ishiguro et al., 2009; Wang et al., 2007; Salomon et al., 2012; Mukherjee et al., 2012)
<i>SLC22A4</i>	OCTN1	qRT-PCR qRT-PCR, WB RT-PCR, qRT-PCR RT-PCR, qRT-PCR, WB, IH	BEAS-2B 16HBE14o- hBEpC A549	LCC LCC LCC	(Courcot et al., 2012) (Salomon et al., 2012 ; Courcot et al., 2012)) (Endter et al., 2009; Corucot et al., 2012) (Endter et al., 2009; Ishiguro et al., 2009; Wang et al., 2007; Salomon et al., 2012; Mukherjee et al., 2012; Courcot et al., 2012)
		RT-PCR, WB, IH	Calu-3	AIC and LCC	(Endter et al., 2009; Salomon et al., 2012; Mukherjee et al., 2012)
		RT-PCR, qRT-PCR	BEAS-2B,	LCC	(Nakamura et al. 2010; Endter et al., 2009; Courcot et al., 2012)
		RT-PCR, qRT-PCR, WB	16HBE14o-	LCC	(Endter et al., 2009; Salomon et al., 2012; Courcot et al., 2012)
		qRT-PCR RT-PCR, WB, IH	hBEpC A549	LCC	(Courcot et al., 2012) (Endter et al., 2009; Wang et al., 2007; Salomon et al., 2012; Courcot et al., 2012)
<i>SLC22A5</i>	OCTN2	IH	Human airway epithelial cells	AIC localisation: apical	(Horvath et al., 2007)
		RT-PCR, WB, IH	Calu-3	AIC and LCC	(Endter et al., 2009; Salomon et al., 2012;

		RT-PCR, qRT-PCR	BEAS-2B	LCC	Mukherjee et al., 2012) (Nakamura et al. 2010; Endter et al., 2009; Courcot et al., 2012) (Endter et al., 2009; Salomon et al., 2012; Courcot et al., 2012) (Endter et al., 2009; Courcot et al., 2012) (Endter et al., 2009; Wang et al., 2007; Salomon et al., 2012, Courcot et al., 2012) (Horvath et al., 2007)
		RT-PCR, qRT-PCR, WB	16HBE14o-	LCC	
		RT-PCR, qRT-PCR	hBEpC	LCC	
		RT-PCR, qRT-PCR, WB, IH IH	A549 Human airway epithelial cells	LCC AIC, localisation:apical, co-localised with cilia	
<i>SLC29A1</i>	ENT1	RT-PCR, qRT-PCR, IH RT-PCR, qRT-PCR, WB qRT-PCR qRT-PCR	A549 BEAS-2B 16HBE14o- hBEpC	LCC	(Hubeck et al., 2008 ; Courcot et al., 2012) (Allen-Gipson et al., 2009; Courcot et al., 2012) (Courcot et al., 2012) (Courcot et al., 2012)
<i>SLC29A2</i>	ENT2	RT-PCR, qRT-PCR, WB qRT-PCR	BEAS-2B 16HBE14o-, hBEpC, A549		(Allen-Gipson et al., 2009; Courcot et al., 2012) (Courcot et al., 2012)
<i>SLC29A4</i>	PMAT (ENT4)	qRT-PCR	BEAS-2B, 16HBE14o-, hBEpC, A549		(Courcot et al., 2012)
<i>SLC44A1</i>	CTL1	RT-PCR, WB RT-PCR, WB, IH	Calu-3 A549	LCC LCC	(Ishiguro et al., 2007; Wang et al., 2007) (Ishiguro et al., 2007; Wang et al., 2007)

<i>SLC47A1</i>	MATE1	qRT-PCR	BEAS-2B, 16HBE14o-, A549		(Courcot et al., 2012)
<i>SLC47A2</i>	MATE2	qRT-PCR	EAS-2B hBEpC		(Courcot et al., 2012)

RT-PCR: reverse-transcriptase-polymerase chain reaction; qRT-PCR: quantitative RT-PCR; WB: Western blot; IH: immunohistochemistry;
AEC: abraded epithelial cells; AIC: air-interfaced culture; LCC: liquid-covered conditions; n.s. not specified
hBEpC: human bronchial epithelial primary cell

Table 4. Negative data on transporter expression in human respiratory epithelial cell cultures.

Gene name	Protein	Method	Cell line	Comments	References
<i>SLC5A7</i>	CHT1	RT-PCR, WB	A549	LCC	(Wang et al., 2007)
<i>SLC7A7</i>	y ⁺ LAT1	qRT-PCR	16HBE14o-, BEAS-2B		(Courcot et al., 2012)
<i>SLC22A1</i>	OCT1	RT-PCR RT-PCR, qRT-PCR	A549 BEAS-2B	LCC LCC	(Ishiguro et al., 2007) (Nakamura et al., 2010, Courcot et al., 2012)
<i>SLC22A2</i>	OCT2	RT-PCR, WB	Calu-3	LCC	(Endter et al., 2009; Ishiguro et al., 2009; Salomon et al., 2012; Mukherjee et al., 2012)
		RT-PCR, qRT-PCR	BEAS-2B	LCC	(Endter et al., 2009; Nakamura et al., 2010, Courcot et al., 2012)
		RT-PCR, qRT-PCR, WB	16HBE14o-	LCC	(Endter et al., 2009; Salomon et al., 2012, Courcot et al., 2012)
		RT-PCR, qRT-PCR	hBEpC	LCC	(Endter et al., 2009; Courcot et al., 2012)
		RT-PCR, qRT-PCR	A549	LCC	(Endter et al., 2009; Ishiguro et al., 2009; Courcot et al., 2012)
<i>SLC22A3</i>	OCT3	RT-PCR	Human airway epithelial cells	AIC	(Horvath et al., 2007)
		RT-PCR	BEAS-2B	LCC	(Endter et al., 2009; Nakamura et al., 2010)
<i>SLC22A4</i>	OCTN1	qRT-PCR	hBEpC		(Endter et al., 2009)
<i>SLC47A2</i>	MATE2	qRT-PCR	16HBE14o-, A549		(Courcot et al., 2012)

1.4.3.1 PHYSIOLOGICAL SUBSTRATES FOR CATION TRANSPORTERS IN THE LUNG

Organic cations are pivotal for lung function; they act as neurotransmitters, regulate lung fluid homoeostasis, stress response and immune reactions, and serve as important antioxidants (Table 5). These functions often require translocation of cationic molecules across epithelial membranes. One relatively well investigated example in this regard is the putative non-neuronal (acetyl-) cholinergic system (Hollenhorst et al., 2012). CHT1 and CLT1 are involved in the uptake of the precursor, choline, into lung epithelial cells, whereas OCT1 and OCT2 have been suggested to mediate the luminal release of acetylcholine after its synthesis (Lips et al., 2005; Kummer et al., 2008).

Table 5. Selection of physiological substrates of organic cation transporters in the lung

Endogenous substrate	Physiological function	Confirmed transporter	References
Acetylcholine	Neurotransmitter	OCT1, OCT2, CHT1, CTL1	(Lips et al., 2005; Koepsell et al., 2004; Nies et al., 2011; Kummer et al., 2008)
Adenosine	Nucleoside	ENT1, ENT2, PMAT	(Duan and Wang, 2010; Elwi et al., 2006)
Choline	Metabolite	CHT1, CTL1	(Kummer et al., 2008)
Corticosterone	Hormone	OCT1, OCT2, OCT3, MATE1	(Koepsell et al., 2004; Nies et al., 2011)
Dopamine	Neurotransmitter	OCT2, PMAT	(Duan and Wang, 2010; Elwi et al., 2006; Koepsell et al., 2004; Nies et al., 2011)
Epinephrine	Neurotransmitter	OCT2, OCT3	(Koepsell et al., 2004; Nies et al., 2011)
Ergothioneine	Antioxidant	OCTN1	(Koepsell et al., 2004; Nies et al., 2011)
Histamine	Neurotransmitter	OCT3	(Koepsell et al., 2004; Nies et al., 2011)
L-Carnitine	Metabolite	OCTN1, OCTN2	(Koepsell et al., 2004)
Prostaglandin E ₂	Hormone	OCT1, OCT2	(Koepsell et al., 2004; Nies et al., 2011)
Prostaglandin F _{2α}	Hormone	OCT1, OCT2	(Koepsell et al., 2004; Nies et al., 2011)
Serotonine	Neurotransmitter	OCT2, MATE1, PMAT	(Duan and Wang, 2010; Elwi et al., 2006; Koepsell et al., 2004; Nies et al., 2011)

Monoamine neurotransmitter transporters (*SLC6* family), in particular the sodium- and chloride-dependent neutral and basic amino acid transporter B(0,+)*(SLC6A14)*, have been suggested to transport essential cationic amino acids across epithelial cells of the trachea to bronchioles and in alveolar type I cells. Thus, by removing amino acids from the airway lumen, the transporter contributes to protein clearance and, by maintaining a low nutrient environment, plays a role in lung defence (Sloan et al., 1999; Uchiyama et al., 2008). Furthermore, CAT and system L transporters of the *SLC7* family are involved in the translocation of cationic amino acids and regulation of NO synthesis (Rotoli et al., 2005). Other examples of endogenous organic cations, their physiological function in the lung and their confirmed transporters are summarised in Table 5.

1.4.3.2 TRANSPORTER INTERACTIONS WITH XENOBIOTICS

1.4.3.2.1 Substance class of interest: β_2 -receptor agonists

Short- and long-acting β_2 -adrenoceptor agonists (SABA and LABA, respectively) as well as inhaled corticosteroids are the cornerstones of asthma and COPD treatment due to their bronchodilating and bronchoprotective effects (van der Woude et al., 2001).

Salbutamol, fenoterol and terbutaline are representatives of SABAs commonly used as rescue medication to relieve acute symptoms. LABAs such as formoterol and salmeterol are used in single or combination therapy with corticosteroids for long-term control of chronic asthma (Walthers et al., 2007). The target structures of these compounds are the β_2 -adrenoceptors which are widely distributed in the airway smooth muscle cells (30,000-40,000 per cell) (Johnson, 2001). It is of note that the density of β_2 -receptors is considerably lower in the proximal airspaces, when compared to the distal lung (Barnes et al., 1982). The physicochemical properties of β_2 -agonists determine how they interact with the β_2 -receptor. In Figure 5, the chemical structures of beta-agonists are shown.

Salbutamol ($\log P = -2.15$) is hydrophilic in nature and has a 3 - 4 fold lower affinity to the beta-receptor, whereas in comparison, formoterol ($\log P = 0.4$) and salmeterol ($\log P = 3.88$) have relatively high affinities (Johnson, 2001). Therefore, salbutamol possesses a short duration of action (4 - 6 h) in comparison to a sustained effect of salmeterol (about 12 h). However, the potency of beta-2-agonists is also determined by the efficacy of their binding to the receptor. Formoterol is a compound with a high binding efficacy, whereas salbutamol is of moderate and, interestingly, salmeterol is of low binding efficacy (Anderson et al., 1994). Experimental findings suggested a slow and non-competitive binding of the receptor by salmeterol due to the lipophilic chain (Johnson, 2001). Along with the pulmonary effect of bronchodilatation, the β_2 -agonists

lead to dilatation of blood vessels, relaxation of the uterine and increase of heart rate and tremor.

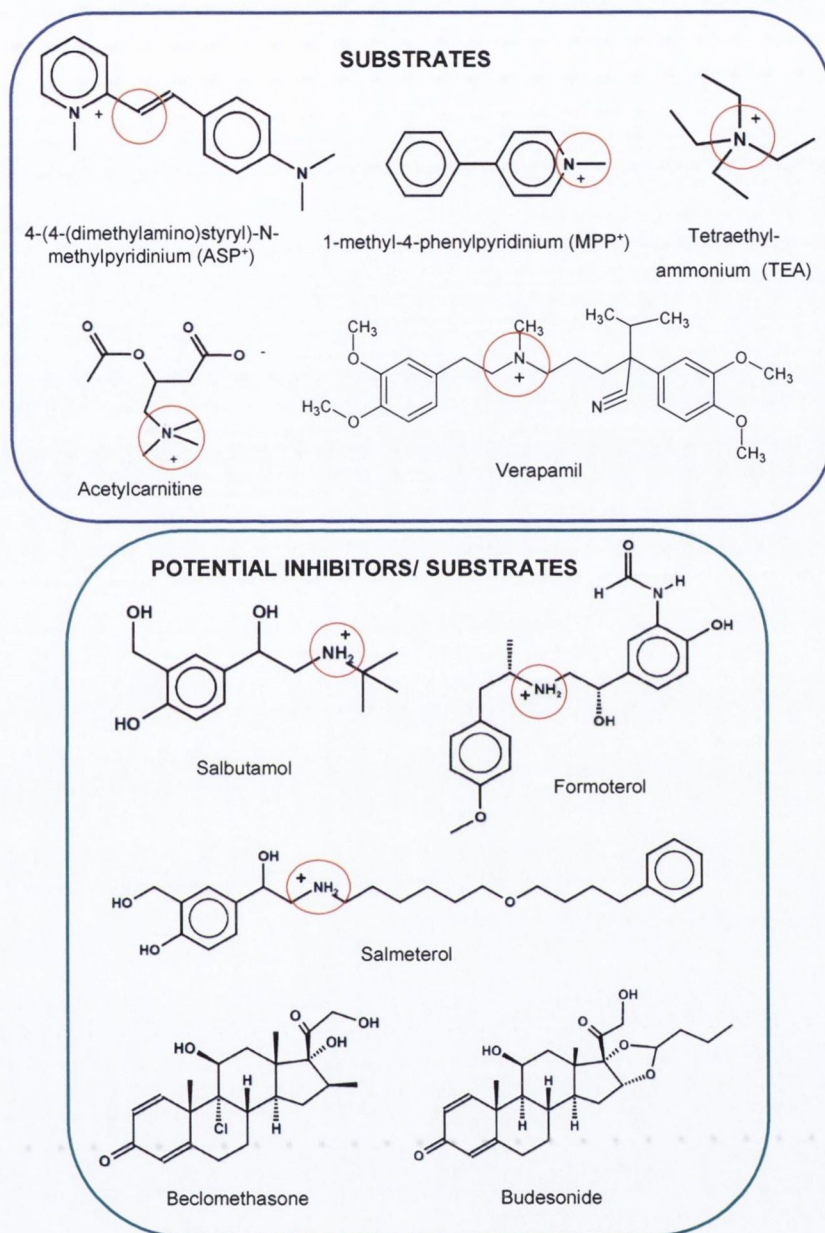


Figure 5. Chemical structures of substrates and inhibitors of organic cation/carnitine transporters. Substrates such as ASP⁺, MPP⁺, TEA, acetylcarnitine and verapamil have been widely used in previous studies to characterise functionality of OCT/Ns and P-gp in other organs (Koepsell et al., 2007). Beta-agonists (e.g., salbutamol, formoterol and salmeterol) carry a net positive charge and hence are thought to interact with OCT/Ns. Corticosteroids (e.g., beclomethasone and budesonide) are known OCT inhibitors (Horvath et al., 2007; Ehrhardt et al., 2005).

1.4.3.2.2 Drug-Transporter interactions

Approximately 40% of all drugs including β_2 -receptor agonists and anti-muscarinergics, are positively charged at physiologic pH values. Thus, absorption across the lung epithelial barrier often represents a limiting step for these drugs in reaching their pharmacological target sites, e.g., the smooth airway muscle cells, in the case of bronchodilators (see Figure 6).

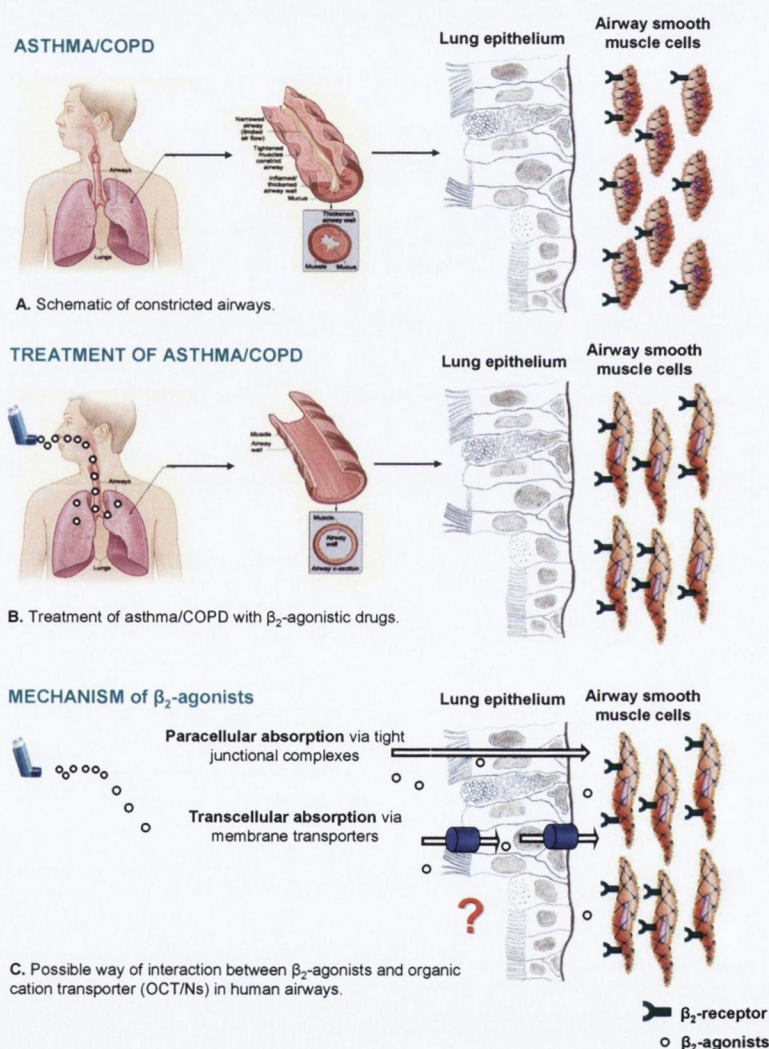


Figure 6. **A** In an asthmatic event, the airways are extremely constricted and the patient suffers from e.g., chest tightness and coughing. **B** After inhalation of, for example, beta-agonists, the airways relax and the patient overcomes the symptoms. **C** The molecular mechanism of how beta-agonists such as formoterol, salbutamol and salmeterol cross the lung epithelium (actively or via passive diffusion) in order to reach the airway smooth muscle cells where beta-2-receptors are located is a topic of great debate.

This led recently to the hypothesis that transporters associated with organic cation translocation in other organs, e.g., OCTs and OCTNs (*SLC22A1-A5*) might also be involved in absorption and clearance processes in the lung (Ehrhardt et al., 2005, Horvath et al., 2007). The chemical structures of model cations used in this thesis and pharmacological substrates such as beta-agonists and corticosteroids are shown in Figure 5.

The existence of carrier-mediated organic cation transport was first observed in 1999 by Shen *et al.* in cultured rabbit alveolar epithelial cells (Shen et al., 1999). In 2005, our group was the first to hypothesise that active transport mechanisms are involved in the translocation of an inhaled medicine [i.e., salbutamol (albuterol in the US)], after a significant net absorption of the drug was observed across monolayers of bronchial epithelial cells *in vitro* (Ehrhardt et al., 2005). Although, we postulated an involvement of an OCT-type transporter and confirmed the presence of OCT1-3 and OCTN1/2 by RT-PCR, direct evidence to support the hypothesis was still outstanding. Horvath and colleagues subsequently proposed OCTN1 and -N2 as the responsible transporters for the uptake of β_2 -agonists into human airway epithelial cells. Whilst they convincingly showed that uptake of the cationic fluorophore, 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) can be inhibited by formoterol and salbutamol, their report failed to provide direct evidence of OCT/N-mediated transport of the β_2 -agonists (Horvath et al., 2007). Intriguingly, in the same study, salmeterol did not have an effect, although the drug was used at concentrations significantly exceeding its solubility in the uptake medium. ASP^+ was also used in another current report by Mukherjee *et al.*, who evaluated Calu-3 cell monolayers for investigation of inhaled drug interactions with organic cation transporters *in vitro* (Mukherjee et al., 2012). They found that ASP^+ uptake was decreased in the presence of TEA, L-carnitine, L-ergothioneine and

decynium-22. Regrettably, no kinetic analyses were shown in that paper. In addition, transport of formoterol across the bronchial epithelium was investigated and found to be increased in the presence of organic cations. Cells from normal and polyps nasal tissues were used in a study by Agu *et al.* (2011) investigating the expression and functionality of OCT/Ns in the upper respiratory tract (Agu *et al.*, 2011). They observed high mRNA expression levels of OCT3 and OCTNs. Again, ASP^+ was used as an OCT/N probe and an active absorption process of the compound was confirmed. Intriguingly, the chosen inhibitors did not show significant attenuation of ASP^+ uptake. Another recently published study by Umwallah *et al.* investigated the cellular uptake and the permeability of salbutamol across human bronchial epithelial cells grown under air-interface culture (AIC) conditions. The uptake of salbutamol was found to be saturable and only inhibited by lysine and histidine (Umwallah *et al.*, 2012). Interestingly, the transepithelial flux of salbutamol was not saturable, decreased under acidic conditions and increased when tight junctions were disrupted. This suggests a paracellular pathway as the main contributor to the translocation of salbutamol across the lung mucosa. Interactions of β_2 -agonists with transporter systems of the respiratory epithelial barrier have been studied for several years now. Indirect evidence conclusively shows the inhibitory potential of β_2 -agonists on organic cation uptake, and a number of transporters have been proposed to be involved. Without the use of RNAi technique or relevant knock-out animals, however, no ultimate conclusions can be drawn. Nakamura *et al.* investigated the transport of ipratropium bromide in BEAS-2B human bronchial epithelial cells (Nakamura *et al.*, 2010). Whilst evidence was presented that ipratropium bromide uptake into OCTN1 and OCTN2 expressing HEK transfectants was saturable and inhibitable, the responsible uptake system in the organotypic *in vitro* model could not be identified. A possible contribution of OCTs was discussed, but not

further investigated. Table 6 summarises uptake and transport data which has been obtained using *in vitro* models of human respiratory epithelial barrier and organic cation transporter substrates.

Table 6. Overview of xenobiotic uptake and transport studies using human respiratory epithelial cells *in vitro*.

Cell model	Substrate	Transporter	References
Calu-3	ASP ⁺	OCT/Ns, OCTs	(Chapter 3, Mukherjee et al., 2012)
16HBE14o-	Salbutamol, TEA ASP ⁺	OCT/Ns OCTs	(Ehrhardt et al., 2005) Chapter 3
BEAS-2B	Ipratropium	OCTN2	(Nakamura et al., 2010)
H441	ASP ⁺	OCT/Ns	Chapter 4
A549	Choline ASP ⁺	Mainly CTL1, OCT3, OCTNs, CTL1, CTL2 OCTs	(Ishiguro et al., 2007; Wang et al., 2007) Chapter 3
Human airway epithelial cells	ASP ⁺	OCTN2	(Horvath et al., 2007)

In terms of the *in vivo* relevance of organic cation transporters in pulmonary drug disposition even less is known. In 2005, Kummer *et al.*, following on from a study by Lips and co-workers, demonstrated a high acetylcholine accumulation in the bronchial epithelium in OCT1/2 double knock-out mice and proposed that OCT1 and OCT2 play essential roles in the release of acetylcholine into the airway lumen (Kummer et al.; 2006). A very recent study by Gnadt *et al.* (2012) used the isolated human lung reperfusion model to study a possible involvement of drug transporters on pharmacokinetics of salbutamol and a novel long-acting β_2 -agonist, GW597901. It is of note that the study found a delayed pulmonary absorption of β_2 -agonists after administration of methacholine and carnitine (Gnadt et al., 2012). Thus, an involvement

of the organic carnitine transporter 2 was concluded. Although the study has a number of limitations, e.g., high variability of transporter tissue levels and extent of delaying effect, it was a first step to identify the contribution of transport systems to the distribution of an aerosolised drug in the lung in an *ex vivo* model. The contribution of active transport to the pulmonary absorption of the OCT/OCTN substrates, L-carnitine and the anti-muscarinergic drug, ipratropium bromide, was also investigated in an isolated perfused rat lung (IPRL). Whilst *in vitro* studies showed significant impact of OCT/OCTN transporter activity upon the cellular accumulation of both molecules, with preferential uptake of ipratropium by OCTs, and of L-carnitine uptake by OCTNs, OCT/OCTN inhibition did not alter the pulmonary absorption of these molecules in the IPRL (Mark Gumbleton – personal communication).

Table 7 gives an overview of inhaled drugs and their confirmed interactions with organic cation transporter proteins. At the moment, the contribution of active transport processes in the lung to the pulmonary absorption and systemic pharmacokinetics of organic cations remains an issue of debate.

Table 7. Current and future drugs for pulmonary administration and their confirmed or potential interaction with organic cation transporters.

Drug substance	Class	Action on organic cation transporter	References
Formoterol	β ₂ -agonist	Inhibitor, substrate (?)	(Chapter 3 and 6, Horvath et al., 2007; Mukherjee et al., 2012)
Salbutamol	β ₂ -agonist	Inhibitor, substrate (?)	(Chapter 3 and 6, Horvath et al., 2007; Ehrhardt et al., 2005)
Salmeterol	β ₂ -agonist	Inhibitor (controversial)	(Chapter 3 and 6; Horvath et al., 2007)
Ipratropium	anticholinergic	Inhibitor and substrate	(Nakamura et al., 2010)
Tiotropium	anticholinergic	Inhibitor and substrate	(Nakamura et al., 2010)
Budesonide	corticosteroid	Inhibitor	Chapter 3
Beclomethasone	corticosteroid	Inhibitor	Chapter 3

1.4.3.2.3 Nanoparticle-Transporter interactions

Nanotechnology has expanded rapidly during the last decade and begins to revolutionise the scientific landscape. The use of nanoparticles, nanometre ranged drug delivery systems, offers opportunities across all areas of medicine. Significant new advances in cancer diagnostics, imaging and therapy have been developed based on the nanomedicine approach (Bhaskar et al., 2010). To improve the biodistribution of (anti-cancer) drugs, nanoparticles have been designed for optimal size and surface characteristics to increase their circulation time in the bloodstream. In addition, active targeting strategies using surface-bound ligands directed against selected cellular targets amplify the specificity of these therapeutic nanoparticles (Cho et al., 2008). McCarthy et al. identified an activation of ion channels in airway cells after exposure to polystyrene-based nanomaterials and concluded that polystyrene nanoparticles cannot be only considered as a neutral vehicle for drug delivery for the treatment of lung diseases, due to the fact that they may have the ability to affect epithelial cell function and physiological processes on their own (McCarthy et al., 2011). It has also been suggested that nanoparticles have the ability to accumulate in cells without being recognised by P-glycoprotein, one of the main mediators of multidrug resistance, resulting in the increased intracellular concentration of drugs (Lee et al., 2005).

1.5 AIMS OF THE THESIS

A need for greater understanding of membrane transporters expression and their functional characterisation in the lung epithelium led us to the studies discussed in this thesis.

The specific aims of this work were:

- I To investigate the expression levels of organic cation/carnitine transporters (*SLC22A1-5*) in different *in vitro* models of the lung epithelial barrier using alveolar (A549), bronchiolar (H441) and bronchial (Calu-3, 16HBE14o-) epithelial cell lines, and to compare these data with freshly isolated human alveolar epithelial cells in primary culture (hAEpC). Furthermore, to determine protein expression of other members of the *SLC* super family such as *SLCO* and *SLC47A1* (Chapter 2 and 4).
- II To characterise the translocation processes of organic cations into and across respiratory epithelial cells using a fluorescently-labelled cationic compound (ASP^+) as well as radiolabelled substrates, e.g., the model cation [^{14}C]-TEA and the OCTN probe [^3H]-acetylcarnitine, in order to specifically determine cationic pathways in the lung (Chapter 3 and 5).
- III To establish the bronchiolar epithelial cell line, H441 as an *in vitro* model for biopharmaceutical studies of the mucosa of the distal lung (Chapter 4).

- IV To study the susceptibility of organic cation pathways to the presence of β_2 -adrenergic drugs (e.g., salbutamol, formoterol and salmeterol) and to explore the uptake and transport of [^3H]-salbutamol across the lung epithelium (Chapter 6).
 - V To elucidate a transporter for the organic cation verapamil in the human respiratory mucosa (Chapter 7).
 - VI To assess the suitability of new analogues (isomeric 3-desoxy 3-NBD-amino derivatives of UDCA and DCA) to investigate the distribution of bile acids into intestinal epithelial Caco-2 cells in comparison to human alveolar epithelial A549 cells (Chapter 8).
 - VII To investigate to what extent nanoparticulates affect the functionality of transport systems, in particular P-gp (Chapter 9).
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CHAPTER 2

Expression of drug transporter proteins in human respiratory epithelial cells

Parts of this chapter have been published in:

Salomon JJ, Endter S, Tachon G, Falson F, Buckley ST, Ehrhardt C. (2012) Transport of the fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺) in human respiratory epithelial cells. *Eur J Pharm Biopharm* 81(2): 351-

ABSTRACT

The spatial expression of drug transporters in the lung epithelium remains mainly uncharted terrain, albeit their potential importance with regard to the disposition of inhaled drugs. Here, we studied the expression of transport proteins of the SLC super family, in particular, the polyspecific organic cation transporters (OCT/N, *SLC22A1-5*), multidrug and toxin extrusion transporter (MATE1/*SLC47A1*) and the organic anion transporting polypeptides (OATP/*SLCO*), being involved in the uptake and release of a variety of cationic and anionic endogenous substances and xenobiotics.

The expression of transporter proteins was studied by immunoblotting and by confocal laser scanning microscopy (CSLM) in human bronchial (16HBE14o-, Calu-3), bronchiolar (H441) and alveolar (A549) epithelial cell lines as well as in human alveolar epithelial cells in primary culture. Intestinal Caco-2 cells were used as a control due to their well published nature. Protein abundance was comparable for all OCT/Ns in the respiratory epithelial cell lines studied; only OCT2 was exclusively observed in A549 cells. OCT1 and -2 were present at significantly higher levels in Caco-2 cells, compared with the respiratory epithelial cell types; OCTN1 and -2 were also more abundant in Caco-2; only OCT3 was expressed evenly across all cell lines investigated. Presence of MATE1 was confirmed in A549, H441 and Calu-3 cells by Western blot analysis and CLSM. The protein expression pattern of OATPs revealed high expression levels of OATP2A1, OATP3A1 and OATP4A1 in alveolar (primary cell culture and A549) cells, whereas low levels of transport proteins were detected in 16HBE14o- and Calu-3 cells. OATP1B3 was only weakly expressed in all investigated cell types. CLSM mostly confirmed data obtained by Western blot analysis. Pulmonary epithelial cells express several SLC and SLCO transporters. These transporters are likely to have an effect on the disposition of pulmonary delivered cationic and anionic xenobiotics.

2.1 INTRODUCTION

In recent years, the molecular and functional identification of transporters and their substrates have increasingly been recognised. Drug transporters are expressed in many tissues such as the intestine, liver, kidney, and brain, and play key roles in drug absorption, distribution, and excretion. The functional characterisation of drug transporters hence provides important information that allows improvement of drug delivery and drug design. Nevertheless, many clinical implications of these new discoveries have not yet been fully established. The lung is increasingly been recognised as an organ of (systemic) drug absorption, however, our knowledge about the distribution and functionality of pulmonary drug transporters is rather poor (Bosquillon, 2010; Gumbleton et al., 2011).

Investigations on transporter expression and activity in respiratory epithelial cells are emerging, but the picture is still far from complete. We are starting to understand better some of the more prominent drug transporters in the lung, e.g., P-glycoprotein and BCRP, belonging to the ABC super family, whereas most of the more than 400 known molecules remain uninvestigated. The SLC transporter family includes many transporter proteins that are responsible for the transport of cationic and anionic endogenous and exogenous compounds across various epithelia (Koepsell, 2004). The electrogenic cation transporters OCT1-3 (*SLC22A1-3*), carnitine transporters OCTN1 and OCTN2 (*SLC22A4-5*) and proton/cation antiporters of the MATE family (*SLC47A1-2*) have overlapping substrate and inhibitor affinities (Nies et al., 2011; Ciarimboli, 2010, Kouji et al., 2009). Moreover, MATE1 has only been recently identified as a proton/cation antiporter efflux pump (Otsuka et al., 2005) and only little information is available about the molecule. A recently published review underlines the existing gaps in cation transporter research in the lung (Salomon and Ehrhardt, 2012).

Another interesting transporter group are the organic anion transporting peptides (OATP). Although over 40 different OATP family members have been identified in the kidney and liver, only little is known about their expression and localisation in the lung (Roth et al., 2011; Bosquillon, 2009). Several members of the OATP family, e.g., OATP2A1, OATP3A1 and OATP4A1 have been confirmed by gene array analysis and mRNA expression to be highly expressed in the lung (Bleasby et al.; 2006; Endter et al., 2009; Seki et al., 2009).

Hence, it was one of the aims of this chapter to investigate the expression levels of the five cloned human OCT/N members in alveolar epithelial A549 and bronchial epithelial 16HBE14o- and Calu-3 cell lines; all three cell lines are well established *in vitro* models used in biopharmaceutical sciences (Sporty et al., 2008; Forbes and Ehrhardt, 2005). Additionally, we sought to determine, whether MATE1 and four selected members of the OATP family (OATP2A1, OATP3A1, OATP4A1 and OATP1B3) were expressed as proteins by means of Western blotting and CLSM. Expression studies were also carried out in freshly isolated human alveolar epithelial cells in primary culture (ATII and ATI-like cells) and in the human bronchiolar epithelial cell line, H441. Of note, H441 epithelial cells are a bronchiolar cell culture model which is still rather unexplored in terms of transporter expression and functionality. Chapter 4 will particularly focus on this topic, and furthermore expression of OCTs will be compared to expression in human alveolar epithelial cells in primary culture. Hence, only limited expression data is shown in the current chapter.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

A549 (American Type Culture Collection CL-185; LGC Standards, Teddington Middlesex, UK) cells are derived from a human pulmonary adenocarcinoma (Giard et al., 1973). Due to phenotype similarity to type II alveolar epithelial cells this cell line has been widely utilised in studies of alveolar epithelium function (Sporty et al., 2008). A549 cells (passage numbers 65 to 84) were seeded at a density of 40,000 cells/cm². Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F-12, Sigma-Aldrich, Dublin, Ireland) supplemented with 5% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. NCI-H441 (American Type Culture Collection, HTB-174) cells were obtained from LGC Standards and cultured at a seeding density of 75,000 cells/cm². Cells were maintained at 37°C in 5% CO₂ atmosphere in Gibco RPMI-1640 medium (Biosciences, Dun Laoghaire, Ireland) supplemented with 5% FBS, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich). 16HBE14o- cells were a gift from Dr. Dieter C. Gruenert (University of California, San Francisco, CA, USA) and were used from passage 2.57 to 2.61. Calu-3 cells (passage numbers 42 to 51) were purchased from ATCC (Manassas, VA, USA). Seeding density of 16HBE14o- cells was 100,000 cells/cm² and for Calu-3 cells 75,000 cells/cm², respectively. Cell lines were maintained in minimum essential medium (MEM, Sigma-Aldrich, Dublin, Ireland) supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1% non essential amino acids, 1% sodium pyruvate and 0.5% glucose.

The human colon carcinoma Caco-2 cell line was also purchased from ATCC (Manassas, VA, USA). In this case, cells (passage numbers 23 to 41) were seeded at a

density of 66,000 cells/cm² in DMEM supplemented with 20% FBS, 1% penicillin and streptomycin, 1% non-essential amino acids and 1% sodium pyruvate. All cell types were cultured at 37°C in 5% CO₂ atmosphere and the culture medium was exchanged every 48 h. Cells were grown to confluent monolayers for 5 (A549), 8 (16HBE14o-, H441), 12 (Calu-3) and 21 (Caco-2) days on 6-well plates (Greiner BioOne, Frickenhausen, Germany) or Lab-Tek chamber slides (Nunc, Roskilde, Denmark).

2.2.2 Human alveolar epithelial cell isolation and culture

Human alveolar type II (ATII) epithelial cells were freshly isolated from non-tumour lung tissue obtained from patients undergoing lung resection. The use of human material was approved by Saarland State Medical Board, Germany. Isolation of ATII was performed according to a protocol modified from Demling *et al.* (2006). Purified ATII cells were seeded at a density of 600,000 cells/cm² on collagen (Sigma-Aldrich, Dublin, Ireland)/fibronectin (BD Biosciences, Oxford, UK)-coated plastics using SAGM supplemented with penicillin, streptomycin and 1% FBS (Lonza BioResearch, Dublin, Ireland). ATII cells were either used after 24 h of culture or after transdifferentiation into monolayers of type I-like (ATI-like) phenotype, following 8 days of culture.

2.2.3 Commercially available antibodies used in the experiments

Antibodies used in the studies are listed in Table 1. It is of note that in the current chapter expression of OCT1, OCT2 and OCT3 was detected using antibodies from Sigma-Aldrich in Western blot and from Santa Cruz in immunostaining experiments, respectively.

Table 1. Properties of anti-SLC and anti-SLCO antibodies used in studies.

protein	company	product name/ catalog no.	isotype	epitope	concentration used (WB/CLSM)	application (s)
OCT1	Santa Cruz Biotechnology	sc-19809	goat IgG	N-terminus (N-12)	1:200/ 1:100	CLSM
OCT2		sc-19814	goat IgG	C-terminus (C-13)	1:200/ 1:100	CLSM
OCT3		sc-19815	goat IgG	N-terminus (N-12)	1:200/ 1:100	CLSM
OCTN1		sc-19819	goat IgG	C-terminus (C-13)	1:200/ 1:100	WB/CLSM
OCTN2		sc-19822	goat IgG	C-terminus (H-13)	1:200/ 1:100	WB/CLSM
OCT1	Sigma- Aldrich	AV41516	rabbit IgG	extracellular loop	1:500/1:250	WB
OCT2		AV43847	rabbit IgG	N-terminus	1:500/1:250	WB
OCT3		AV44026	rabbit IgG	extracellular loop	1:2000/1:1000	WB
OCT1	Aviva Systems Biology	ARP41640_T100	rabbit IgG	C-terminal region	1:200/ 1:50	WB/CLSM
OCT2		ARP4384_T100	rabbit IgG	N-terminal region	1:200/ 1:50	WB/CLSM
OCTN1		OAAB03385	rabbit IgG	C-terminus	1:200/ 1:50	WB/CLSM
OCTN2		OAAB05449	rabbit IgG	Center region	1:500/ 1:50	WB/CLSM
MATE1		OAAB02770	rabbit IgG	C-terminus	1:500/ 1:50	WB/CLSM
OATP2A1	Santa Cruz Biotechnology	sc-103086	goat IgG	N-terminus	1:200/ 1:100	WB/CLSM
OATP3A1		sc-66566	goat IgG	C-terminus	1:200/ 1:100	WB/CLSM
OATP4A1		sc-51169	goat IgG	N-terminus	1:200/ 1:100	WB/CLSM
OATP1B3		sc-47273	goat IgG	C-terminus	1:200/ 1:100	WB/CLSM

For OCTN1 and OCTN2 detection, antibodies of Santa Cruz have been used. In the following chapters of this Thesis, experiments were performed using antibodies obtained from Aviva Systems Biology. A protein sequencing of a selection of antibodies was furthermore performed (see Appendix III).

2.2.4 Western blotting

These experiments were partially carried out by Simona Etzbach (Saarland University, Germany) and Julia Gausterer (Universität Wien, Austria).

A549, H441, 16HBE14o-, Calu-3 and Caco-2 cell monolayers were grown as described above. To obtain cell lysates, cells were washed with ice-cold phosphate saline buffer (PBS) twice and scraped off in 300 µl freshly prepared lysis buffer, containing aprotinin (0.06 mg/ml) and leupeptin (0.01 mg/ml). Cell samples were sonicated twice for 10 s using a Microson Ultrasonic Cell Disruptor (Misonix) and centrifuged at 10,000 rpm for 20 min at 4°C. The total protein amount was determined by Bio-Rad protein assay (Hercules, USA) with bovine serum albumin (BSA) as a standard. Samples were standardised to equal protein concentrations, loading buffer was added and the mixture was heated up at 95°C for 5 min, before sample were loaded onto SDS gels.

Polyacrylamide gel electrophoresis was performed at 120 V, followed by transfer onto immunoblot polyvinylidene fluoride membranes (Bio-Rad, Hemel Hempstead, UK) at 25 V for 30 min. Membranes were blocked in 5% (w/v) BSA in Tris-buffered saline with Tween 20 (pH 7.4) for at least one hour at room temperature. Incubation with the desired primary antibody was carried out overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody (1:12,500 [anti-rabbit] and 1:22,500 [anti-goat], respectively) at room temperature for 1 h.

Horseradish peroxidase-conjugated anti-rabbit and anti-goat antibodies were purchased from Promega (Medical Supply Company, Dublin, Ireland) or Sigma-Aldrich (Dublin, Ireland), respectively. Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). Relative levels of protein abundance were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system (Bio-Rad). When appropriate, blots were stripped and analysed for β -actin (Sigma-Aldrich) as internal control.

2.2.5 Immunohistochemistry

These experiments were partially carried out by Simona Etzbach (Saarland University, Germany) and Julia Gausterer (Universität Wien, Austria).

Cells were grown on Lab-Tek chamber slides before being fixed for 10 min with 4% (w/v) paraformaldehyde and incubated for 10 min in 50 mM NH_4Cl , followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100 in PBS. The relevant primary antibodies dissolved in PBS containing 1% (w/v) BSA were incubated for 60 min at 37°C. The cell layers were then washed three times with PBS, before indirect labelling was performed by using a 1:300 dilution of the corresponding Alexa Fluor-labelled F(ab')_2 fragment in PBS containing 1% (w/v) BSA. Propidium iodide (1 $\mu\text{g/ml}$ in PBS) was used to counterstain cell nuclei. Alexa Fluor-conjugated secondary antibodies were obtained from Biosciences, Dun Laoghaire, Ireland. After 30 min of incubation, the specimens were again rinsed three times with PBS and embedded in FluorSave anti-fade medium (Merck, Nottingham, UK). Images were obtained using a confocal laser scanning microscope (CLSM, Zeiss LSM 510, Göttingen, Germany) with the instrument's settings adjusted so that no positive signal was observed in the channel corresponding to the fluorescence of the isotypic controls.

2.3 RESULTS

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

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

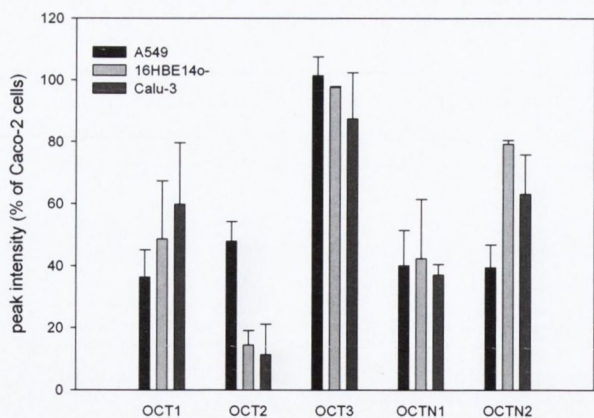


Figure 1. Densitometric analysis of organic cation transporter abundance. OCT/N expression levels have been corrected for beta-actin. Protein bands are shown as mean peak intensities relative to the amount found in Caco-2 cells $\pm$ SD, $n = 3$.

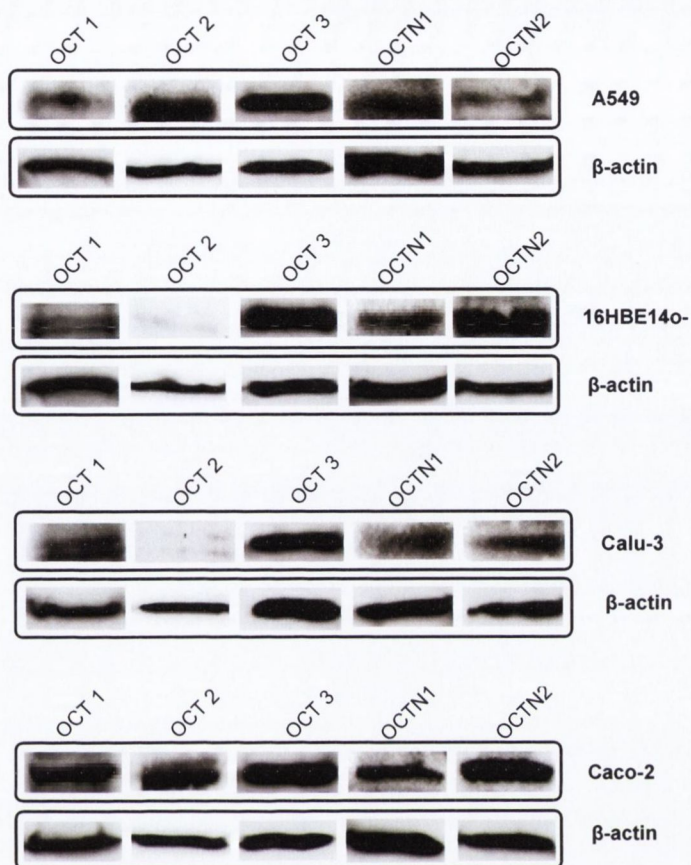


Figure 2. Protein abundance of OCT1 (66 kDa), OCT2 (65 kDa), OCT3 (70 kDa), OCTN1 (65 kDa) and OCTN2 (70 kDa) in human respiratory epithelial cells. Representative Western blots of cell lysates isolated from A549 alveolar epithelial cells, 16HBE14o- and Calu-3 bronchial epithelial cells and intestinal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

Confocal laser scanning microscopy was carried out to localise OCT/N protein signals within the cells (Figures 3 and 4). However, none of the antibodies used allowed us to reliably determine the spatial localisation of transporter proteins, even though antibodies were purchased from different companies and targeted various epitopes on the antigen (Table 1).

CLSM showed strong signals for OCT1 and OCT3 in all cell lines investigated (Figure 3; left and right panel, respectively), except for Calu-3, where OCT3-immunoreactivity exhibited lower signals. OCT1 might be localised to the cell membranes in 16HBE14o-, Calu-3 and Caco-2 cells, whilst OCT3 was found more in the cytosol. In A549 cells, both OCT1 and OCT3 proteins were localised to vesicular structures in the cytosol. OCT2 was absent in Calu-3 and 16HBE14o- cells, while weak staining was observed in Caco-2 cells (Figure 3; middle panel). OCTN1 and OCTN2 immunolabels were strong in A549 cells and recognisable in 16HBE14o- cell layers (Figure 3 and 4). For Caco-2 monolayers no positive signals were obtained for organic carnitine transporters (Figure 4). In the case of Calu-3 cells, weak signals were detected for OCTN1, whereas strong immunoreactivity was observed for OCTN2.

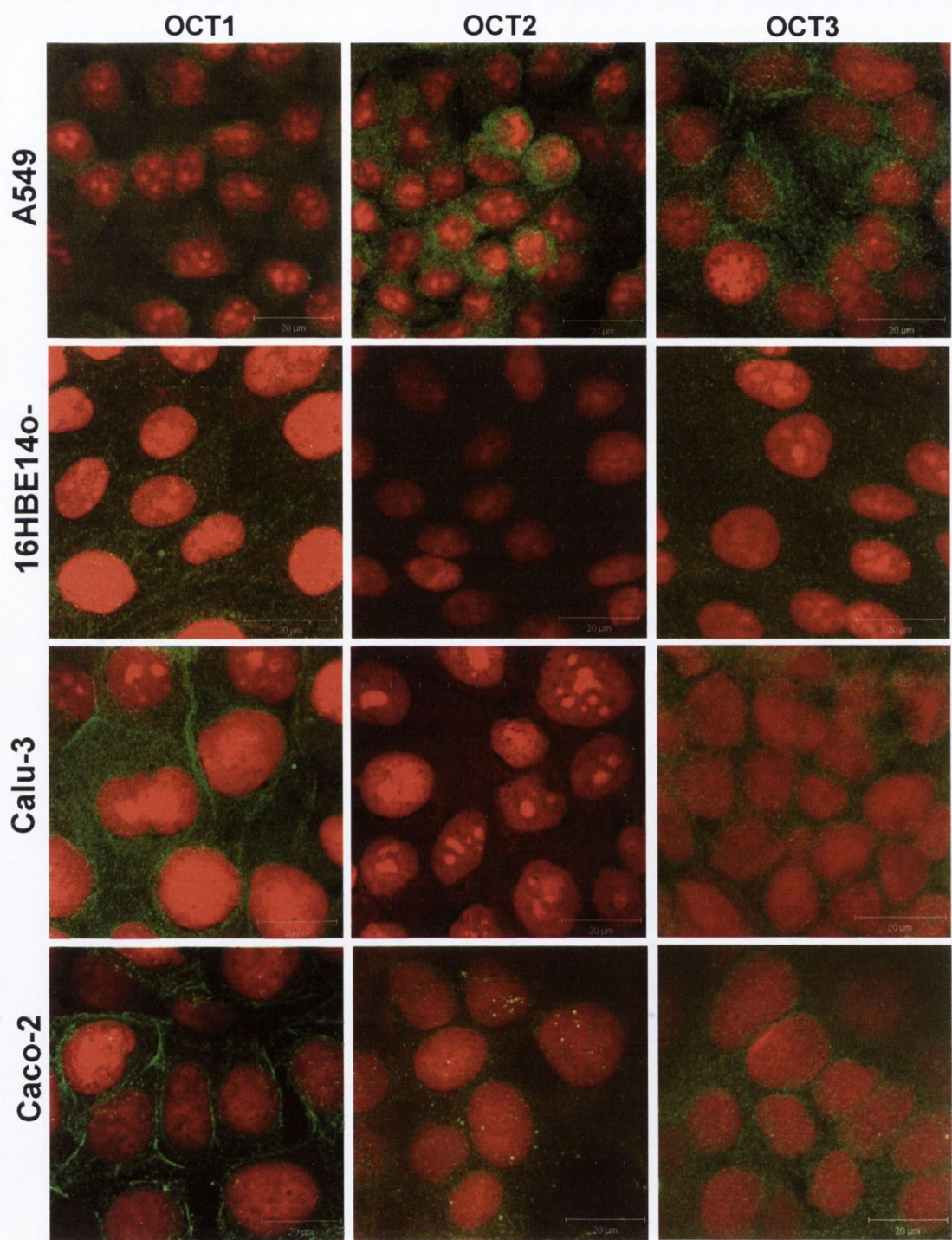


Figure 3. Immunostaining of organic cation transporters, OCT1, OCT2 and OCT3 (all *green*) in human respiratory epithelial cells and Caco-2 monolayers. A549, 16HBE14o-, Calu-3 and Caco-2 monolayers were grown on chamber slides as indicated. Nuclei were counterstained with propidium iodide (*red*). Bars represent 20 µm.

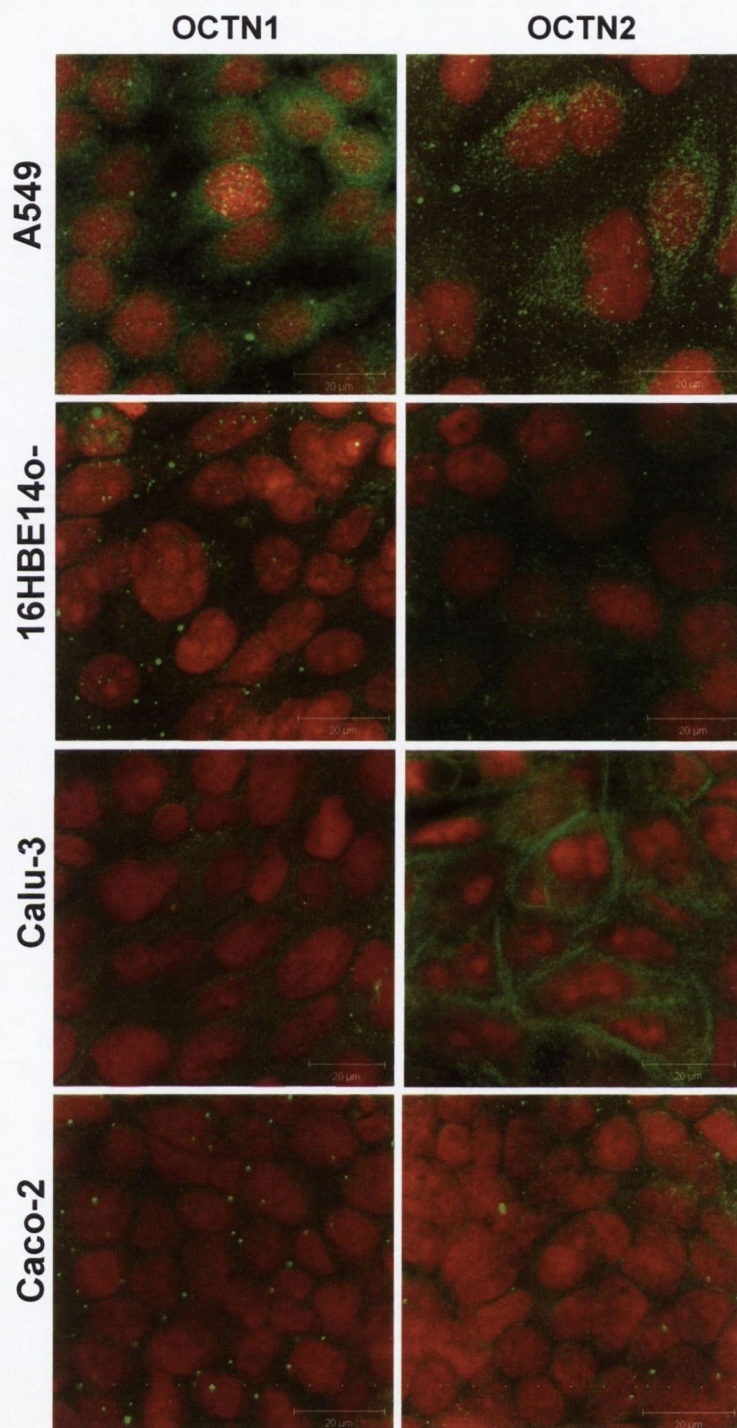


Figure 4. Immunohistochemistry of novel organic cation transporters, OCTN1 and OCTN2 (both *green*) in human respiratory epithelial cells and Caco-2 monolayers. A549, 16HBE14o-, Calu-3 and Caco-2 monolayers were grown on chamber slides as indicated. Nuclei were counterstained with propidium iodide (*red*). Bars represent 20 μm.

2.3.2 Expression of MATE1 in human respiratory epithelial cells

Immunoblot confirmed the expression of MATE1 in A549, H441 and Calu-3 cell lines and densitometric analysis revealed similar abundance in the different cell types (Figure 5A and 5B). Furthermore, the expression of the transporter protein was investigated by CLSM (Figure 5C). CLSM data were consistent with what was found in Western blot, confirming high expression of MATE1 in all three respiratory epithelial cell lines. As shown in Figure 5C, the transporter protein was localised to the cytosol in A549 alveolar epithelial cells, whilst a more membranous localisation was apparent in the case of H441 and Calu-3 cells.

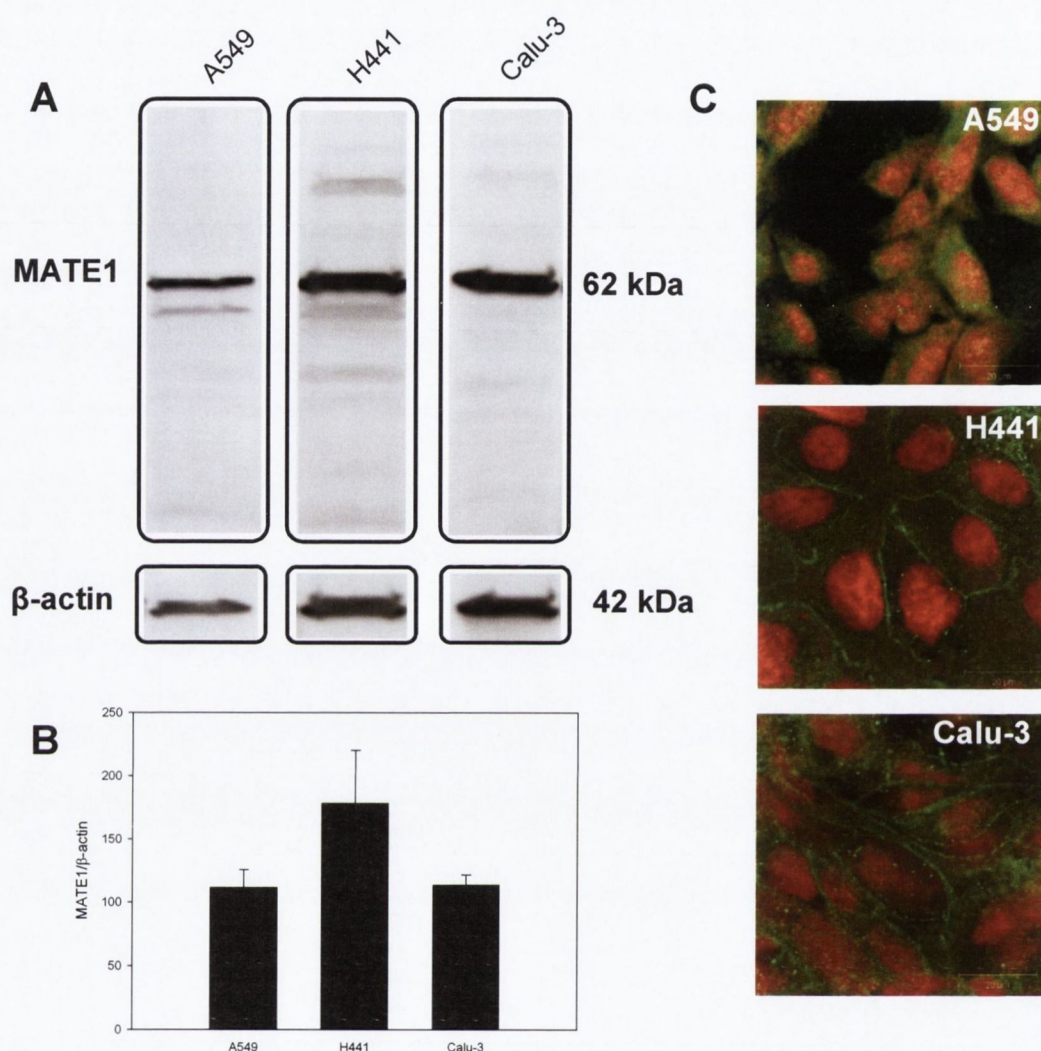


Figure 5. Expression of MATE1 was investigated on protein level in human respiratory epithelial cells. Representative immunoblot for MATE1 in A549, H441 and Calu-3 epithelial cell lines (A). Expression levels of MATE1 were evaluated by densitometric analysis and standardised by comparison to beta-actin (B). Each bar represents means $\pm$ SD from at least 3 independent experiments. Furthermore, immunostaining of MATE1 (green) in human alveolar epithelial cells (A549), bronchiolar (H441) and bronchial (Calu-3) cell line is shown (C). Nuclei were counterstained with propidium iodide (red). Bars represent 20 μ m.

2.3.3 Expression of OATPs in the respiratory epithelium

We aimed to investigate the expression pattern of four members of the OATP super family in human respiratory epithelial cell lines in comparison to the intestinal cell line, Caco-2. Immunoblotting confirmed the expression of OATP2A1 in all cell lines, however, lower levels were found in the two bronchial epithelial cell types, 16HBE14o- and Calu-3 (Figure 6A). In line with Western blot data, high levels of immunoreactivity were obtained for OATP2A1 in all studied cell lines (see Figure 6B). Interestingly, the transporter protein was localised perinuclearly in human alveolar epithelial primary cells (ATII and ATI-like), whereas the signal was detected in the cytosol in the remaining cell lines.

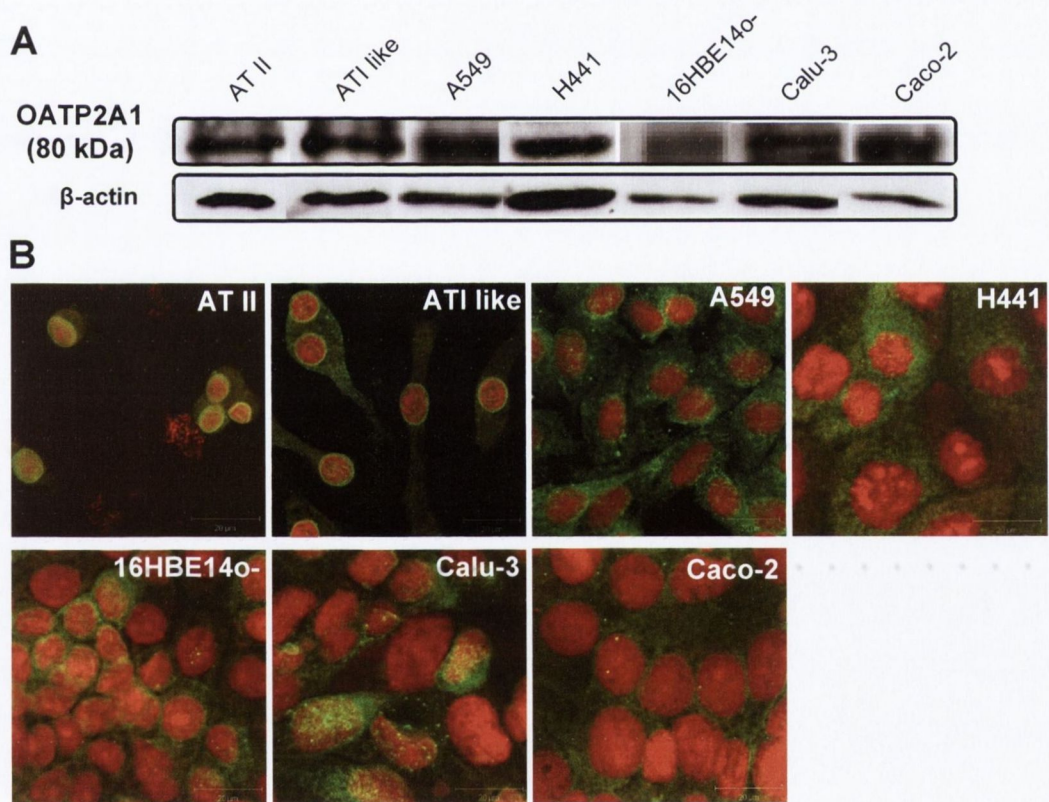


Figure 6. Representative immunoblot for OATP2A1 in respiratory and intestinal epithelial cells *in vitro* (A). CLSM of OATP2A1 transporter (green) in human alveolar epithelial cells in primary cell culture (ATII and ATI-like) and continuously grown (A549), bronchiolar (H441), bronchial (16HBE14o-, Calu-3) and intestinal (Caco-2) cell lines (B). Nuclei were counterstained with propidium iodide (red). Bars represent 20 μ m.

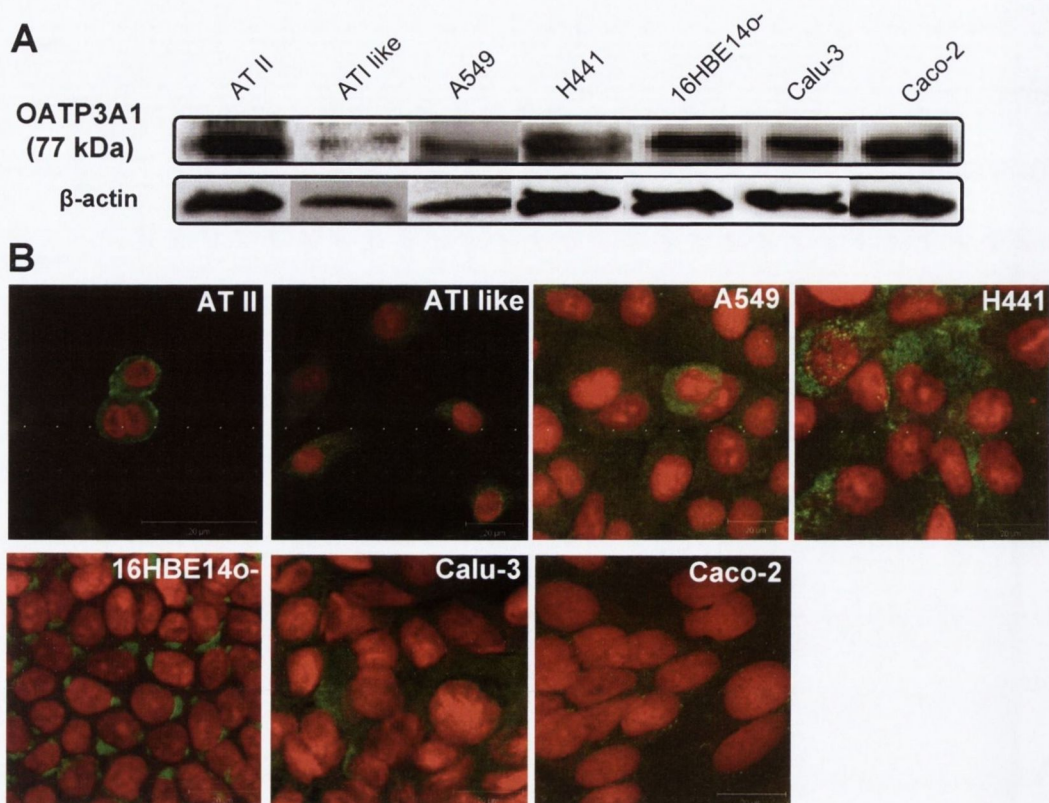


Figure 7. Expression of OATP3A1 in human respiratory epithelial cells in comparison to the intestinal Caco-2 cell line. Representative immunoblots for OATP3A1 in lung and intestinal epithelial cell types *in vitro* (A). CLSM of OATP3A1 transporter (green) in human alveolar epithelial cells in primary cell culture (ATII and ATI-like) and continuously grown (A549), bronchiolar (H441), bronchial (16HBE14o-, Calu-3) and intestinal (Caco-2) cell lines (B). Nuclei were counterstained with propidium iodide (red). Bars represent 20 μ m.

In the case of the OATP3A1, Western blotting revealed strong bands in all cell lines, with the exception of weak protein expression in primary alveolar epithelial cells (ATI like) and continuously grown A549 cells (Figure 7A). The expression of the protein was also investigated by CLSM (Figure 7B). Generally, CLSM data were consistent with Western blot results, even though signals were overall weaker. A cytosolic localisation was found in A549 cells; in contrast, ATI-like cells presented the transporter protein in the apical membrane. An apically localised signal was also obtained in H441 and Calu-3 cells. Of note, the localisation of OATP3A1 in 16HBE14o- cells was significantly

different from the other epithelial cells, localised to the Golgi apparatus or the endoplasmatic reticulum.

Protein abundance of OATP4A1 was also investigated by means of Western blot and CLSM (Figure 8). Immunoblot analysis confirmed the presence of the transporter at low levels in all cell lines studied (Figure 8A). No band, however, was obtained in the case of ATII epithelial cells. Furthermore, CLSM studies confirmed these data and weak signals were obtained throughout with the exception of ATI-like cells, where the highest immunoreactivity was evident. OATP4A1 was localised to perimembranous domains along the apical aspect of the most cell types.

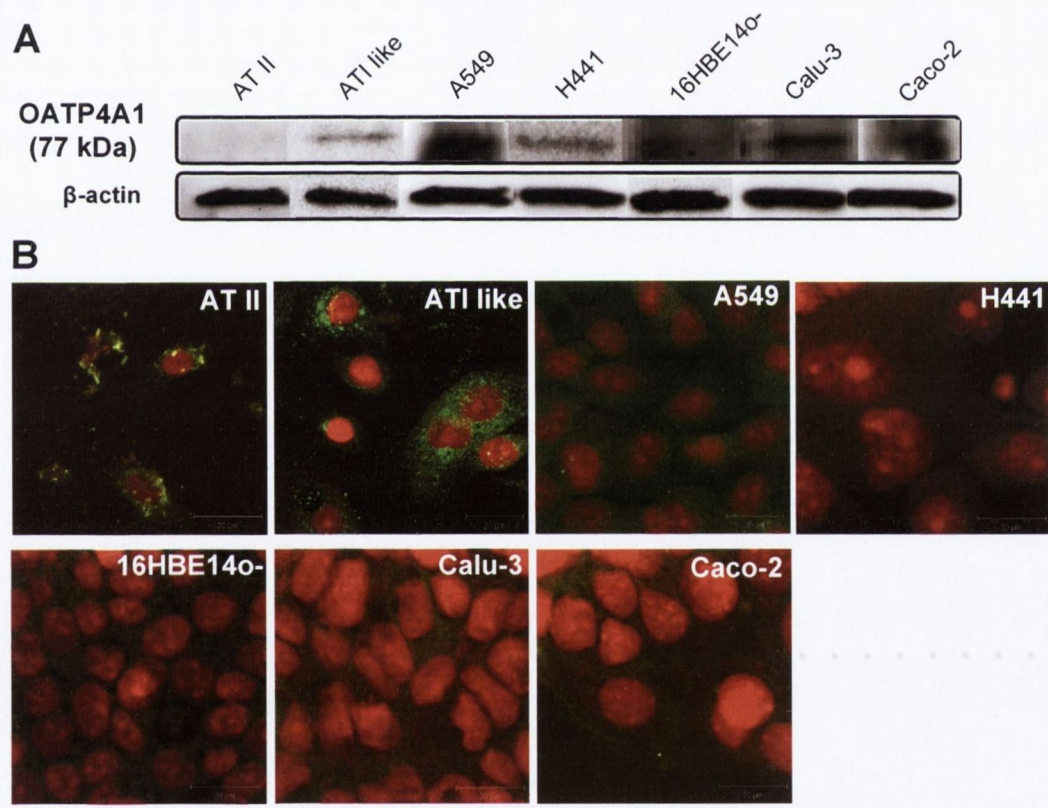


Figure 8. Expression of OATP4A1 in human respiratory epithelial cells in comparison to the intestinal Caco-2 cell line. Representative immunoblots for OATP4A1 transporter protein in lung and intestinal epithelial cell types *in vitro* (A). Confocal laser scanning microscopy of OATP4A1 (*green*) in human alveolar epithelial cells in primary cell culture (ATII and ATI-like) and continuously grown (A549), bronchiolar (H441), bronchial (16HBE14o-, Calu-3) and intestinal (Caco-2) cell lines (B). Nuclei were counterstained with propidium iodide (*red*). Bars represent 20 μ m.

Protein expression of OATP1B3 was investigated in human respiratory and intestinal epithelial cell lines. Western blot data demonstrated faint bands in alveolar (A549), bronchial (16HBE14o- and Calu-3) and intestinal (Caco-2) cells (Figure 9). However, no bands were obtained in alveolar epithelial primary and H441 cells. In contrast, CLSM studies showed significant immunoreactivity in ATII and ATI-like cells, mainly localised to the cytosol. Weak signals were obtained in A549, H441 and Calu-3 cells. No expression was detected in 16HBE14o- and Caco-2 cells. With the exception of alveolar primary cells, these results were consistent with the respective Western blot data.

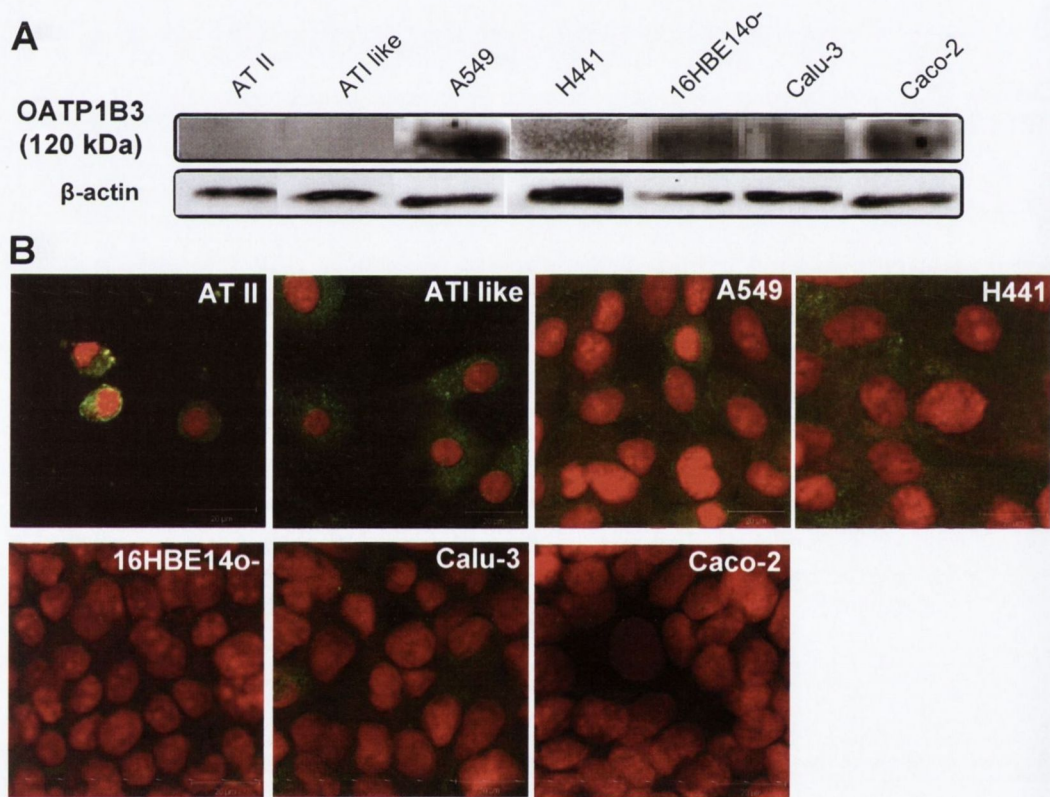


Figure 9. Expression of OATP1B3 in human respiratory epithelial cells in comparison to the intestinal Caco-2 cell line. Representative immunoblots for OATP1B3 transporter in lung and intestinal epithelial cell types *in vitro* (A). CLSM of OATP1B3 transporter (green) in human alveolar epithelial cells in primary cell culture (ATII and ATI-like) and continuously grown (A549), bronchiolar (H441), bronchial (16HBE14o-, Calu-3) and intestinal (Caco-2) cell lines (B). Nuclei were counterstained with propidium iodide (red). Bars represent 20 μ m.

2.4 DISCUSSION

In this Chapter, the presence of specific transporters responsible for the uptake and efflux of organic cations and anions in continuously growing cell lines of respiratory epithelium and freshly isolated human alveolar epithelial cells in primary culture was investigated. Moreover, we confirmed transporter abundance in intestinal Caco-2 cell monolayers. It is of note that previous literature on OCT/N and OATP expression in *in vitro* models of pulmonary origin was almost entirely limited to genomic studies (Endter et al., 2009; Ishiguro et al., 2008; Wang et al., 2007; Nakamura et al., 2010; Tamai et al., 2000; Adachi et al., 2003; Seki et al., 2009; Sai et al., 2006). For example, in a gene expression study of human whole lung tissue based on a reference set of 19,000 genes, OCTN1 and OCTN2 appeared to be strongly expressed transcripts at the 50-75% quartile of gene expression intensity; whereas OCT1 and OCT3 were in the 25-50% quartile and OCT2 even lower at 0-25%. Furthermore, OATP2A1 expressed highest transcripts at the 75-100% quartile, OATP3A1 and OATP4A1 appeared to be expressed in the 50-75% quartile, whereas OATP1B3 was found to appear in the 0-25% quartile of gene expression (Bleasby et al., 2006).

SLC22A1-5 transporter expression. We confirmed by immunoblot that the five cloned isoforms of the human *SLC22* organic cation/carnitine transporter family, that is, OCT1, -2, -3 and OCTN1 and -N2 were present in the alveolar epithelial cell line, A549 as well as in intestinal epithelial Caco-2 cells. Quantitatively, OCT1 and OCT2 were detected at significantly higher levels in Caco-2 cells compared to the respiratory cell types. Only OCT3 was expressed at equivalent levels in all cell lines studied. Abundance of OCTN1 and OCTN2 was lower in the lung derived cells, but these differences were not as pronounced as in the case of OCT1 and -2.

This is consistent with previous observations of OCT1 (More et al., 2010), OCT3 (Ishiguro et al., 2008; Wang et al., 2007) and OCTN1 and OCTN2 protein expression (Wang et al., 2007) in the A549 cell line. Müller *et al.* using RT-PCR found mRNA encoding OCT1, -2 and -3 in Caco-2 cells, however, immunocytochemistry in their study showed only faint signals for hOCT1 (Müller et al., 2005). Immunoreactivity for hOCT2 and hOCT3 in Caco-2 cells was more pronounced and predominantly localised to the cytoplasm and to the lateral cell membranes. In a separate study, Lamhonwah and colleagues (Lamhonwah et al., 2005) demonstrated the presence of OCTN1 and -2 in Caco-2 cells. Whilst OCTN1 was observed by confocal laser scanning microscopy to be predominantly subcellularly localised, OCTN2 was found at both the apical and basolateral cell membranes of Caco-2 cells cultured on Transwell filters. Data from another study showed that Caco-2 cells, when grown to differentiated monolayers on glass coverslips, localise OCTN2 protein exclusively to the brush-border membrane, whereas no signal was detected in basolateral membrane fractions by Western blot (Elimrani et al., 2003).

By Western analysis, we found all OCT/N gene products to be present in the bronchial epithelial cell lines, 16HBE14o- and Calu-3, with the exception of OCT2. Lips and co-workers (Lips et al., 2005) previously observed immunoreactivity of OCT1 mainly within human bronchial epithelial cells *in situ*, however, weak staining of the apical membrane of ciliated cells was also detected. hOCT2 was mainly located in apical membranes of ciliated cells and to a lesser extent also in basal cells. Immunoreactivity of hOCT3 was localised to plasma membranes of basal cells, basolateral membranes of intermediate cells and apical membranes of ciliated cells. To our knowledge, no data is available on the spatial expression pattern of OCT1, -2 and -3 in human distal lung. In

another study, Horvath *et al.* (Horvath et al., 2007) identified OCTN1 and OCTN2 in human airway epithelium *in situ* and freshly isolated airway epithelial cells in primary culture. Whilst OCTN1 was primarily localised to the apical surface of tracheal epithelia, expression was also observed in alveolar type II epithelial cells, albeit at lower levels. OCTN2 was found in apical membranes of airway epithelial cells and also in alveolar type I and type II cells in the lung parenchyma.

As aforementioned, the next chapter will focus on the functional expression of transporter proteins in H441 cells in comparison to pneumocytes in primary culture. Briefly, immunoblot revealed that OCT1, OCT2 and OCT3 were expressed in alveolar epithelial type II and type I-like cells as well as in the H441 cell line at comparable high levels. On the other hand, OCTN1 was detected at low levels, whilst OCTN2 was only expressed in ATII cells. CLSM studies were generally consistent with Western blot data, except for OCTN2, confirming expression of all five investigated SLC22 gene products.

SLC47A1 transporter expression. Additionally, we confirmed strong protein expression of MATE1 in all investigated cell lines by immunoblot and cytohistochemistry.

Recently, this transporter protein was reported to be highly expressed on mRNA level in A549 and BEAS-2B cell culture models by Courcot *et al.* (2012). MATE1 was not detected in human bronchial epithelial primary cells, but at moderate levels in various lung tissues (Courcot et al., 2012).

SLCO transporter expression. Variations in the expression profiles of OATP2A1, OATP3A1, OATP4A1 and OATP1B3 were noted amongst human respiratory epithelial *in vitro* models in comparison to intestinal epithelial cells and previous findings. In good agreement with Bleasby *et al.* (2006) and Tamai *et al.* (Tamai), we found high

levels of OATP2A1 protein in all cell lines investigated, including the human alveolar primary epithelial cells. In contrast to our findings, Endter *et al.* did not detect mRNA transcripts of OATP2A1 in human respiratory cells (2006). OATP3A1 was found to be moderately expressed in both bronchial and intestinal epithelial cells, which was in good agreement with literature data (Endter *et al.*, 2009; Adachi *et al.*, 2003, Tamai *et al.*, 2000). Expression levels were lower in alveolar epithelial cell lines consistent with findings by Seki *et al.* (Seki *et al.*, 2009). OATP4A1 was expressed at consistently low levels in all cell lines which was supported by CLSM data. By contrast, other reports found higher expression levels of this OATP family member (Endter *et al.*, 2009, Bleasby *et al.*, 2006, Tamai *et al.*, 2000), whilst Sai and colleagues confirmed the low expression of OATP4A1 in Caco-2 cells (Sai *et al.*, 2006). In the case of OATP1B3, we found very low protein abundance by means of immunoblot and CLSM, consistent with previous reports (Bleasby *et al.*, 2006; Endter *et al.*, 2009). By contrast, Seki *et al.* reported a strong OATP4A1 mRNA expression in A549 cells (Seki *et al.*, 2009).

As mentioned above, data on the expression of drug transporters in the lung is mainly based on findings on mRNA levels. Only a few groups reported protein expression such as Horvath *et al.* (2007), Wang *et al.* (2007) and Lips *et al.* (2005). The latter research group used affinity purified antibodies against rOCT1 (Alpha Diagnostics) which only cross-reacted with hOCT1. In the case of OCT2 and OCT3, not commercially available antibodies were used (Lips *et al.*, 2005). Horvath *et al.* and Wang *et al.* purchased goat anti-hOCT3, anti-hOCTN1 and anti-hOCTN2 IgG antibodies from one of the companies we mentioned (Santa Cruz Biotechnologies). Our immunoblot data was in good agreement with the findings by Wang *et al.* (2007), whereas we could not confirm the distinct localisation of OCTN1 and OCTN2 as shown by Horvath *et al.* in CLSM

studies (2007). One reason might be the different concentration of the used antibodies (1:100 in our case vs. 1:40 reported by Horvath *et al.*).

2.5 CONCLUSIONS

Pulmonary epithelial cells express several SLC transporters (*SLC22*, *SLC47* and *SLCO*) in a distinct and complex fashion. We provided first evidence for the expression of MATE1 protein in the respiratory epithelium. CLSM data revealed a localisation to the membrane in H441 and Calu-3 cells. Furthermore, we report pioneering data on the expression of various OATPs in human alveolar epithelium, using freshly isolated pneumocytes. These transporters are likely to have an effect on the disposition of inhaled cationic or anionic drugs (e.g., formoterol, salbutamol or cromolyn).

The data summarised in this chapter clearly showed that subcellular localisation of transporters depends on the quality of the used antibodies. We have studied a variety of antibodies from different commercial sources. Despite attempts to optimise the antibodies' performance, we could not pin point exact cellular localisation for a number of proteins. Gumbleton *et al.* (2011) proposed that a panel of antibodies should be used for immunohistochemical analysis and a semi-quantitative scoring for expression levels is needed to gain agreement on the spatial expression of drug transporters in the lung. The next step now is to measure the transporters' functionality and the potential impact on pulmonary drug disposition.

CHAPTER 3

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

Parts of this chapter have been published in:

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ABSTRACT

Organic cation/carnitine transporters (OCT/N) mediate the uptake of positively charged molecules. Their role in lung epithelium, however, is not well understood. OCT/N functional activity was studied by means of 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺) uptake in cell lines of human alveolar (A549), bronchial (16HBE14o- and Calu-3) and intestinal (Caco-2) epithelium.

Uptake of ASP⁺ was dependent on concentration, temperature, membrane potential and pH. In 16HBE14o-, Calu-3 and Caco-2 monolayers substrate saturation of ASP⁺ uptake was not reached within the concentration range studied. Alveolar A549 cells, however, showed saturable ASP⁺ uptake via two transporter sites with K_m values of $12.5 \pm 4.0 \mu\text{M}$ and $456.9 \pm 164.5 \mu\text{M}$, respectively. TEA and verapamil markedly inhibited ASP⁺ uptake in A549 cells, whereas the effect of amantadine and HC-3 was less pronounced. In the bronchial epithelial models, verapamil and amantadine most efficiently inhibited ASP⁺ uptake, however, TEA and HC-3 exhibited only weak inhibitory potential. Formoterol and salbutamol showed a concentration-dependent inhibitory effect on ASP⁺ uptake in A549 and 16HBE14o- cells. Moreover, efflux of ASP⁺ was a saturable process in A549 cells, indicating one transporter site. This efflux was sensitive to pH and inhibition by verapamil. On the other hand, efflux was not saturable in the other cell lines, with only marginal or no effect by pH and verapamil being observed.

Uptake and efflux of organic cations appears to be mediated via different pathways in different regions of lung mucosa. Luminally localised OCT2 is exclusively involved in the alveolar epithelium, whereas OCT1 might play a role in alveolar as well as in bronchial epithelial cells. MATE1 can be suggested as the responsible efflux transporter in the alveolar epithelium.

3.1 INTRODUCTION

Although the body of literature on disposition of inhaled medicines is increasing, there is still a surprising lack of mechanistic information on the processes involved (Patton et al., 2010). To further complicate matters, a broad range of different epithelial cell types, which change significantly in phenotype depending on their localisation, is found along the mucosal surface of the lung (Tronde et al., 2008). It is now widely accepted that membrane transporters impact on the absorption and extrusion of drugs, resulting in alterations of their pharmacokinetics and pharmacodynamics, potentially leading to organ toxicity. The study of drug transporters, however, has been mainly focused on the liver, kidneys, gut and also the blood-brain barrier, with the lung remaining largely uncharted terrain (Bosquillon et al., 2010; Gumbleton et al., 2011).

Transporters involved in organic cation translocation are electrogenic cation transporters OCT1-3 (*SLC22A1-3*), cation and carnitine transporters OCTN1 and OCTN2 (*SLC22A4-5*), proton/cation antiporters of the MATE family (*SLC47A1-2*), monoamine neurotransmitter transporters (*SLC6* family), cationic amino acid transporters (*SLC7* family), nucleoside transporters (*SLC28A1-3* and *SLC29A1-4*) and several choline transporters (*SLC5A7* and *SLC44A1-4*) (Ciarimboli 2010; Kouji et al., 2009). Evidence is now emerging that organic cation transporters are involved in transport processes in various cell types of the lung (Endter et al., 2009; Bleasby et al., 2006). Epithelial surface cells (Lips et al., 2005), but also airway smooth muscle cells (Horvath et al., 2007a) have been suggested to functionally express members of the *SLC22A* encoded family of proteins.

The aim of this paper was to use 4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide (ASP⁺) as a non-radioactive OCT/N probe (Stachon et al., 1997; Rytting et al.,

2007), and to study the mechanisms (including uptake and efflux) of organic cation transport across lung epithelia, with particular emphasis on OCT/N-mediated drug-drug interactions. Commonly prescribed inhalation medicines such as β_2 -agonists and anti-cholinergic drugs carry a cationic charge at physiological pH, but have to cross the epithelial barrier in order to reach the β_2 -adrenoceptor localised to the airway smooth muscles. Data from our laboratory and others suggest that β_2 -agonists are transported by OCT/N, or at least interfere with OCT/N function (Ehrhardt et al., 2005; Horvath et al., 2007).

The well characterised human intestinal epithelial cell line, Caco-2, was also included in our study, in order to investigate whether any differences in transporter expression and function exist between the epithelia of the gut and the lung.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Cell culture plastics (i.e., Cellstar[®] 75 cm² flasks [cat. no. 658175] and 24-well tissue culture plates [cat. no. 662160]) were obtained from Greiner BioOne (Frickenhausen, Germany), with the exception of Lab-Tek chamber slides (Nunc, Roskilde, Denmark). 4-(4-(Dimethylamino)-styryl)-N-methylpyridinium iodide (ASP⁺) was supplied by Invitrogen (Bio-Sciences, Dun Laoghaire, Ireland). Enantiomerically pure S-salbutamol (S-Sal), R-salbutamol (R-Sal), SS-formoterol (SS-For) and RR-formoterol (RR-For) were kind gifts from Sunovion (Marlborough, MA). Polyclonal rabbit anti-OCT1 was purchased from Aviva Systems Biology (San Diego, CA), polyclonal rabbit anti-OCT2 and -OCT3 antibodies were from Sigma-Aldrich (Dublin, Ireland). Horseradish peroxidase-conjugated anti-rabbit antibodies were purchased from Promega (Medical Supply Company, Dublin, Ireland). All other chemicals and reagents were either of analytical grade or of the highest purity available and purchased from Sigma-Aldrich unless stated otherwise.

3.2.2 Cell culture

Human alveolar epithelial A549 cells (ATCC CCL-185) were obtained from LGC Promochem (Teddington, UK). Cells of passage numbers 79 to 95 were grown at 40,000 cells/cm² in an 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium (DMEM/F-12) supplemented with 5% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

Passages of 2.51 to 2.79 of the 16HBE14o- human bronchial epithelial cell line, a gift from Dr. Dieter C. Gruenert (University of California, San Francisco, CA), were cultured at 100,000 cells/cm² in minimum essential medium (MEM) supplemented with

10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1% non-essential amino acid solution, 1% sodium pyruvate solution and 0.5% glucose solution. Calu-3 cells (ATCC HTB-55, LGC Promochem) are derived from serous cells of human lung submucosal glands. Passage numbers 44 to 59 were grown at 75,000 cells/cm² in an identical medium as used for 16HBE14o- cultures.

The human colon carcinoma Caco-2 cell line (ATCC HTB-37, LGC Promochem) at passages between 36 and 56 was grown to monolayers at a density of 66,000 cells/cm² in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1% non-essential amino acid solution and 1% sodium pyruvate.

All cell types were cultured at 37°C in 5% CO₂ atmosphere on cell culture treated plastics and the culture medium was exchanged every 48 h. Cells were grown to confluent monolayers for 5 (A549), 8 (16HBE14o-), 12 (Calu-3) and 21 (Caco-2) days, before being used in studies as described below.

3.2.3 Uptake and release studies

Uptake of the cationic fluorescent probe, ASP⁺ was investigated at different concentrations, temperatures, pH values and sodium ion concentrations. The effects of several organic cations (i.e., amantadine, D- and L-carnitine, hemicholinium-3 (HC-3), tetraethylammonium (TEA) and verapamil) on ASP⁺ uptake into cell monolayers were studied. Furthermore, the inhibitory potential of β₂-agonists (i.e., formoterol, salbutamol and salmeterol) as well as inhaled glucocorticosteroids (i.e., budesonide and beclomethasone) on ASP⁺ uptake was investigated. All experiments were performed in bicarbonated Krebs-Ringer buffer (KRB) composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, 0.81 mM MgSO₄, and 5.55 mM glucose, pH 7.4, unless stated otherwise.

Time-dependence of ASP⁺ uptake was measured by incubating cell monolayers with the fluorophore (at 10 μ M final concentration) for up to 90 min at 37°C. Five, 10, 15, 30, 45 and 60 min after the beginning of the experiment, monolayers were rinsed twice with ice-cold KRB, solubilised with 1% (w/v) Triton X-100 and ASP⁺ activity of the cell lysate was measured (see below). Concentration-dependence of ASP⁺ uptake was studied from 10 to 1000 μ M at 37°C and 4°C for 20 min. Uptake experiments were also performed in buffers of varying pH values (i.e., pH 5.5, 6.5 and 8.5). In addition, the effect of membrane depolarisation on ASP⁺ uptake was investigated. This was achieved by increasing the K⁺ concentration of the KRB to intracellular levels with a concurrent reduction of Na⁺ by replacing NaCl, NaH₂PO₄ and NaHCO₃ with equimolar concentrations of KCl, KH₂PO₄ and KHCO₃ (i.e., Na⁺ concentration, 5.4 mM; K⁺ concentration, 142 mM).

Uptake inhibition experiments were carried out in the presence of amantadine (100 μ M and 500 μ M), D-carnitine (10 μ M and 100 μ M), L-carnitine (100 μ M and 500 μ M), verapamil (10 μ M and 200 μ M), TEA (1 mM and 20 mM) and HC-3 (100 μ M and 500 μ M). Concentrations of these organic cations were chosen according to their published K_i or IC₅₀ values (Koepsell et al., 2007). The two β_2 -receptor agonists, salbutamol and salmeterol were used as racemic mixtures (RS-salbutamol in all cell lines studied; salmeterol only in A549 cells) and in addition, as pure enantiomers in A549 and 16HBE14o- cells. In the case of formoterol, the RR- and SS-enantiomers were studied in A549 and 16HBE14o- cells. Salbutamol and formoterol were used at final concentrations of 100 μ M and 500 μ M; salmeterol only at 100 μ M (due to the drug's poor solubility). The inhibitory potential of the inhaled corticosteroids (ICS), budesonide (10 - 30 μ M) and beclomethasone (10 μ M), and additionally selected cationic substrates, acetylcholine (1 mM and 5 mM), corticosterone (100 μ M and 500

μM), L-ergothioneine (100 μM), L-lysine (1 mM) and 1-methyl-4-phenylpyridinium (MPP^+) at final concentrations of 100 - 1000 μM , was studied in A549 cell monolayers. All drugs and compounds were dissolved in KRB (pH 7.4). Salmeterol as well as the ICS drugs were freshly prepared from ethanolic stock solutions. A similar amount of co-solvent was used in the relevant control experiments. In all cases cell monolayers were incubated for 20 min with the compound of interest.

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

In release studies, cell layers were loaded with 10 μM ASP^+ solution for 20 min, which then was replaced by fresh KRB and the ASP^+ release from the cell layers was followed for 90 min at time intervals similar to the ones described above. ASP^+ release was also studied at concentrations ranging from 10 to 1000 μM . These experiments were carried out at 37°C and 4°C for 20 min, to evaluate the activity of ASP^+ translocation across the cell membranes.

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

3.2.4 Kinetic analysis

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

$$v = V_{max} \times (S) / [K_m + (S)] \quad [Eq. 1]$$

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

3.2.5 OCT-siRNA transfection

A549 cells were seeded in 24-well cell culture plates at a density of 40,000 cells/cm² in culture medium containing FBS and antibiotics and grown for 24 h at 37°C in 5% CO₂ atmosphere. Two microliters of HyperFect Reagent (Qiagen, West Sussex, UK), 50 µl of DMEM/ F12 and OCT1-, OCT2-, or OCT3-siRNA (75 nM; Santa Cruz Biotechnology, Heidelberg, Germany) or the control scrambled siRNA (AllStars, Qiagen) were mixed, incubated at room temperature for 5 -10 min and added drop wise to the relevant wells. Cells were then incubated at 37°C for 24 h, before the medium was changed back to the respective normal cell culture medium. After 24, 48 and 72 h, cell monolayers were lysed in cell extraction buffer containing protease inhibitors (Invitrogen Karlsruhe, Germany). Protein sample concentrations were determined using a standard protein concentration assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions and used for Western blot analysis. For uptake studies, cells were used for analysis after 72 h (4 days after transfection).

3.2.6 Western blot analysis

After siRNA transfection, A549 cells were lysed with cell extraction buffer (Invitrogen Karlsruhe, Germany) on ice and sonicated (10 s, 2 cycles, 2 W) using a Microson Ultrasonic Cell Disruptor (Misonix). Protein sample concentrations were determined using a standard protein concentration assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. Samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gels via electrophoresis (SDS-PAGE) and transferred to immunoblot polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (pH 7.4) for 1 h at room temperature. Incubation with the relevant primary antibody (1:200 for OCT1, 1:500 for OCT2 and 1:2000 for OCT3, respectively) was carried out overnight at 4°C, followed by incubation with HRP-conjugated secondary anti-rabbit antibody (1:12,500) at room temperature for 1 h. Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). Relative levels of protein abundance were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system (Bio-Rad). When appropriate, blots were stripped and analysed for β -actin (Sigma-Aldrich, Dublin, Ireland) as internal control.

3.2.7 Statistical analysis

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

3.3 RESULTS

3.3.1 ASP⁺ uptake into human respiratory epithelial and Caco-2 cell monolayers

Transport mechanisms of organic cations across membranes of A549, Calu-3, 16HBE14o- and Caco-2 cell monolayers were studied by performing uptake experiments with the fluorescent probe, ASP⁺. When measuring the time course of ASP⁺ (10 μM) uptake, A549 monolayers exhibited the highest intracellular concentration after 60 min by a significant ($P < 0.05$) margin, that is, 16.54 ± 1.59 nmol/mg protein (Figure 1A). ASP⁺ concentrations in Caco-2, 16HBE14o- and Calu-3 monolayers were lower at 2.43 ± 0.79 , 1.79 ± 0.44 and 1.01 ± 0.23 nmol/mg protein, respectively (Figure 1B). The uptake increased linearly up to 45 min in A549 and up to 60 min in 16HBE14o-, Calu-3 and Caco-2 cells. Consequently, all further uptake studies were carried out for 20 min.

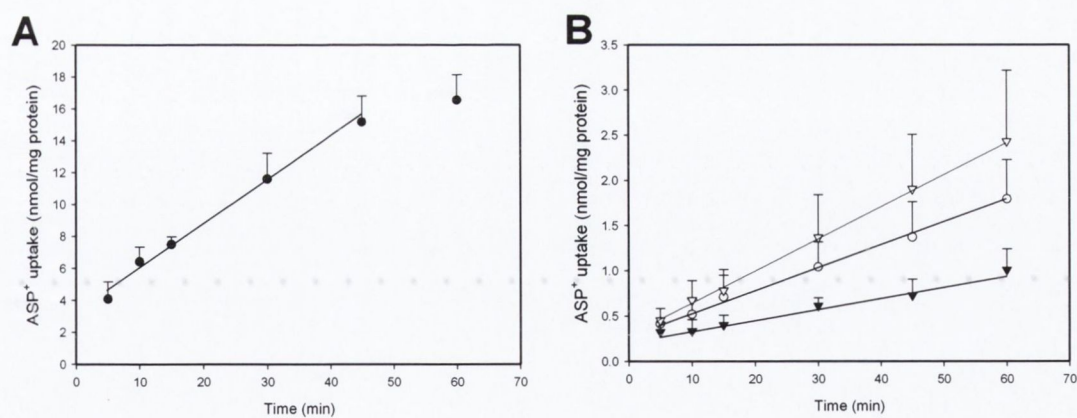


Figure 1. Time-dependent uptake of ASP⁺ into human respiratory epithelial cells and Caco-2 monolayers. (A) Time profile of ASP⁺ uptake in A549 (●) and (B) time profile of ASP⁺ uptake in 16HBE14o- (○), Calu-3 (▼) and Caco-2 (▽) monolayers. Cell layers were incubated with ASP⁺ (10 μM) as indicated. Data are represented as mean ± SD, $n = 3 - 9$.

3.3.2 Kinetics of ASP⁺ uptake

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

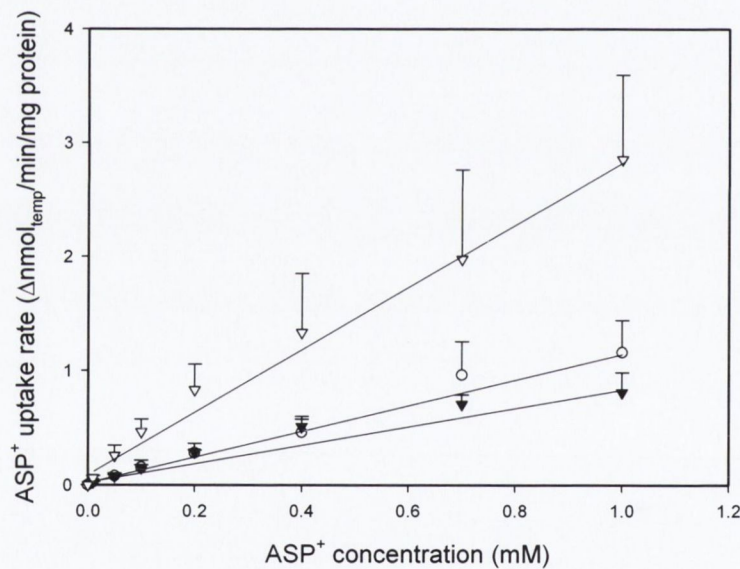


Figure 2. Concentration-dependent uptake of ASP⁺ in human respiratory epithelial cells and Caco-2 monolayers. 16HBE14o- (○), Calu-3 (▼) and Caco-2 (▽) cell layers were incubated at pH 7.4, 37°C and 4°C for 20 min as indicated. Uptake rates were calculated as the difference in ASP⁺ uptake between 37°C and 4°C. Data are represented as mean ± SD, $n = 3 - 6$.

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

	K_m (μ M)	V_{max} (nmol/min/mg protein)
A549 (high affinity)	12.50 $\pm$ 4.00	1.51 $\pm$ 0.12
A549 (low affinity)	456.88 $\pm$ 164.47	4.50 $\pm$ 0.69

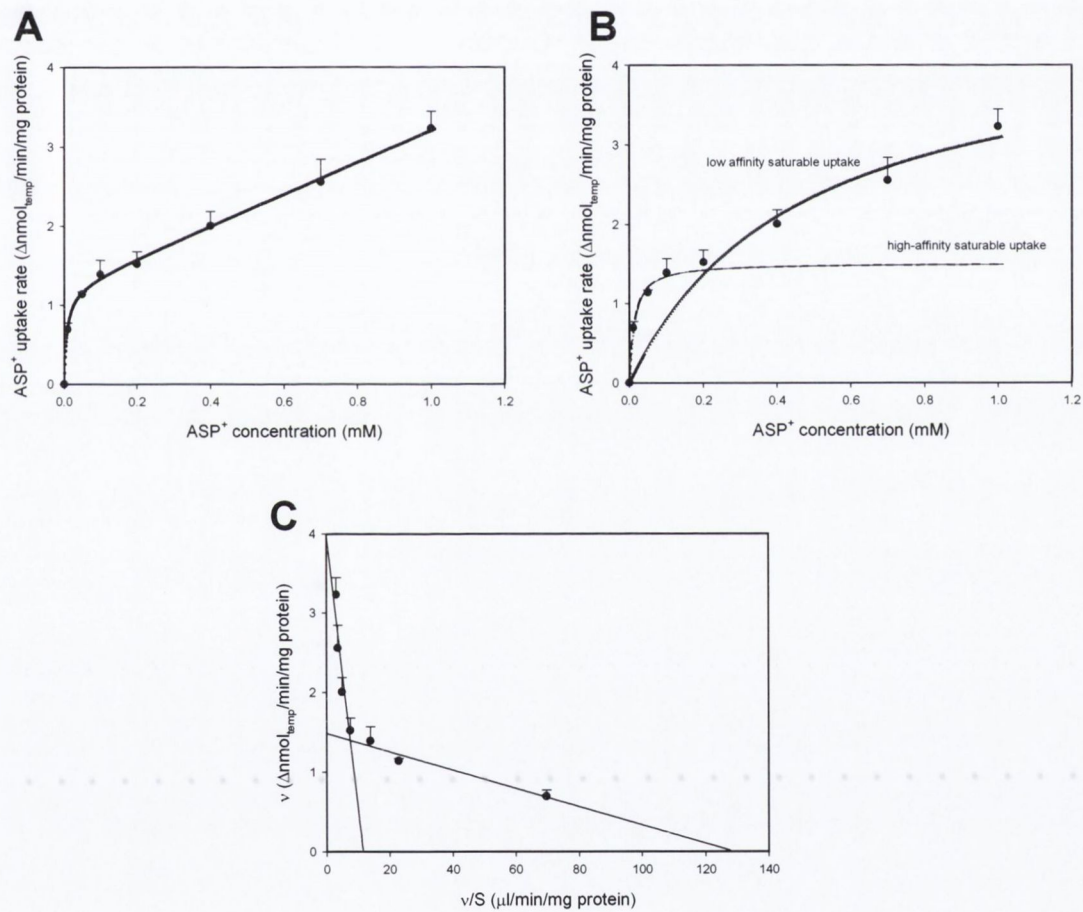


Figure 3. Concentration-dependent uptake of ASP⁺ into A549 (●) cells. Cell layers were incubated at pH 7.4, 37°C and 4°C for 20 min as indicated. Uptake rates were calculated as the difference in ASP⁺ uptake between 37°C and 4°C. The specific (carrier-mediated) uptake by the two distinct transporter sites is shown in (B). Furthermore, Eadie-Hofstee transformations of the data was performed (C): v , uptake rate (nmol/min/mg protein); S , ASP⁺ concentration in mM, v/S (μ l/min/mg protein). In A549 monolayers two distinct transporter sites were identified. Data are represented as mean $\pm$ SD, $n = 3 - 6$.

3.3.3 Effect of extracellular conditions on ASP⁺ uptake

To further characterise the mechanisms involved in organic cation transport at the respiratory epithelium, the effects of extracellular pH and membrane depolarisation on ASP⁺ uptake were studied. The uptake of ASP⁺ was significantly ($P < 0.05$) decreased in acidic conditions in all cell lines (Figure 4A). There was, however, no increased uptake observed at pH 8.2. In fact, uptake at high pH was reduced when compared to control values, that is, pH 7.4 (Figure 4A). Membrane depolarisation, achieved by replacement of extracellular Na⁺ with equimolar K⁺, also resulted in a significantly ($P < 0.05$) reduced ASP⁺ uptake into human respiratory epithelial cells. This effect was most pronounced in the case of A549 cell monolayers (Figure 4B).

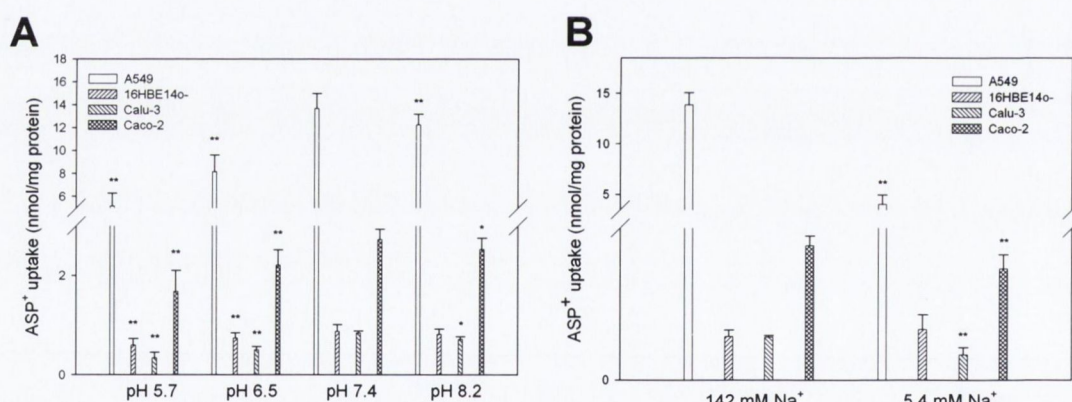


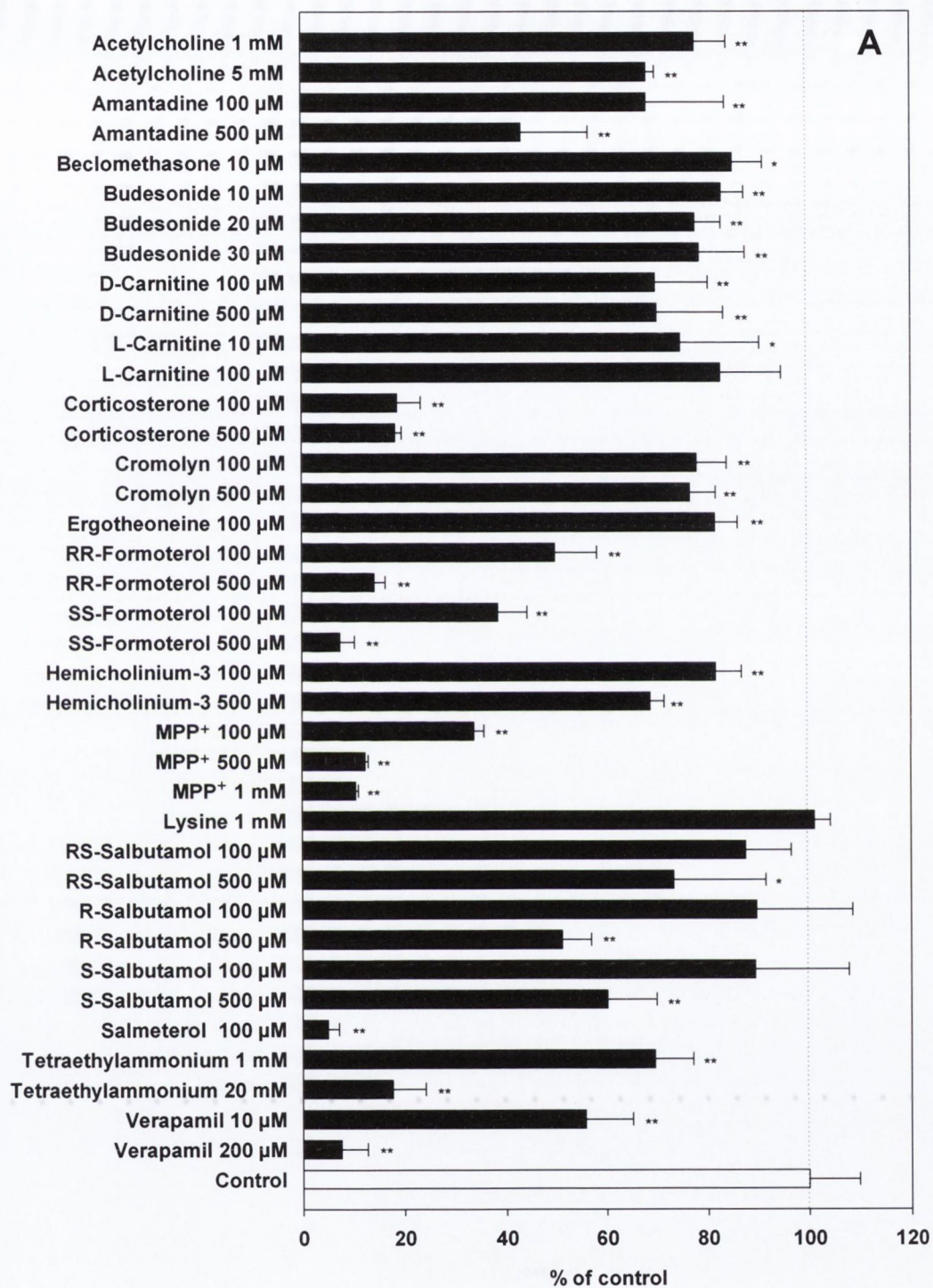
Figure 4. Effect of extracellular pH (A) and membrane potential (B) on uptake of ASP⁺ in human respiratory epithelial cells and Caco-2 monolayers. A549, 16HBE14o-, Calu-3 and Caco-2 monolayers were incubated with ASP⁺ (10 μ M) at pH 5.7 - 8.2, 37°C for 20 min as indicated. ASP⁺ uptake was measured in A549, 16HBE14o-, Calu-3 and Caco-2 monolayers in the presence of increasing and decreasing concentrations of KCl and NaCl, respectively, pH 7.4, 37°C for 20 min. Data are represented as mean $\pm$ SD, $n = 3 - 6$. * $P < 0.05$, ** $P < 0.01$ versus control.

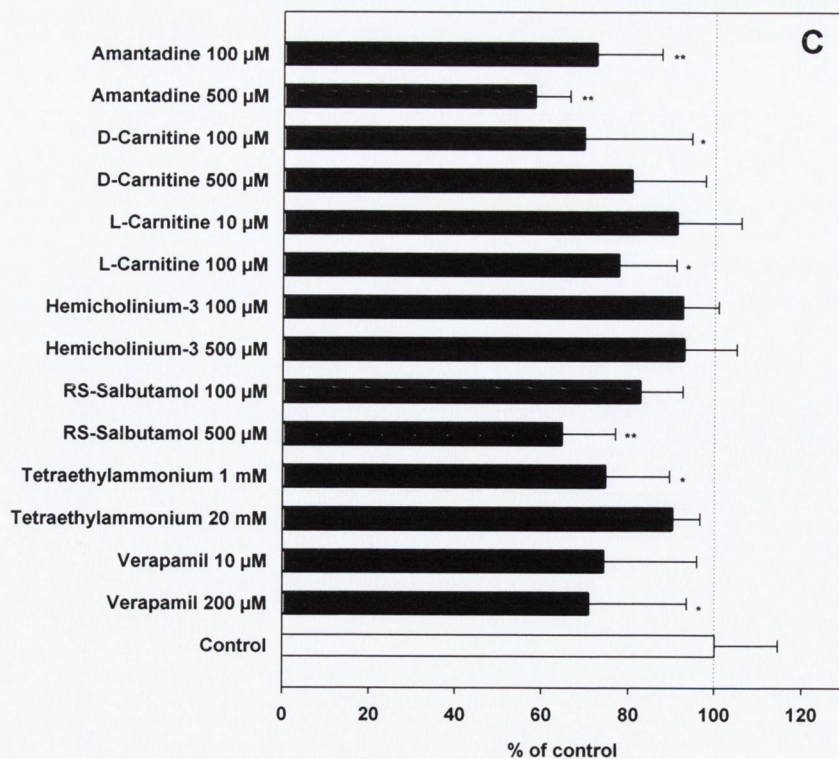
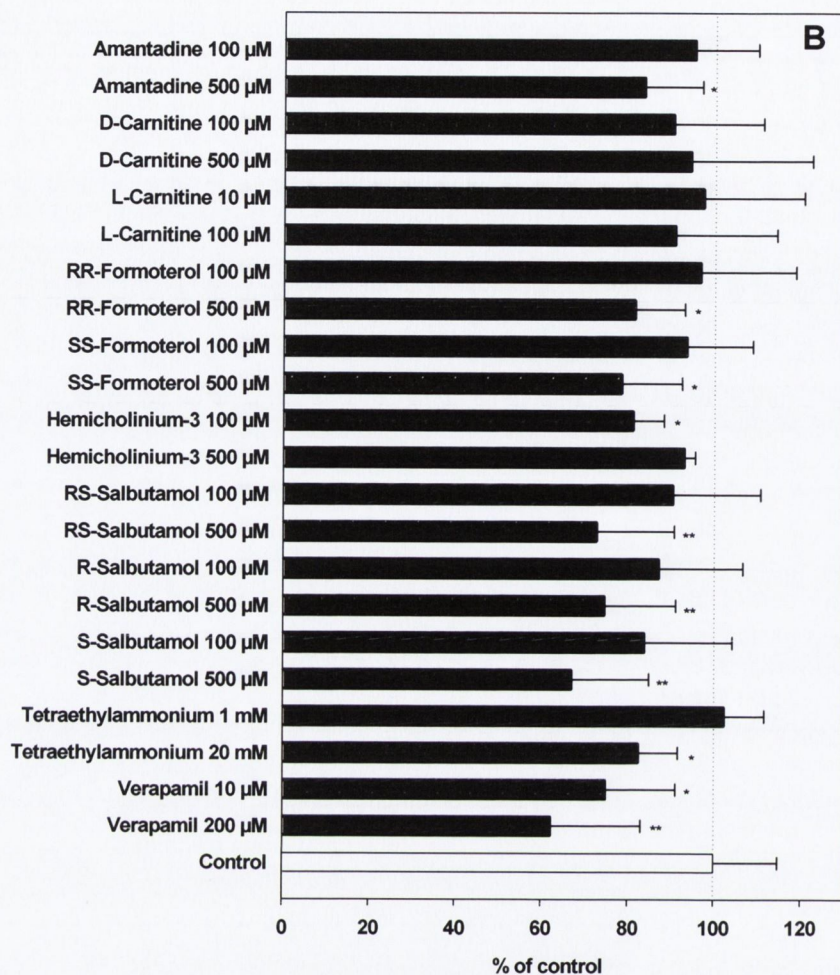
3.3.4 Effect of organic cations on ASP⁺ uptake

The structural analogue, MPP⁺ reduced ASP⁺ uptake into A549 monolayers almost completely at 1 mM (Figure 5A). TEA, resulted in a concentration-dependent decrease of ASP⁺ uptake and also verapamil significantly diminished ASP⁺ uptake to < 10% of control (at 200 μ M). Both substances have the potential to inhibit OCT1-3 and OCTN1 and -N2 at the concentrations used (Koepsell et al., 2007). The steroids, corticosterone, beclomethasone and budesonide showed unequal effects. Corticosterone, which is known to inhibit OCT1 and -2, but most efficiently OCT3, reduced ASP⁺ uptake by almost 80%, whilst both inhaled corticosteroids (ICS) caused a much lower, albeit significant, reduction. Amantadine, like the ICS drugs, an inhibitor of mainly OCT2, had a more pronounced effect, but the concentrations used were also higher. The OCTN substrates, ergothioneine and D- and L-carnitine reduced ASP⁺ uptake to 70-80% of the control values. ASP⁺ uptake was also inhibited by ~35%, when the choline transporter substrates, HC-3 or acetylcholine were present in the transport buffer. L-lysine at 1 mM had no effect on ASP⁺ accumulation.

A concentration-dependent attenuation of ASP⁺ uptake into A549 cells was achieved with the rank order salmeterol (> 90% at 100 μ M) > formoterol (> 80% at 500 μ M) > salbutamol (> 40% 500 μ M) (Figure 5A). R-salbutamol proved to be a slightly, but not significantly, more potent inhibitor of ASP⁺ uptake than S-salbutamol ($P = 0.06$), whilst in the case of the formoterol, the SS-enantiomer was significantly ($P < 0.05$) more effective than the RR-enantiomer. The anionic mast cell stabiliser, cromolyn sodium did not show a concentration dependency, but both tested concentrations (100 μ M and 500 μ M) were able to reduce ASP⁺ uptake by > 20%.

A more limited set of compounds was tested in 16HBE14o- (Figure 5B), Calu-3 (Figure 5C) and Caco-2 (Figures 5D) cell monolayers. Whilst some inhibition of ASP⁺ uptake was observed, the effects were generally much less pronounced when compared to A549 monolayers. In all three cell types, amantadine significantly ($P < 0.05$) inhibited ASP⁺ uptake at the higher concentration (i.e., 500 μ M). A similar, but more pronounced effect was also observed with the universal inhibitor, verapamil. Intriguingly, TEA inhibition was not very distinct (i.e., 20 - 25%), and inversely correlated to concentration in the case of Calu-3 and Caco-2 cells. The choline transporter blocker, HC-3, was only significantly ($P < 0.05$) effective in 16HBE14o- cells, and only at the lower concentration used. D- and L-carnitine did not inhibit ASP⁺ uptake in 16HBE14o- cell layers. In Caco-2 cells only D-carnitine had a significant ($P < 0.05$) effect, whilst L-carnitine did not attenuate ASP⁺ uptake. In Calu-3 monolayers, only the lower concentration of D-carnitine and the higher concentration of L-carnitine exhibited significance, albeit the difference between values obtained for the respective concentrations was not significantly different from each other. Racemic salbutamol showed a concentration dependent inhibition only in the bronchial cell types. Therefore, the enantiomers of salbutamol and formoterol were only tested in 16HBE14o- cells. In all cases, a concentration dependent inhibition of ASP⁺ uptake was observed. No distinction, however, could be made between the relevant enantiomers.





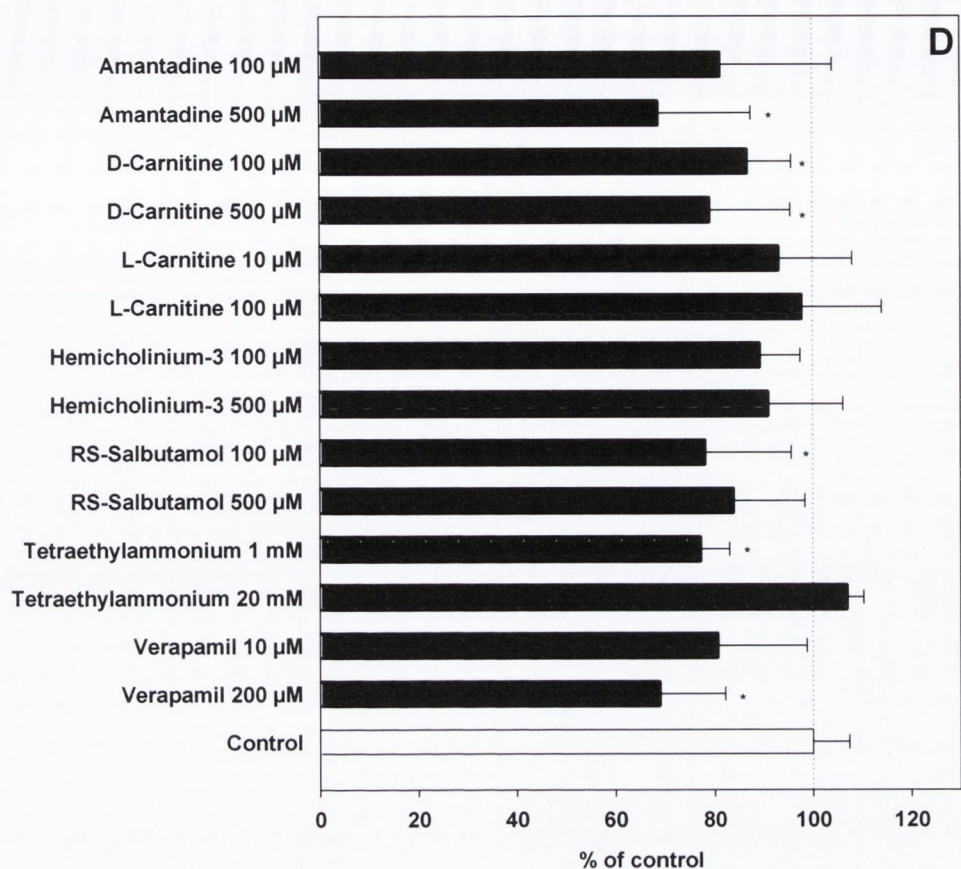


Figure 5. Inhibitory effects of organic compounds including β_2 -receptor agonists on ASP^+ uptake in human respiratory epithelial cells and Caco-2 monolayers. ASP^+ uptake (10 μ M) was measured in the presence of amantadine (100 μ M and 500 μ M), D-carnitine (100 μ M and 500 μ M), L-carnitine (10 μ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

3.3.5 Contribution of tight junctions to ASP⁺ uptake

Uptake of ASP⁺ into Calu-3 monolayers was studied after the tight junctions were opened using EDTA complexation of calcium ions. When transporter sites localised to the basolateral membranes were accessible to ASP⁺, uptake was significantly increased (Figure 6).

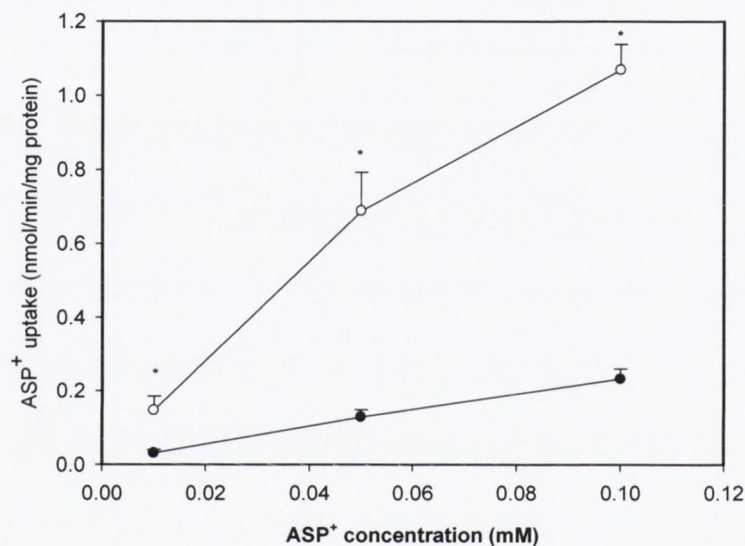
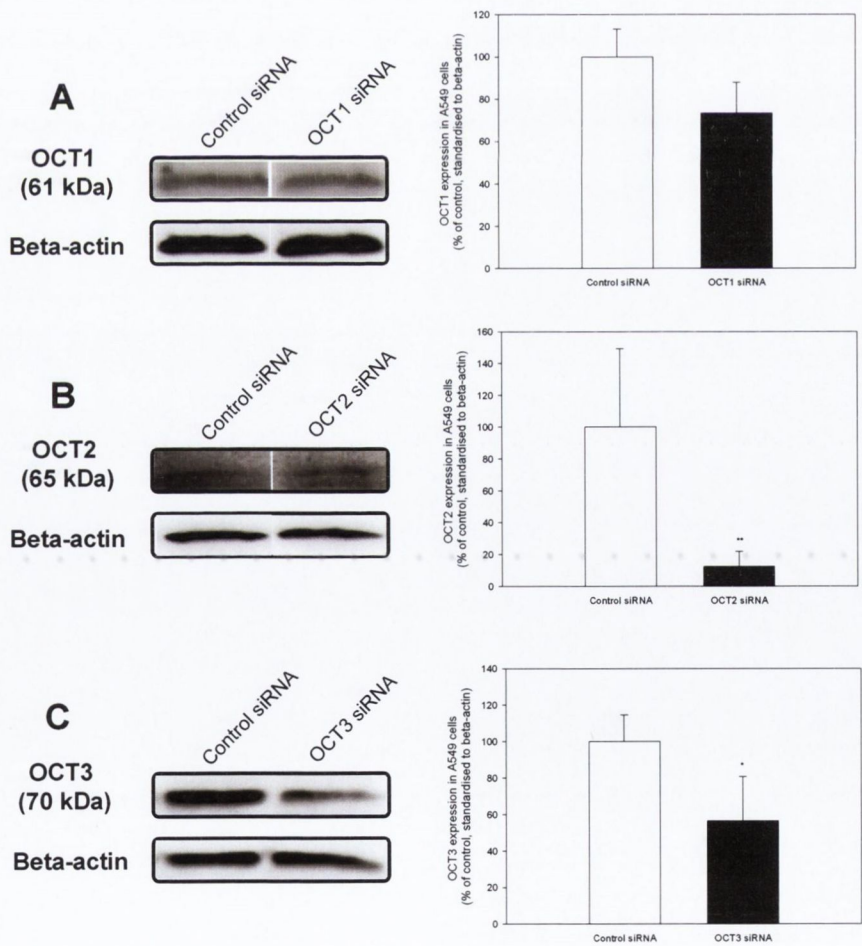


Figure 6. Contribution of the tight junctional barrier to uptake of ASP⁺ into human respiratory epithelial cells. Calu-3 cell monolayers were incubated with ASP⁺ as described previously (●) or in the presence of 5 mM EDTA (○). Data are represented as means ± SD, *n* = 3. * *P* < 0.01 versus control.

3.3.6 Effect of RNAi on OCT protein expression and ASP⁺ uptake in A549 cells

To investigate, whether knockdown of OCT1, OCT2 or OCT3 led to changes in ASP⁺ uptake, A549 cells were transfected with OCT1-, OCT2- and OCT3-siRNA. Protein expression levels were determined 3 days after transfection and results are shown in Figure 7 A-C. To assess if RNAi had an effect on transporter function, ASP⁺ uptake studies were carried out under similar conditions as described above. All experiments

were performed in non-treated, mock-transfected and OCT siRNA-transfected A549 cells. No significant differences were observed between non-treated and mock-transfected A549 cells. Nevertheless, all data obtained from OCT-siRNA transfected cells were compared to the relevant mock-transfected controls. Knockdown of OCT1 or OCT2 significantly inhibited ASP⁺ uptake to $53.3 \pm 11.6\%$ and $38.1 \pm 19.7\%$, respectively, whilst knockdown of OCT3 did not show an effect on ASP⁺ uptake (Figure 7D). Hence, knockdown of OCT proteins resulted in decreased uptake of ASP⁺. There were, however, some discrepancies when comparing effect on uptake and protein quantities measured by Western blot.



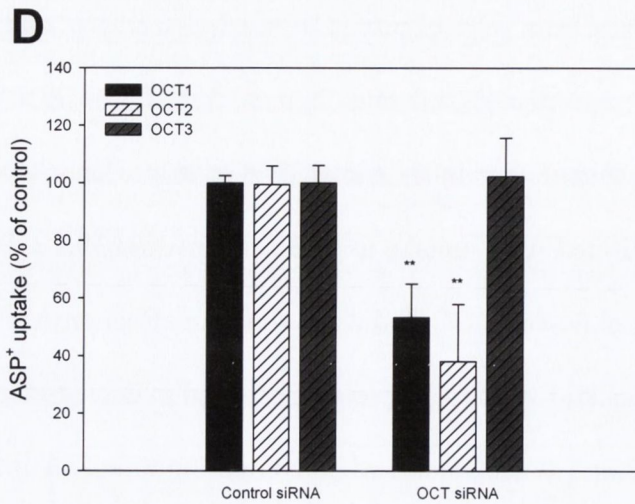


Figure 7. Effects of RNAi on OCT1-3 protein expression in human alveolar epithelial cells. A549 cells were transfected with 75 nM OCT1-, OCT2- and OCT3-siRNA. Three days after transfection, total cell protein was isolated and analysed by Western blotting. Beta-actin was used as a loading control. Scrambled siRNA was used in parallel to test potential non-specific effects of siRNA complexes (control siRNA). A densitometric analysis of OCT expression corrected for β -actin was carried out. Western blots of OCT1, OCT2 and OCT3 are shown in (A), (B) and (C), respectively. Furthermore, effects of OCT1-3-siRNA were studied on ASP⁺ uptake into A549 cells (D). Uptake studies were performed 3 days after siRNA transfection at pH 7.4 and at 37°C. Data represent means $\pm$ SD ($n = 3 - 6$). ** $P < 0.01$ indicates a significant difference from the uptake at pH 7.4 by mock-transfected cells.

3.3.7 ASP⁺ release from human respiratory epithelial and Caco-2 cells

The time course of ASP⁺ release (after 20 min of loading) from cell layers was also studied. Figure 8A shows the absolute decrease of intracellular fluorescence signals. In addition, the percentage of residual ASP⁺, relating to the initial amount is shown in Figure 8B. It is noted that A549 cell layers showed the highest absolute and relative ASP⁺ release; the remaining intracellular ASP⁺ concentration after 60 min was calculated to be $26.3 \pm 5.2\%$ (Figure 8B). Calu-3 cell monolayers contained $31.8 \pm 10.8\%$ of the initial ASP⁺ amount. Caco-2 and 16HBE14o- cells, however, exhibited significantly lower release rates, with $56.0 \pm 9.1\%$ and $108.7 \pm 39.9\%$ ASP⁺ being retained, respectively (Figure 8B).

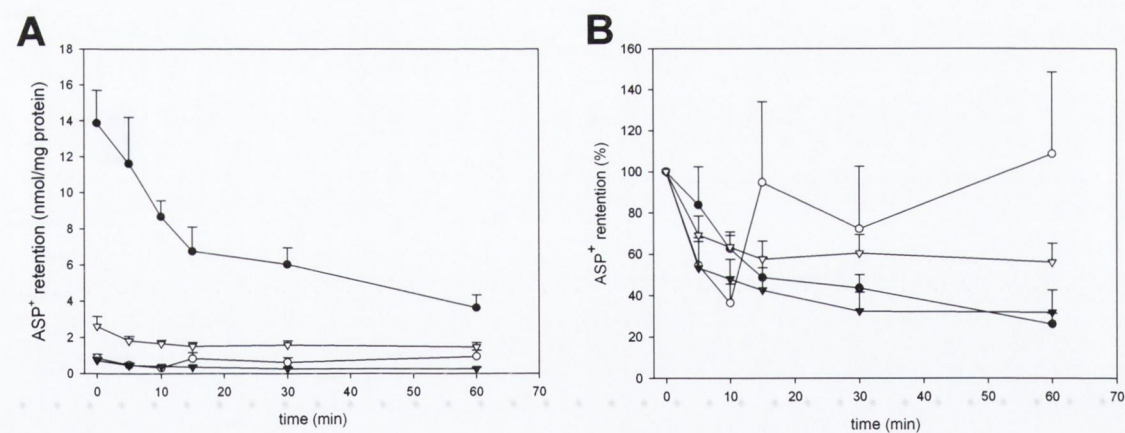


Figure 8. Time-dependent release of ASP⁺ from human respiratory epithelial cells and Caco-2 monolayers. Time profiles of ASP⁺ retention in A549 (●), 16HBE14o- (○), Calu-3 (▼) and Caco-2 (▽) monolayers (A). Cells were loaded with ASP⁺ (10 μ M) for 20 min, washed twice with fresh buffer and cellular retention was measured for 90 min. Relative retention of ASP⁺ as percentage of the compound's intracellular concentration at the end of the incubation period (B). Data are represented as mean $\pm$ SD, $n = 3 - 9$).

3.3.8 Kinetics of ASP⁺ release

ASP⁺ efflux was likewise a saturable (A549, Figure 9A) and not-saturable (Calu-3, 16HBE14o- and Caco-2 cells, Figure 9B) process. Again, marked differences were observed between A549 and the other cell lines.

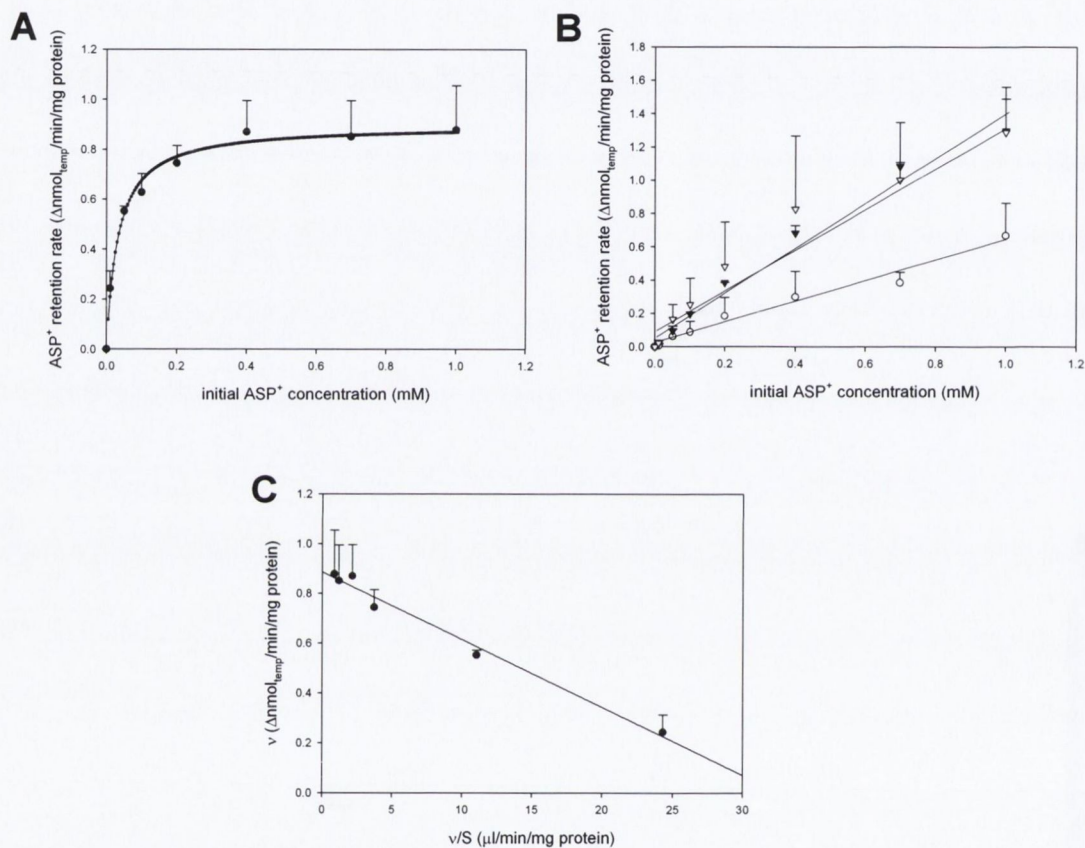


Figure 9. Concentration-dependent release of ASP⁺ from human alveolar (A), bronchial (B) and Caco-2 (B) monolayers. A549 (●), 16HBE14o- (○), Calu-3 (▼) and Caco-2 (▽) cell layers were loaded at pH 7.4, 37°C for 20 min. After washing twice with KRB, cellular retention was measured after 20 min. Release rates were calculated as the difference in ASP⁺ retention between 37°C and 4°C. (C) Eadie-Hofstee transformations of the data: v , retention rate; S , ASP⁺ concentration in mM, v/S ($\mu\text{l}/\text{min}/\text{mg protein}$). ASP⁺ release could be described as a linear function of ASP⁺ concentration. Data are represented as mean $\pm$ SD, $n = 3$.

Pharmacokinetic analysis of the saturable process in A549 cells was performed according to Eadie-Hofstee transformation ($[S]$ was the initial ASP^+ concentration) and indicated a single transport system being responsible for the release of ASP^+ (Figure 9C). The kinetic parameters were calculated as an estimation of the release process with a hypothetical K_m value of $32.92 \pm 4.57 \mu M$ and a V_{max} of $0.89 \pm 0.02 \text{ nmol/min/mg}$ protein.

3.3.9 Influence of extracellular cations and verapamil on ASP^+ release

The release of ASP^+ was differently affected by extracellular pH in the various cell types (Figure 10A). In 16HBE14o- and Caco-2 cell layers, ASP^+ release was not affected with increasing pH, whereas A549 and, to a lesser extent, Calu-3 cell layers showed an enhanced level of ASP^+ release at lower pH values.

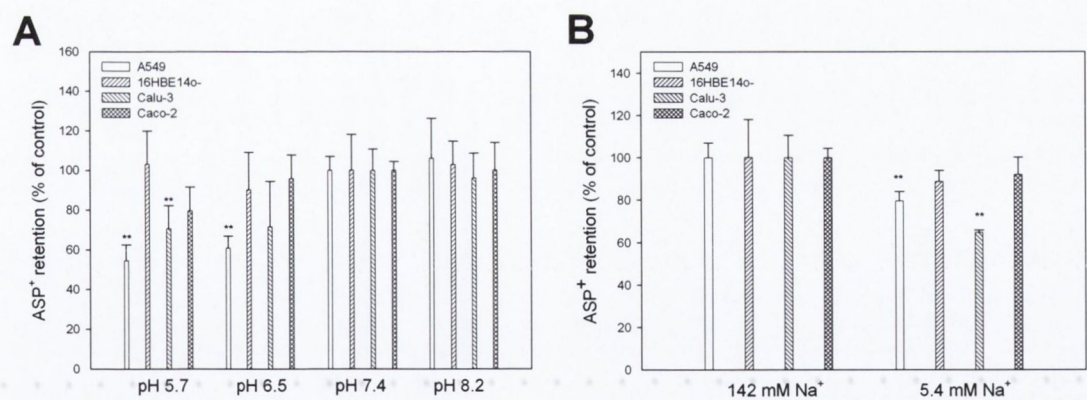


Figure 10. Effect of extracellular cations on release of ASP^+ from human respiratory epithelial cells and Caco-2 monolayers. (A) A549, 16HBE14o-, Calu-3 and Caco-2 monolayers were loaded at pH 7.4, 37°C for 20 min. Cells were then washed twice with fresh buffer (pH 5.7 - 8.2) and cellular retention was measured after 20 min. (B) ASP^+ release was measured in A549, 16HBE14o-, Calu-3 and Caco-2 monolayers in the absence and presence of Na^+ salts, at pH 7.4, 37°C for 20 min Data are represented as mean $\pm$ SD, $n = 3 - 6$. * $P < 0.05$, ** $P < 0.01$ versus control.

ASP⁺ release after membrane depolarisation showed results similar to those obtained after changing extracellular pH (Figure 10B), i.e., no effect on ASP⁺ release in 16HBE14o- and in Caco-2 cell layers, and an increased level of ASP⁺ release in A549 and Calu-3 cells. Next, ASP⁺ release was investigated in the absence (control) and presence of the P-gp efflux inhibitor, verapamil (Figure 11). No statistically significant effect was obtained in 16HBE14o-, Calu-3 and Caco-2 cell monolayers, whereas in A549 cells, the amount of retained ASP⁺ was remarkably increased to 136.1 ± 17.1 % of control in the presence of 200 μ M verapamil.

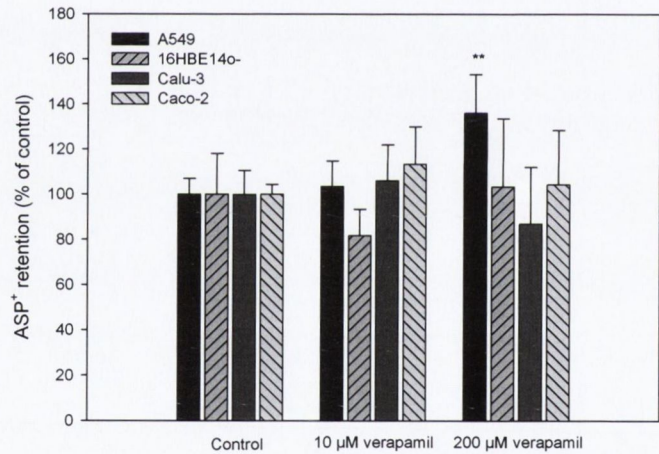


Figure 11. Effects of verapamil on ASP⁺ release from human respiratory epithelial cells and Caco-2 monolayers. ASP⁺ release (10 μ M) was measured in the absence and presence of verapamil (10 μ M and 200 μ M) in A549, 16HBE14o-, Calu-3 and Caco-2 cell monolayers at pH 7.4, 37°C for 20 min as indicated. Data are represented as mean $\pm$ SD, $n = 3 - 6$. * $P < 0.05$, ** $P < 0.01$ versus control.

3.4 DISCUSSION

Dependence of ASP⁺ uptake on extracellular conditions. ASP⁺ has been used as a non-radioactive substrate for monoamine and organic cation/carnitine transporters (Stachon et al., 1997; Rytting et al., 2007; Horvath et al., 2007b; Rohlicek et al., 1994; Hohage et al., 1998; Mason et al., 2005; Oz et al., 2010). In our hands, ASP⁺ uptake was concentration and temperature-dependent and sensitive to extracellular pH and membrane potential in all cell lines studied.

Horvath *et al.* (Horvath et al., 2007b) previously reported that ASP⁺ uptake into human airway epithelial cells in primary culture on collagen-coated coverslips had a pH-profile, but was independent of extracellular Na⁺ concentration. ASP⁺ uptake into placental BeWo cells was mediated by a low-affinity, carrier-associated process exhibiting a K_m of 580 ± 110 μ M, and asymmetric transport was observed, with greater permeability in the apical to basolateral direction (Rytting et al., 2007). Whilst the authors of that study did not test for the presence of OCT3, OCTN1/2 or indeed any monoamine transporter, they concluded that ASP⁺ uptake in their hands was not facilitated by OCT1 or OCT2. Bleasby and co-workers (Bleasby et al., 2006) reported that absorption of 1-methyl-4-phenylpyridinium (MPP⁺; a structural analogue to ASP⁺) by Transwell-grown Caco-2 cells was mediated by a Na⁺-dependent transport mechanism, which was inhibited at acidic pH values. Another study from the same year, however, described uptake of [³H]-MPP⁺ by Caco-2 monolayers to be metabolic energy-dependent and Na⁺-, pH- and potential-independent (Martel et al., 2000). Of note, Caco-2 monolayers in the latter study were cultured on tissue-treated plastic. The differences observed between published studies reporting organic cation uptake by cultured epithelial cells might be explained by the choice of culture conditions or indeed by clonal variation between the cells used by various groups (Hayeshi et al., 2008).

Kinetics and inhibition of ASP⁺ uptake. Eadie-Hofstee transformation of ASP⁺ uptake into alveolar epithelial-like A549 cell layers indicated two differential transporter sites; one of which exhibited a relatively high affinity ($K_m = 12 \mu\text{M}$), whereas the other one had a lower affinity ($K_m = 450 \mu\text{M}$). ASP⁺ uptake into the bronchial (i.e., 16HBE14o-, Calu-3) and the intestinal (i.e., Caco-2) cell models did not reach substrate saturation. However, concentrations above 1 mM for ASP⁺ could not be dissolved in the buffer system used. These observations are consistent with a previous study that likewise concluded that total initial uptake rates for ASP⁺ in an OCT expression system (at 1 mM) were not saturable, and only time-dependent saturation for maximal cellular fluorescence could be measured (Biermann et al., 2006). In contrast, Horvath *et al.* (Horvath et al., 2007b) reported airway epithelial cells in primary culture to exhibit a single transporter site for ASP⁺ uptake with an estimated K_m of 394 μM .

Inhibition of ASP⁺ uptake into A549 monolayers was most pronounced with MPP⁺, TEA, verapamil and corticosterone. A common trait shared by these compounds is their capacity to interact with OCT1-3 (Nies et al., 2011). TEA and verapamil also have a reported affinity for OCTN1 and -N2 (Koepsell et al., 2007). OCTN substrates, ergothioneine (OCTN1), D-carnitine (OCTN2) and L-carnitine (OCTN2 > OCTN1), also attenuated ASP⁺ uptake, but only by ~ 20%. This effect was similar to that observed with budesonide and beclomethasone, both known OCT2 inhibitors (Koepsell et al., 2007). A slightly higher inhibition was observed for choline analogues, HC-3 and acetylcholine, and the NMDA-receptor antagonist, amantadine (OCT2 > OCT1). Moreover, β_2 -receptor agonists formoterol and salmeterol exerted very strong inhibitory effects. Previous reports from our laboratory and others already hypothesised an interaction between β_2 -adrenoceptor agonists and organic cation/carnitine transporters

(Ehrhardt et al., 2005; Horvath et al., 2007b, Valenzuela et al., 2001). Of note, the inhibitory potential closely followed the lipophilicity of the substances (log *P* salbutamol -2.15; formoterol 0.4; salmeterol 3.88).

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

Our results showed that in alveolar A549 cells, significantly higher OCT-mediated ASP⁺ uptake occurred than in the other cell types. Sensitivity of this uptake to MPP⁺, TEA, verapamil, corticosterone, amantadine and the ICS compounds indicated an involvement of OCT2 and probably OCT1 (Koepsell et al., 2007; Valenzuela et al., 2001). Interestingly, uptake rates in Calu-3 monolayers reached almost comparable levels to values obtained in A549 cells, after opening the tight junctions with EDTA (Koval et al., 2009). This strongly supports the notion that at least one of the responsible transporter sites is localised at the basolateral cell aspect. Under normal conditions, ASP⁺ diffusion to the basolateral membrane will be restricted by the tight and adherens junctions; hence, it is unlikely that sufficiently high concentrations are achieved to saturate the transporter site. This is consistent with our observation that no saturation of ASP⁺ absorption was observed in polarised Calu-3, 16HBE14o- and Caco-2 monolayers. The observation that cellular junctions in A549 cells are compromised, resulting in an inability of this cell line to form electrically tight monolayers when cultured on semi-permeable membranes, offers further support to this hypothesis

(Sporty et al., 2008). Most pharmacological inhibitors showed only relatively weak activities in 16HBE14o-, Calu-3 and Caco-2 monolayers. The exception to this was the highly lipophilic compound, verapamil. It is therefore possible that passive diffusion of verapamil into the cells occurred, resulting in the inhibition of these basolaterally localised transporters.

Effect of RNAi on ASP⁺ uptake. Significant knockdown of OCT2 and OCT3 was shown on protein level, whilst transfection with OCT1-siRNA did not reveal significant reduction. OCT knockdown studies revealed that ASP⁺ uptake was reduced to values of 50% and 40% when cells were transfected with OCT1- or OCT2 siRNA, respectively. These results suggest that the transporters mainly involved in the translocation of ASP⁺ in A549 cells were OCT1 and OCT2 ($P > 0.05$). The knockdown of OCT3 did not affect the uptake of the organic cations in A549 cells.

Involvement of OCT/Ns. This study identified clear differences between the processing of ASP⁺ by A549 cell monolayers and bronchial 16HBE14o- and Calu-3 as well as the intestinal Caco-2 cell models. Our observations suggest variations in organic cation transporter expression level and localisation between the various cell types. Whilst OCT1, -3, -N1 and -N2 were ubiquitously found, OCT2 was only present in A549 and Caco-2 cells. In the latter cell type, Müller *et al.* (Müller et al., 2005) previously reported hOCT2 and -3 localised to the lateral cell membranes. No information on the cellular localisation of any of the OCT in A549, Calu-3 and 16HBE14o- cells is currently available. We have evaluated several commercially available antibodies, and in our hands, it was difficult to obtain reproducible results in confocal laser scanning microscopical analysis (please refer to Chapter 2: Expression of drug transporter

proteins in human respiratory epithelial cells). From the immunohistochemistry data of Lips and colleagues, however, it can be speculated that OCT2, if present, is localised to the apical membrane, whilst OCT3 is found in the basolateral membranes (Lips et al., 2005). It was previously suggested by us (Salomon et al., 2012) that OCT3 was the low affinity, high throughput transporter for ASP^+ , due to the fact that it was expressed at similar levels across all cell types, and had previously been associated with basolateral expression (Lips et al., 2005; Müller et al., 2005). OCT1, however, was also found in all cell lines studied, but with pronounced differences in protein abundance. In addition, several studies from other labs have described OCT1 predominantly localised to intracellular domains in both Caco-2 cells as well as human bronchial epithelium (Lips et al., 2005; Müller et al., 2005). Of note, OCT2 was only to be found present in A549 epithelial cells ruling out that this transporter protein would play a major role at the bronchial epithelium (Salomon et al., 2012). Here, the exact nature of the transporters involved has to be confirmed.

Cis-inhibition by ergothioneine and D- and L-carnitine only weakly attenuated ASP^+ uptake, suggesting that organic carnitine transporters had only a minor involvement in the process. Horvath and colleagues (Horvath et al., 2007b) previously proposed OCTN2 as the responsible transporter for ASP^+ uptake into the human airway epithelium. Regretfully, their report fails to demonstrate direct evidence of ASP^+ transport by OCTNs. In addition, a previous publication by Grigat and co-workers (Grigat et al., 2009) comes to the conclusion that OCTN2 is not a general drug transporter and hence, does not translocate ASP^+ in HEK-293 cells. Moreover, the data on the structural ASP^+ analogue, MPP^+ reports only a very weak affinity to OCTN

transporters (Koepsell et al., 2007). We therefore believe that OCTNs are not involved in ASP^+ uptake by the cell lines investigated in our work.

Efflux of ASP^+ . Furthermore, the efflux of organic cations from human respiratory epithelium was investigated. Here as well, distinct differences between the pulmonary and intestinal cell culture models were observed. We found a time-dependent release from A549, Calu-3 and Caco-2 epithelial cells, whilst ASP^+ release from 16HBE14o-cell monolayers was seen only up to 10 min. After that, no significant release was detected and ASP^+ retention values remained more or less at the same level. However, up to 70% of intracellularly accumulated ASP^+ was released from A549 and Calu-3 cells, indicating the presence of an efficient efflux pump. In the case of ASP^+ release from A549 monolayers, only one high-affinity transporter site was identified with a similar K_m value as found in the uptake studies (i.e., 33 μM). ASP^+ release from A549 cell layers exhibited an about 50% lower V_{max} value than seen for the uptake of the fluorophore. No saturation of ASP^+ efflux could be reached in Calu-3, 16HBE14o- and Caco-2 cells. Our data demonstrated a remarkable pH-dependency of ASP^+ release in A549 cells, which was less pronounced in Calu-3 cells. A sodium-sensitive ASP^+ release could be identified in A549 and Calu-3 epithelial cells, which was, in the case of A549 cells, also susceptible to verapamil. Verapamil has been reported to inhibit P-glycoprotein functionality (Altenberg et al., 1993), but furthermore, is an inhibitor for MATE1 (Koepsell et al., 2007; Nies et al., 2011). It has been shown in small intestine, liver and kidney that uptake of organic cations was mediated by OCTs as the first step of excretion, concomitant with a second step involving the active efflux of organic cations (Koepsell et al., 2007). This second step is thought to be mediated by transporter proteins such as P-glycoprotein (*MDR1*, ABC transporter family) or MATE1 (*SLC47A1*,

SLC family) (Yonezawa and Inui, 2011). A bilateral interplay of OCTs and P-gp/MATE1 is essential for transcellular movements of organic cations (Nies et al., 2011). Otsuka *et al.* (Otsuka et al., 2005) and Terada *et al.* (Terada et al., 2006) reported a pH-dependent transport activity in MATE1-expressing HEK-293 cells. Accordingly, we speculate that MATE1 is involved in the efflux of ASP^+ at the alveolar epithelium and possibly also at the bronchial epithelium. Evidently, Caco-2 cells express P-gp, hence the efflux of ASP^+ might be accounted for by P-gp, since a pH-independent release was demonstrated (Hilgendorf et al., 2007; Altenburg et al., 1993). On the other hand, verapamil did not affect ASP^+ release.

3.5 CONCLUSIONS

In summary, respiratory epithelial cells functionally express a complex array of organic cation transporters. These transporters facilitate ASP^+ uptake into human respiratory epithelial cell monolayers. The major findings of this chapter were that alveolar A549 cell monolayers showed an absorption pattern different from bronchial 16HBE14o- and Calu-3 as well as intestinal Caco-2 monolayers. Figure 12 shows the hypothetical absorption pathways in the different cell types. The uptake of ASP^+ into A549 cells was consistent with active uptake of ASP^+ across the apical membrane mediated by organic cation transporters, most likely OCT2.

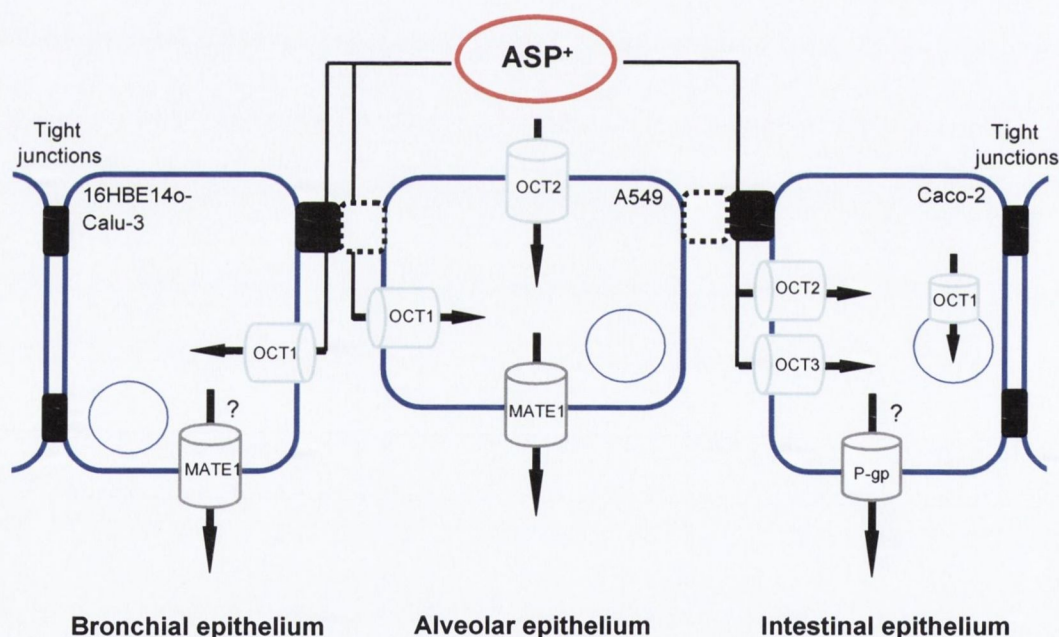


Figure 12. Proposed absorption pathways for ASP^+ in alveolar, bronchial and intestinal epithelial cells.

Beta-2 agonists and ICS were able to attenuate this process, highlighting possible drug-drug interactions at the respiratory epithelial barrier. In addition, concentration- and temperature-dependent uptake of ASP^+ across the basolateral membrane was observed.

It can be speculated that this transport was mediated by OCT1 at the bronchial epithelium. Moreover, we provided evidence for the presence of an efflux transporter in A549 and Calu-3 cell monolayers, possibly MATE1.

Studying drug transport processes in an organotypic *in vitro* model is far more complex than in expression systems transfected with only one or two transporters. Multiple organic cation transporters were found to be expressed at the respiratory epithelium. It cannot be ruled out that transporter proteins other than those encoded by *SLC22A1-5* or *SLC47A1* as well as passive diffusion are involved in the uptake of ASP⁺ (Salomon and Ehrhardt, 2012). Particularly, in the case where the transporter sites in question show a high degree of substrate overlap, and where no specific inhibitors are available, ultimate proof might be obtained using RNAi technique, but the paucity of good commercially available antibodies limits the application of this approach.

CHAPTER 4

The cell line H441 is a useful *in vitro* model for transport studies of distal lung epithelial barrier

Parts of this chapter have been submitted as:

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The cell line NCI-H441 is a useful *in vitro* model for transport studies of distal lung epithelial barrier. *Mol Pharm*, under revision

ABSTRACT

No continuously growing cell line of human alveolar epithelial phenotype with the ability to form polarised, electrically tight monolayers has been described to date. This constitutes a serious limitation in the area of inhalation biopharmaceutics, particularly, in the context of transepithelial transport studies. Here we investigated if the human lung adenocarcinoma cell line, H441, has potential to serve as an *in vitro* model of human distal lung epithelium. The development of epithelial barrier properties was studied by immunocytochemistry (ICC) against the tight junction protein, ZO-1, and measurement of transepithelial electrical resistance. The expression of P-glycoprotein (P-gp) and organic cation/carnitine transporters (OCT/Ns; *SLC22*) was investigated by ICC in addition to immunoblot. Uptake of ASP^+ was carried out, in order to assess organic cation transporter function *in vitro*. Furthermore, the effect of several organic cations on ASP^+ uptake was studied. H441 cells, when grown under liquid covered conditions, formed confluent, electrically tight monolayers with peak resistance values of approximately $1000 \Omega \cdot \text{cm}^2$, after 8-12 days in culture. The presence of P-gp, OCT1, OCT2, OCT3, OCTN1 and OCTN2 was confirmed by Western blot and ICC, similar to data from freshly isolated alveolar epithelial cells in primary culture. The uptake of ASP^+ was time- and temperature-dependent with $K_m = 881.2 \pm 195.3 \mu\text{M}$ and $V_{\max} = 2.07 \pm 0.26 \text{ nmol/min/mg protein}$. TEA, amantadine, quinidine and verapamil significantly inhibited ASP^+ uptake into H441 cells, whereas the effect of D-carnitine and ergothioneine, two OCTN substrates was less pronounced. H441 cells are the first cell line of human distal lung epithelial origin with the ability to form monolayers with appreciable barrier properties. Moreover, drug transporter expression and activity is

consistent with what has been reported for human alveolar epithelial cells in primary culture.

4.1 INTRODUCTION

The lung offers great potential as a portal into the systemic circulation for drugs with difficult oral pharmacokinetics or stability issues (Patton and Byron, 2007). Recent examples using this pulmonary route are aerosolised monomeric insulin (Afrezza[®]) and dihydroergotamine (Levadex[®]). Due to the complex structure of the respiratory tract, the fate of inhaled medicines after reaching the pulmonary mucosa thus far remains poorly understood (Patton et al., 2010). One of the less well conceived issues is epithelial transport, particularly in the bronchiolar and alveolar segments. One reason why the study of transport processes across the epithelial barriers of the distal lung has been seriously compromised is the absence of reliable, continuously growing cell culture models (Kim et al., 2001; Forbes and Ehrhardt, 2005). For other biological barriers, e.g., the intestine or indeed the proximal respiratory tract, a number of well characterised *in vitro* models are available, such as Caco-2, Calu-3 and 16HBE14o- (Ehrhardt et al., 2008a).

The respiratory zone, where the better part of systemic drug absorption is believed to occur, includes the terminal bronchioles and the alveolar sacs. Whilst the bronchioles are mainly populated by ciliated cuboidal epithelial and Clara cells, the alveolar barrier is formed by type I and type II pneumocytes (Weibel, 1963). Alveolar type II (ATII) cells are granular and roughly cuboidal in shape and cover approximately 5% of the total alveolar surface area, albeit they contribute to ~60% of the cell population by numbers (Stone et al., 1992). ATII cells participate in lung fluid homoeostasis and are responsible for the synthesis and secretion of surfactant (Kawada et al., 1989). The extremely thin, squamous alveolar type I (ATI) cell, on the other hand, forms the bulk (~95%) of the alveolar wall and also contributes to water and ion transport (Williams, 2003). A number of protocols for the

isolation and culture of alveolar cells from many species, including human, have been established over the years (Ehrhardt et al., 2008b). Whilst these primary cultures best reflect the situation *in situ*, they come with the downside that they are time consuming, expensive and require ethical consent (and a well developed tissue supply chain in the case of isolation from human specimens). The use of lung tissue from laboratory animals qualifies some of these disadvantages, but at the cost of yet unknown species differences and animal welfare concerns.

Immortal, continuously growing cell lines, in contrast, are much easier to handle, their use can be validated and they are suitable for high throughput assays (Li et al., 2008). The most commonly used cell line of human alveolar origin is A549. A549 cells were isolated from a human pulmonary adenocarcinoma and exhibit a morphologic phenotype similar to alveolar type II cells due to the presence of lamellar bodies and surfactant proteins (Lieber et al., 1976; Sporty et al., 2008). Whilst routinely used as a surrogate for human pneumocytes *in vitro*, A549 cells lack the ability to form electrically tight monolayers of polarised cells, due to their compromised tight junctions (Foster et al., 1998; Elbert et al., 1999).

A cell line with the potential to overcome some of the issues mentioned above is NCI-H441. The continuously growing NCI-H441 cell line was derived from the pericardial fluid of a patient with papillary adenocarcinoma of the lung and shows characteristics of Clara-like bronchiolar pulmonary epithelium (Hermanns et al., 2004; Vuong et al., 2002). Expression of markers typical of human alveolar type II cells like TTF-1 and the surfactant proteins SP-A, SP-B, SP-C and SP-D has also been confirmed in H441 cells (Gazdar et al., 1990). Hermanns and colleagues reported that H441 monolayers, when grown on permeable

supports, were able to obtain reasonable transepithelial electrical resistance (TEER) values in monoculture and in co-culture with microvascular endothelial cells (Hermanns et al., 2004).

The aim of this work is to characterise H441 cells as an *in vitro* model for transport studies of distal lung epithelial barrier. For this purpose the influence of different culture conditions on cell monolayer formation was measured. Moreover, the expression and function of prominent drug transporter molecules which have previously been associated with human alveolar barrier such as P-glycoprotein (P-gp) and the five members of the polyspecific organic cation transporter family (OCT1-3, OCTN1, OCTN2) was investigated (Endter et al., 2007; Salomon and Ehrhardt, 2012).

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

NCI-H441 (American Type Culture Collection, HTB-174) cells were obtained from LGC Standards (Teddington, UK) and cultured at 37°C in 5% CO₂ atmosphere in Gibco RPMI-1640 medium (Biosciences, Dun Laoghaire, Ireland) supplemented with 5% foetal bovine serum (FBS), 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich, Dublin, Ireland). In preliminary studies the optimal seeding density was established to be 75,000-250,000 cells/cm². H441 cells were routinely cultured in 75 cm² growth area tissue culture flasks (Greiner BioOne, Frickenhausen, Germany) and passaged when approximately 80% confluence was reached. Twenty-four hours post-seeding, the medium was replaced with RPMI medium which in addition contained dexamethasone (200 nM, Sigma-Aldrich) and ITS supplement (Roche Diagnostics Limited, West Sussex, UK). For liquid-covered culture (LCC) of cells were grown on Transwell Clear filter inserts (12 mm in diameter, pore size 0.4 µm; Corning, VWR, Dublin, Ireland), and apical and basolateral fluid volumes were 500 µl and 1500 µl, respectively. For cells grown under air-interfaces culture (AIC) conditions, the apical fluid volume was removed completely and the volume of the basolateral compartment was adjusted to 700 µl. H441 cells were used for up to 30 passages after receipt and the culture medium was exchanged every other day.

4.2.2 Human alveolar epithelial cell isolation and culture

Human alveolar type II (ATII) epithelial cells were freshly isolated from non-tumour lung tissue obtained from patients undergoing lung resection. The use of human material was

approved by Saarland State Medical Board, Germany. Isolation of ATII was performed according to a protocol adapted from Ehrhardt *et al.* (2005). Purified ATII cells were seeded at a density of 600,000 cells/cm² on collagen (Sigma-Aldrich) and fibronectin (BD Biosciences, Oxford, UK) coated plastics using complete small airways growth medium (SAGM) supplemented with penicillin, streptomycin and 1% FBS (Lonza, Verviers, Belgium). ATII cells were either used after 24 h of culture or after transdifferentiation into monolayers of type I-like (ATI-like) phenotype, following 8 days of culture (Demling *et al.*, 2006).

4.2.3 Bioelectric measurements

Transepithelial electrical resistance (TEER) values were measured daily with STX2 “chopstick” electrodes connected to a Millicell ERS volt-ohm-meter (Millipore, Carrigtwohill, Ireland). TEER values were corrected for the background value contributed by filter inserts and medium. For cells cultured under AIC conditions, fluid volumes were adjusted to 500 µl apically and 1500 µl basolaterally with pre-warmed medium and cell monolayers were then allowed to equilibrate for 10 min prior to TEER measurement. TEER values were obtained from at least three different passages.

4.2.4 Western blot

These experiments were performed in part by Veronika Fidelj (Universität Heidelberg, Germany).

Total cell protein extracts were generated from H441 and human alveolar epithelial cell monolayers grown on 6-well plates (Greiner BioOne, Frickenhausen, Germany) for 8 days.

Cell lysis was performed on ice in cell extraction buffer (Invitrogen, Karlsruhe, Germany) containing protease inhibitors (Sigma-Aldrich). Cell samples were sonicated twice for 10 s and then the lysate was centrifuged (10,000g at 4°C) for 20 min. The resulting supernatants were collected and sample concentrations were determined using a standard protein assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. Protein was loaded at equal concentrations and separated by electrophoresis using 6% (P-gp/MDR1) and 10% (OCTs) polyacrylamide gels, respectively. After electrophoresis, transfer onto immunoblot polyvinylidene fluoride membranes (Bio-Rad) was performed. Membranes were then blocked in 5% bovine serum album (BSA) in Tris-buffered saline with Tween 20 (pH 7.4) for at least 1 h at room temperature followed by incubation overnight at 4°C with the relevant primary antibody. Mouse monoclonal anti-MDR1 antibody (D-11) and polyclonal goat anti-OCTN1 and OCTN2 antibodies were obtained from Santa Cruz (Heidelberg, Germany), polyclonal rabbit anti-OCT1 from Aviva (San Diego, CA), polyclonal rabbit anti-OCT2 and OCT3 antibodies from Sigma-Aldrich. The antibodies were used at the following concentrations: P-gp (1:100), OCT1 (1:200), OCT2 (1:500), OCT3 (1:2000), OCTN1 and OCTN2 (1:200). Incubation with primary antibodies was followed by three washings with PBS containing 0.1% Tween 20 and subsequent incubation with the relevant HRP-conjugated secondary antibodies at concentrations of 1:12,500 (anti-mouse and anti-rabbit, Promega (Medical Supply Company, Dublin, Ireland)) or 1:22,500 (anti-goat, Sigma-Aldrich) at room temperature for 1 h. After additional three washings with PBS containing 0.1% Tween 20, peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland).

4.2.5 Immunocytochemistry

Lab-Tek chamber slides (Nunc, Roskilde, Denmark) were used to grow cell monolayers for immunohistochemistry. Cells were cultured for 24 h (ATII) or 8 (ATI-like, H441) days, before they were fixed with 4% (w/v) paraformaldehyde solution for 10 min and then incubated for 10 min in PBS containing 50 mM NH_4Cl , followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100 in PBS. After 60 min incubation with 200 μl of a 1:50 (OCT1, OCTN1 and OCTN2; all from Aviva), 1:250 (OCT2; Sigma-Aldrich), 1:300 (ZO-1; BD Biosciences, Oxford, UK), 1:500 (P-gp; Santa-Cruz) or 1:1000 (OCT3; Sigma-Aldrich) dilution of the relevant primary antibody, cell monolayers were rinsed three times with PBS, before incubation with 200 μl of a 1:300 dilution of the respective Alexa Fluor-labelled F(ab')_2 fragment (Biosciences, Dun Laoghaire, Ireland) in PBS containing 1% (w/v) BSA. Propidium iodide (1 $\mu\text{g/ml}$ in PBS) was used to counterstain cell nuclei. After 30 min of incubation, the specimens were again rinsed three times with PBS and embedded in FluorSave anti-fade medium (Merck, Nottingham, UK). Images were obtained using a confocal laser scanning microscope (Zeiss LSM 510, Göttingen, Germany) with the instrument's settings adjusted so that no positive signal was observed in the channel corresponding to the fluorescence of the relevant isotypic controls.

4.2.6 Transport studies

These experiments were performed in part by Veronika Fidelj (Universität Heidelberg, Germany).

All transport experiments were performed in freshly prepared bicarbonated Krebs-Ringer buffer (KRB, pH 7.4) composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4 , 25 mM NaHCO_3 , 1.8 mM CaCl_2 , 0.81 mM MgSO_4 , and 5.55 mM glucose,

unless stated otherwise. H441 cells were grown on Transwell Clear filters for at least 8 days and were only used when TEER values exceeded $350 \Omega \cdot \text{cm}^2$ (Ehrhardt et al. 2002). Prior to the actual experiment, both sides of the cell monolayers were washed twice with pre-warmed KRB solution, followed by a 60 min equilibration in KRB at 37°C. To initiate transport studies, the pre-incubation buffer was replaced with rhodamine 123 (Rh123, 50 μM , Sigma-Aldrich), fluorescein-Na (50 μM , Sigma-Aldrich) or ASP⁺ (100 μM , Bio-Sciences, Dun Laoghaire, Ireland) in the respective donor chamber. For apical-to-basolateral transport, 0.52 ml donor solution was added; for basolateral-to-apical transport, 1.52 ml. The initial donor concentration was determined by taking a 20- μl sample directly after addition of the donor fluids. Cell monolayers were kept at 37°C during the experiment and a 200- μl sample was collected from the respective receiver compartments after 90 min. At the end of transport studies, another 20- μl sample was collected from the donor side to allow determination of mass balance. Each experiment was run at least in triplicate. TEER values were recorded before and after the transport studies, in order to verify the cell layer integrity. In P-gp inhibition experiments, verapamil (50 μM) was added to all donor and receiver solutions.

The fluorescence activity of samples was assessed in 96-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 nm and 520 nm, respectively. Samples were diluted with KRB solution, where appropriate.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = (\Delta Q / \Delta t) / (A * C_0) \quad [\text{Eq. 1}]$$

where ΔQ was the change in amount of the compound over a designated period of time (Δt), A was the nominal surface area of the filters (1.13 cm^2) and C_0 was the initial concentration of the drug in the donor fluid.

4.2.7 ASP^+ uptake studies

These experiments were carried out in part by Julia Gausterer (Universität Wien, Austria).

Uptake studies were carried out on cell monolayers grown for 8 days on 24-well plates. The cationic fluorescent probe, ASP^+ was used to investigate time-, concentration-, temperature-, pH- and sodium ion concentration-dependence of organic cation uptake as described before (Chapter 3). All experiments were carried out in KRB solution (pH 7.4) unless described otherwise. Time-dependence of ASP^+ uptake was studied by incubating H441 and ATI-like cell monolayers with ASP^+ (at $10 \text{ }\mu\text{M}$ final concentration) for up to 90 min at 37°C . The uptake reaction was stopped at certain time points by rinsing the cells twice with ice-cold KRB. Subsequently, monolayers were solubilised with 1% (w/v) Triton X-100 and the fluorescence activity of ASP^+ in the cell lysate was measured (*see below*). Concentration-dependence of ASP^+ uptake into H441 cells was studied at a range from 10 to $1000 \text{ }\mu\text{M}$ at 37°C and 4°C for 20 min. The effect of varying pH values (i.e., pH 5.7, 6.5 and 8.2) on ASP^+ uptake into H441 cells was studied in order to assess pH-dependence. In addition, Na^+ -free medium was prepared by replacing NaCl, NaH_2PO_4 and NaHCO_3 salts with equimolar concentrations of KCl, KH_2PO_4 and KHCO_3 (i.e., $5.4 \text{ mM } [\text{Na}^+]$ and $142 \text{ mM } [\text{K}^+]$) or N-methyl-D-glucamine (NMG), KH_2PO_4 and KHCO_3 (i.e., $5.4 \text{ mM } [\text{Na}^+]$ and

137 mM [NMG]), respectively to investigate sodium ion concentration-dependence of ASP^+ uptake into H441 cells.

It was also investigated in how far the presence of organic cations, i.e., acetylcholine (1 mM and 2 mM), amantadine (100 μM and 500 μM), corticosterone (100 μM), D-carnitine (100 μM and 500 μM), L-ergothioneine (100 and 200 μM), hemicholinium-3 (HC-3, 100 μM and 500 μM), quinidine (100 μM and 500 μM), tetraethylammonium (TEA, 1 mM and 20 mM) and verapamil (10 μM and 200 μM) affected ASP^+ accumulation in H441 monolayers. Concentrations of these organic cations were chosen according to their published K_i or IC_{50} values.^{25,26} Furthermore, the effect of two β_2 -receptor agonists (i.e., formoterol and salbutamol) as well as two inhaled glucocorticosteroids (ICS, i.e., budesonide and beclomethasone) on ASP^+ uptake into H441 cells was determined. The β_2 -receptor agonists were used at final concentrations of 100 μM and 500 μM . The inhibitory potential of budesonide and beclomethasone was studied at concentrations of 10 and 30 μM . All drugs and compounds were dissolved in KRB (pH 7.4). The ICS drugs were freshly prepared in KRB containing 0.01% DMSO as co-solvent. A similar amount of DMSO was tested in the relevant controls and found to not significantly affect the study (data not shown). In all cases, cell monolayers were incubated simultaneously with ASP^+ (10 μM) and the compound of interest for 20 min.

In order to open tight junctions and allow better access to basolaterally localised transporters, calcium complexation was carried out by incubation with 5 mM EDTA for 15 min prior to ASP^+ uptake. The fluorescence activity of samples was assessed in 24-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 nm and 590 nm, respectively.

4.2.8 Statistical analysis

All experiments were carried out at least in triplicate using cells from at least three different passages ($n = 3 - 30$). Results, expressed as means $\pm$ S.D. were compared using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test. $P < 0.05$ was considered as significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Barrier properties

Transepithelial electrical resistance of H441 monolayers was consistent with what is commonly reported for monolayers of ATI-like cells in primary culture (Elbert et al., 1999; Cheek et al., 1989). When grown under LCC condition on permeable polystyrene supports, H441 cells were able to form electrically tight monolayers, reaching peak TEER values of $1010 \pm 105 \Omega \cdot \text{cm}^2$ after 13 days in culture (Figure 1A). A very regular signal for ZO-1 along the cell-cell contacts was visible in the confocal laser scanning micrographs (Figure 1B), confirming well organised tight junctions and cell polarisation. TEER was significantly lower, when cell monolayers were cultured at an air-liquid interface; the ZO-1 signal, nevertheless, remained unchanged (data not shown). There was no clear peak in monolayer resistance, instead a plateau with values ranging from 280 to $315 \Omega \cdot \text{cm}^2$ was observed between days 9 and 17 in culture (Figure 1). In a preliminary study, we tested a cell culture medium without dexamethasone, but neither LCC nor AIC grown cells were able to develop TEER above $100 \Omega \cdot \text{cm}^2$ under these conditions (data not shown). In a previous study, Hermanns *et al.* reported TEER of $218 \pm 83 \Omega \cdot \text{cm}^2$ for H441 cell monolayers in monoculture (Hermanns et al., 2004). The significantly higher values in our work can be explained by the use of different filter materials (polystyrene *vs.* polycarbonate), a higher cell density ($250,000 \text{ cells/cm}^2$ *vs.* $20,000 \text{ cells/cm}^2$) or a lower dexamethasone concentration (200 nM *vs.* 1 μM). The observation that AIC gives rise to cell layers exhibiting lower bioelectric properties has been made before on a number of occasions. For Calu-3 human airway cells, which have many similarities to submucosal gland serous cells, TEER values for cells grown in LCC were also approximately three

times larger than for cells grown in AIC (Stentebjerg-Andersen et al. 2011). The two immortalised human bronchial epithelial cell lines, 16HBE14o- and CFBE41o-, on the other hand, fail to generate electrically tight cell layers, when grown air-liquid interfaced (Forbes et al., 2003; Ehrhardt et al., 2002; Ehrhardt et al., 2006). Respiratory epithelial cells might have to be of a surfactant or mucus producing phenotype to cope with AIC conditions, but this is purely a speculation and beyond the scope of this work.

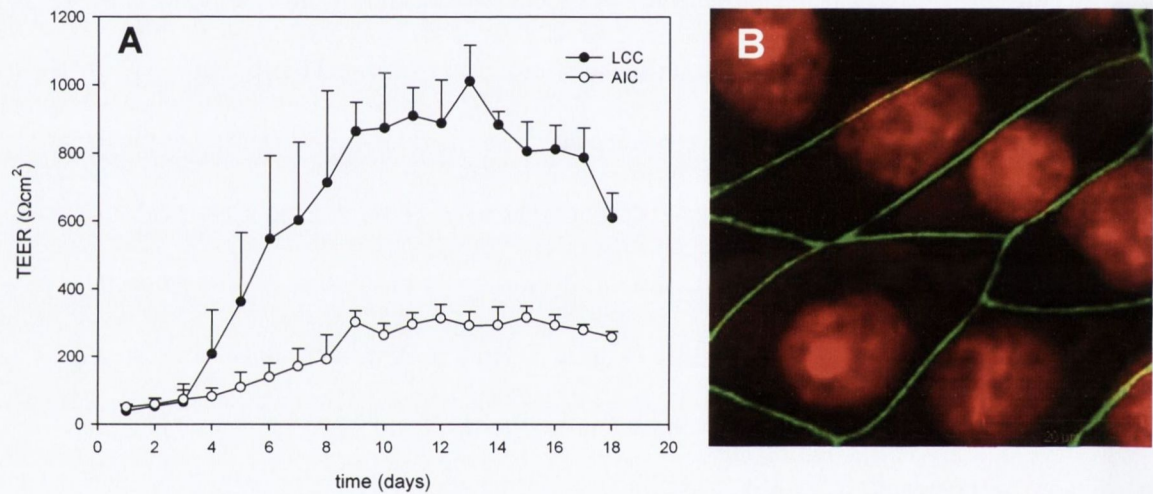


Figure 1. A Time course of TEER development in H441 cell monolayers. Cells were seeded at a density of 2.5×10^5 cells/cm² on Transwell Clear filter inserts and grown under liquid-covered culture (LCC, ●) or under air-interfaced culture (AIC, ○) conditions from day 1 onwards. Each data point represents means $\pm$ SD ($n > 12$) from at least three different passages. **B** Immunostaining of tight junctional protein, ZO-1 (green) in H441 cell monolayers. Cells were seeded on chamber slides and cultured for 8 days. Cell nuclei were counterstained with propidium iodide (red). Bar represents 20 μm.

4.3.2 P-glycoprotein expression and function

TEER is very closely related to trans-monolayer permeability of passively diffusing solutes (Ehrhardt et al., 2002a). It can thus be assumed that passive transport processes across H441 monolayers are comparable to those across primary alveolar epithelial cells. In any case, determination of passive diffusion does not require the additional work and expense of tissue culture, as several phospholipid-based alternatives are available (Flaten et al. 2006; Reis et al., 2010). The main feature of a *bona fide* organotypic cell-based *in vitro* model is that it most closely resembles the biological barrier of interest. And whilst passive permeation is fairly comparable across, e.g., epithelial cell of the lung and the intestine (Tronde et al., 2003), major differences have to be acknowledged when it comes to drug transporter mediated permeability (Bosquillon, 2010). Drug transporters have only recently moved into the centre of attention of pulmonary drug delivery research (Gumbleton et al., 2011; Endter et al., 2009), and of the over 400 identified transporter proteins, P-glycoprotein (MDR1) is probably the most prominent one. In Figure 2A, a representative immunoblot of P-gp in H441 cell monolayers is shown. Bands from LCC and AIC grown cells showed similar appearances and intensities, thus culture conditions have only negligible effects on abundance of the transporter protein. A punctate staining pattern for P-gp, mostly localised along the apical membrane, was observed by CLSM (Figure 2B). Intensity and pattern of the signal were comparable to previously published data of human alveolar epithelial barrier *in vitro* (Campbell et al., 2003; Endter et al., 2007). Expression levels of P-gp in the lung are generally well below what is found in other organs, e.g., the liver, intestine or blood-brain-barrier (Campbell et al., 2003). The lower expression levels

of P-gp in lung epithelium are paralleled by a lower transporter activity (by means of efflux ratio) in respiratory epithelial cells *in vitro* (Ehrhardt et al., 2003; Madlova et al., 2009).

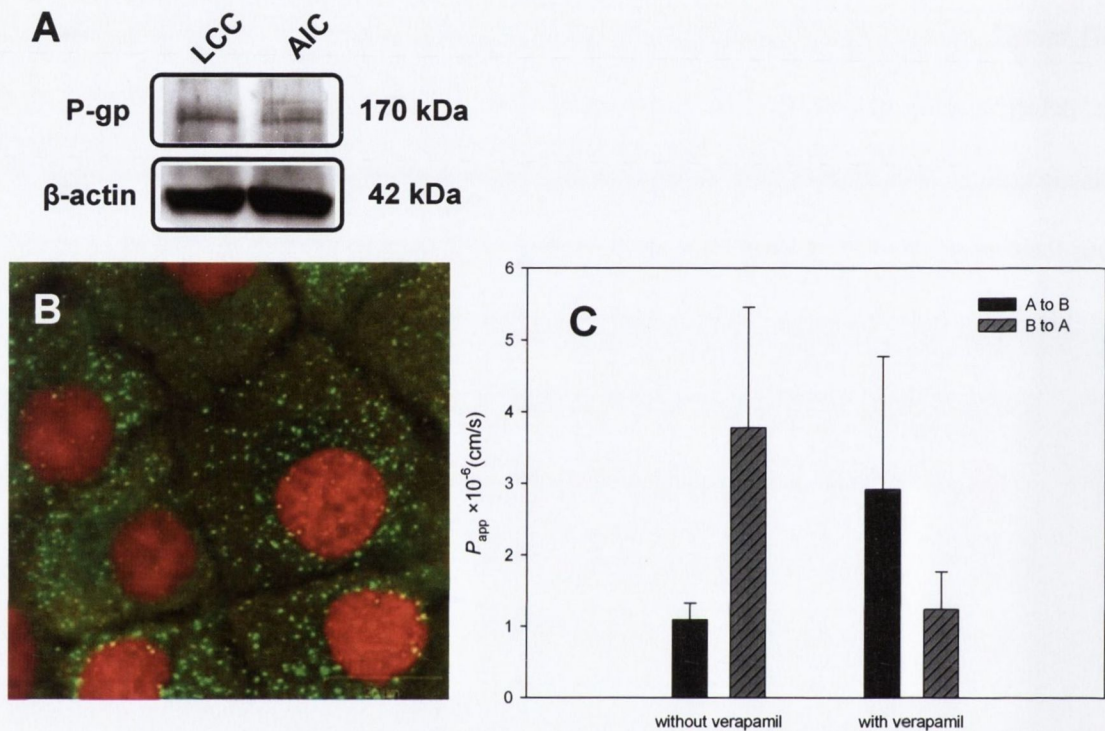


Figure 2. **A** Representative Western blot of P-glycoprotein (P-gp) in H441 human bronchiolar epithelial cells. Cells were cultured under LCC or AIC conditions for 8 days on Transwell Clear filter inserts. Beta-actin was used as loading control. **B** Immunostaining of P-gp (green) in H441 cell monolayers. Cells were grown on chamberslides and stained on day 8. Cell nuclei were counterstained with propidium iodide (red). Scale bar represent 20 μ m. **C** Permeability of rhodamine 123 across H441 cell monolayers. Cells were cultured for at least 8 days on Transwell Clear filter inserts. Transport studies were performed in the presence and absence of verapamil (50 μ M). Black bars represent absorptive and grey bars secretory direction. Data are represented as mean $\pm$ SD, $n = 9 - 12$.

Here, bidirectional transport studies of rhodamine 123 showed a significant net secretion, which was sensitive to inhibition by verapamil (Figure 2B). The calculated rhodamine 123 efflux ratio (at 50 μ M) was 3.43 in H441 cells, which was very similar to a value of 3.09

obtained in monolayers of human ATI-like cells (Endter et al., 2007), but significantly lower than an efflux ratio of 10.5, published in the case of Caco-2 cells (Troutman and Thakker, 2003). Of note, in all three studies rhodamine 123 was used at similar concentrations, i.e. 50 μ M.

Table 1. P_{app} values of Fluorescein-Na and ASP⁺ across H441 cell monolayers are shown. Transport was measured at 37°C for 90 min. Cells were seeded on Transwell Clear inserts and grown under AIC and LCC conditions for at least 12 days. Values are represented as mean $\pm$ SD (n=3-6), **P< 0.01 significantly different from a-to-b direction (AB).

compounds	P_{app} (x10 ⁻⁶ cm*s ⁻¹)			
	LCC		AIC	
	AB	BA	AB	BA
Fluorescein-Na	2.07±1.08	2.71±0.61	3.27±0.37	7.14±1.43**
ASP ⁺	3.89±0.24	4.39±0.90	0.78±0.06	2.62±0.84**

To assess the paracellular permeability of H441 cells, transport experiments were performed with the anionic hydrophilic marker, Fluorescein-Na (Table 1). Interestingly, LCC-grown cells exhibit no significant transport direction, whilst AIC-grown cells show a significant net secretion. Calculated P_{app} values were distinctly different from values obtained in hAEpC (1.0x10⁻⁷ (AB) and 1.2x10⁻⁷ (BA) cm*s⁻¹) with TEER values being higher found for the hAEpC monolayers (Endter et al., 2007). The P_{app} values were higher for AIC- than LCC-grown cells, indicating the leakiness of the cell layer. Transport of the

organic cation ASP^+ revealed a net secretion for LCC- and AIC-grown cells with a higher flux rate under LCC conditions.

4.3.3 Organic cation transporters expression and function

This is the first report detailing expression of the five cloned members of the human polyspecific organic cation transporter (OCT/N) family in human alveolar epithelial cells and in the H441 cell line. These transporters might have therapeutic relevance, as many drugs used in inhalation therapy (e.g., bronchodilators) are organic cations and thus, potential substrates of OCT/Ns (Ehrhardt et al., 2005; Salomon and Ehrhardt, 2012). In addition, it was shown that inhaled corticosteroids (ICS) although they are uncharged molecules, are inhibitors of the polyspecific organic cation transporter 2 (hOCT2) (Lips et al., 2005). In Figure 3, representative immunoblots and the corresponding confocal laser scanning micrographs for OCT1, OCT2, OCT3, OCTN1 and OCTN2 are shown. Generally, expression of all transporter proteins was confirmed in all cell types investigated. Robust bands were observed in Western blots from H441, ATII and ATI-like cells in the case of OCT1 (Figure 3A). In CLSM of H441 monolayers, OCT1 showed a comparably weaker signal with predominant localisation along the lateral membranes and a number of additional intracellular spots. OCT1 immunostaining in freshly isolated ATII cells and ATI-like cell monolayers was considerably stronger. In both primary cell types the exact determination of the protein locus by CLSM was impossible, as freshly isolated ATII pneumocytes are non-polarised and ATI-like cells are extremely thin. OCT2 was also found in the three cell models (Figure 3B). This is in agreement with recently published data on OCT2 expression in alveolar A549 cells (chapter 2). In the same study OCT2 was absent

from cell lines of the bronchial origin (i.e., Calu-3 and 16HBE14o-). A relatively weak and diffuse CLSM signal was again obtained in the case of H441 cells. Nonetheless, bands for OCT2 were clearly visible for H441, ATII and ATI-like cells. Data for OCT3 were very similar to OCT2 (Figure 3C). In the case of the two carnitine transporters, OCTN1 and OCTN2, Western blot analysis revealed rather weak bands in all cells, which was probably related to the quality of the antibodies used (Figure 3D and 3E). Immunocytochemistry, on the other hand, yielded micrographs of reasonable quality, confirming the expression of both transporters in all three cell types studied. Horvath and colleagues previously presented immunohistological evidence of OCTN1 and OCTN2 expression in alveolar epithelial cells of human parenchyma *in situ* (Horvath et al., 2007). However, using immunofluorescence technique in an intricate tissue such as lung parenchyma renders it difficult to allocate the staining to a specific cell type. Our data supports Horvath's findings and moreover, suggest that OCTN1 and OCTN2 are localised to both alveolar epithelial cell types.

ASP⁺ was only recently used as fluorescent substrate in the functional expression analysis of OCT/N transporters in a number of respiratory epithelial cell models (Mukherjee et al., 2012; Horvath et al., 2007). Here, we determined, if ASP⁺ uptake was time- and concentration-dependent, and if it was controlled by extracellular proton and Na⁺ concentrations. As shown in Figure 4A, accumulation of ASP⁺ in H441 cells was linear over 90 min. Uptake of ASP⁺ into H441 cells, when studied after the tight junctional complex was opened using EDTA and thus transporter sites localised to the basolateral membranes became accessible to ASP⁺, was increased ~6-fold (Figure 4B).

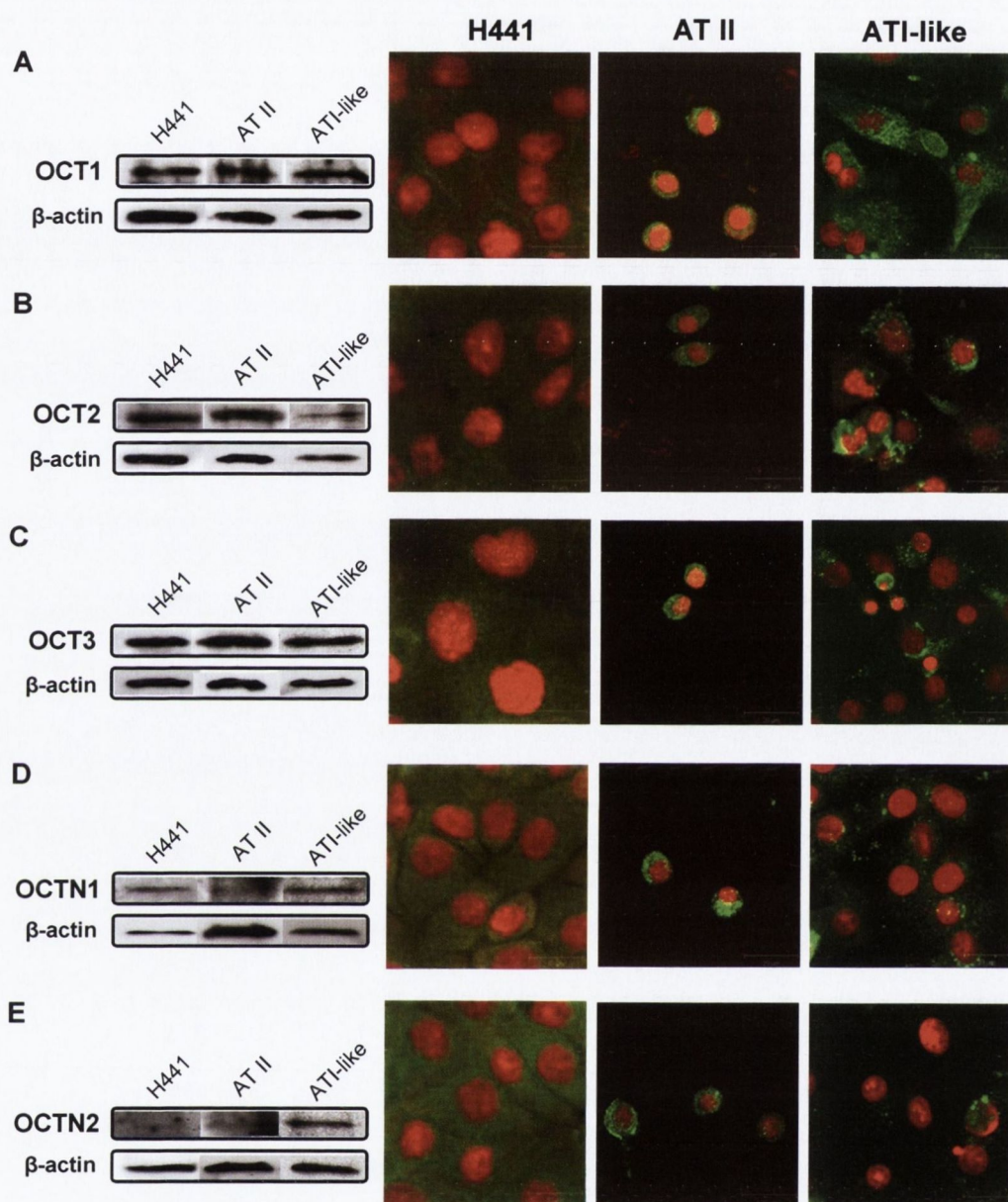


Figure 3. Expression of organic cation transporter proteins in H441 human bronchiolar cell line and human alveolar epithelial cells in primary culture (ATII and ATI-like). The left panel shows representative Western blot and the right panel immunohistochemistry of organic cation transporters, (A) OCT1 (66 kDa), (B) OCT2 (65 kDa), (C) OCT3 (70 kDa), (D) OCTN1 (65 kDa) and (E) OCTN2 (70 kDa). Cells were either used 24 h post isolation (ATII) or cultured for 8 days (H441 and ATI-like). The relevant protein of interest displays a *green* signal. Nuclei were counterstained with propidium iodide (*red*). Bars represent 20 μ m. Beta-actin was used as loading control.

Uptake rates into H441 cells, as well as the tight junction-related phenomenon were very similar to what was previously observed in Calu-3 cell monolayers, whereas non-polarised A549 cells showed much higher accumulation (chapter 3). In Figure 4C, the concentration-dependence of ASP⁺ uptake into H441 cell monolayers is illustrated. ASP⁺ uptake was a saturable process with $K_m = 881.2 \pm 195.3 \mu\text{M}$ and $V_{\text{max}} = 2.07 \pm 0.26$ nmol/min/mg protein. Horvath *et al.* calculated a K_m of 394 μM for ASP⁺ uptake into airway epithelial cells in primary culture (Horvath et al., 2007). In Chapter 3, a K_m of 457 μM for the low-affinity ASP⁺ uptake into A549 cells was reported. ASP⁺ uptake into H441 cells was further found to be significantly reduced at lower extracellular pH values (Figure 4D) and decreased Na⁺ concentrations (Figure 4E), as well as to be significantly reduced at 4°C (Figure 5). Also in Figure 5, data from inhibition studies using various modulators of organic cation transport are shown. TEA resulted in a ~35% decrease of ASP⁺ uptake and verapamil diminished ASP⁺ accumulation by ~70% (at 200 μM). Both substances have the potential to inhibit all five putative OCT/Ns at the concentration range used (Koepsell et al., 2007). Quinidine also showed a concentration dependent inhibition of more than 50%. The steroids, corticosterone, beclometasone and budesonide did not have much of an effect. Amantadine, like the steroids an inhibitor of mainly OCT2 function, showed more pronounced inhibition, but the concentrations used were also considerably higher. The OCTN substrates, ergothioneine and D-carnitine, did not reduce ASP⁺ uptake. However, ASP⁺ uptake was attenuated by 20-30%, when the choline transporter substrates, HC-3 and acetylcholine were present in the transport buffer. A concentration-dependent inhibition of ASP⁺ uptake into A549 cells was also achieved with formoterol and salbutamol.

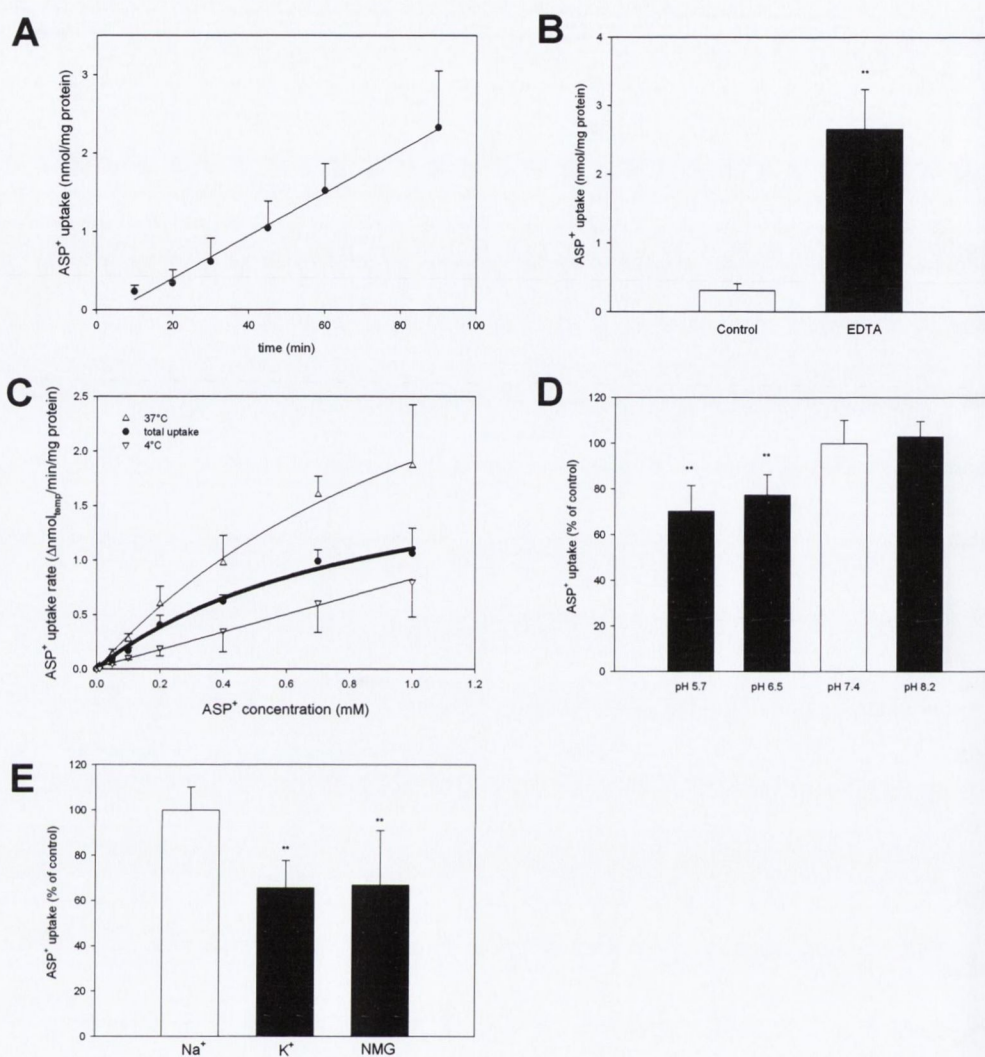


Figure 4. **A** Time-dependent uptake of ASP⁺ into H441 cell monolayers. Cell layers were grown on plastic for 8 days and then incubated with ASP⁺ (10 μM) as indicated. Uptake values are corrected for the uptake at 4°C. **B** Effect of tight junctional barrier on ASP⁺ uptake (10 μM) was investigated. H441 cell monolayers were incubated with ASP⁺ in the presence and absence of 5 mM EDTA. **C** Concentration-dependent uptake of ASP⁺ into human bronchiolar epithelial cells. Cell layers were incubated at pH 7.4, 37°C and 4°C for 20 min as indicated. Total uptake rates (●) were calculated as the difference in ASP⁺ uptake between 37°C (Δ) and 4°C (▽). **D** Effect of extracellular pH on uptake of ASP⁺ in H441 cells. H441 cells were incubated with ASP⁺ (10 μM) at pH 5.7 - 8.2. **E** Effect of extracellular cations on ASP⁺ uptake by H441 cells. ASP⁺ uptake was measured at pH 7.4, 37°C for 20 min. Na⁺ was replaced with K⁺ or N-methyl-D-glucamine (NMG). Data are represented as mean ± SD, *n* = 3 - 9. * *P* < 0.05, ** *P* < 0.01 versus control.

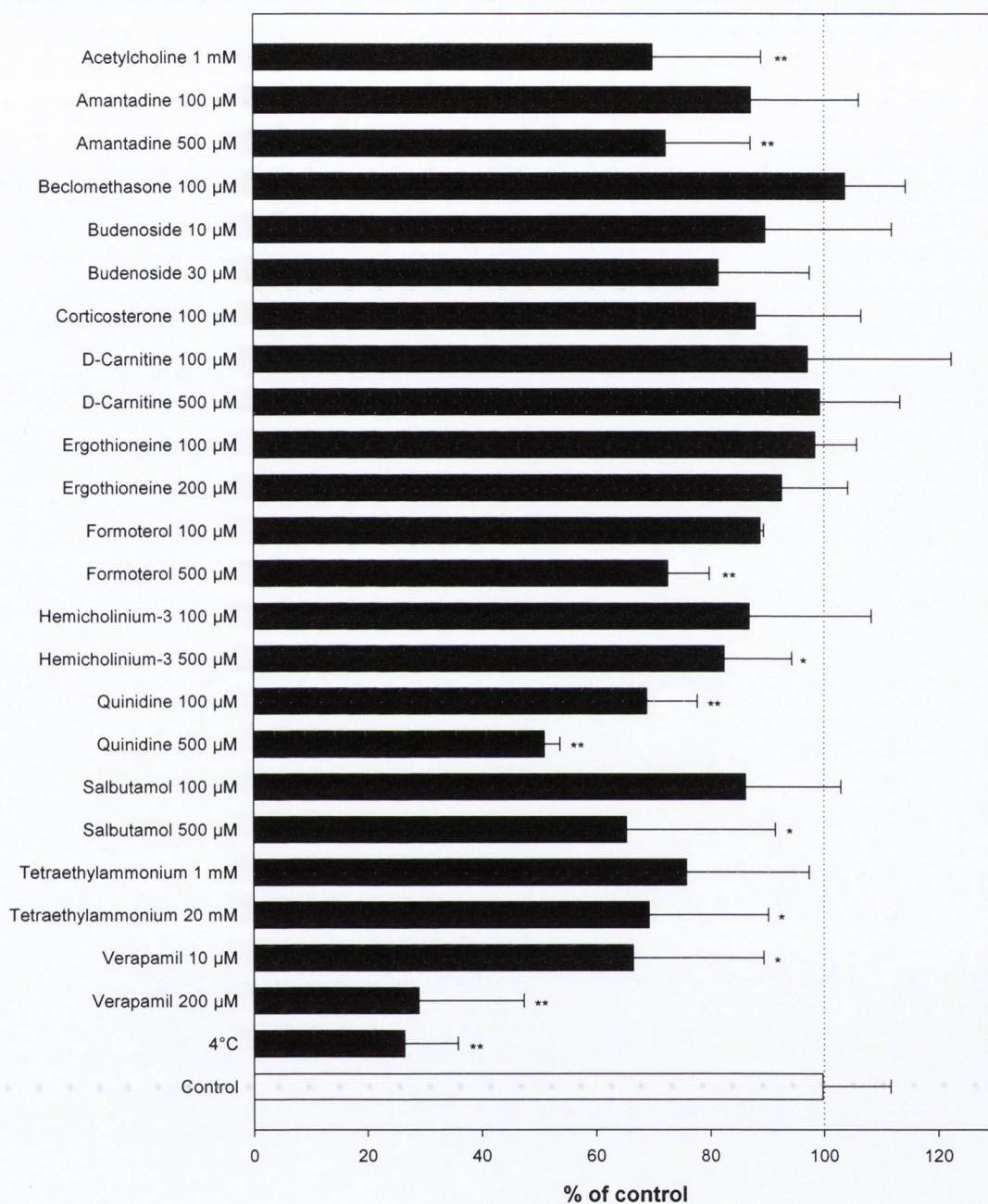


Figure 5. Inhibitory effects of model organic cations and pulmonary administered drugs on ASP^+ uptake into H441 cells. ASP^+ uptake (at 10 μ M) was measured in cell layers grown on plastic for 8 days at pH 7.4, 37°C for 20 min in the presence of the indicated concentration of the respective compound. Data are represented as mean $\pm$ SD, $n = 3 - 9$. * $P < 0.05$, ** $P < 0.01$ versus control.

We previously reported that ASP⁺ accumulation into A549 cells occurred via two distinct transporter sites, whereas comparably lower uptake levels and no saturation of ASP⁺ absorption were observed in polarised Calu-3 and 16HBE14o- monolayers. In the same study, most pharmacological inhibitors showed only relatively weak activities in the bronchial models. The exception to this was the highly lipophilic compound, verapamil. The observations in H441 cells fall in between of what was seen in the alveolar and bronchial cell lines. OCT-mediated uptake reached saturation (albeit with rather high K_m), but opening of tight junction also showed an enhancement effect. Moreover, most organic cations investigated as inhibitors were significantly less active than in A549 cells (see Chapter 3). This allows the speculation that in the bronchiolar region a transition of organic cation transport occurs. In the upper airways, it is believed that mostly basally localised OCT1 (and probably apically expressed OCTN2) are the main transporters, and in the alveolar epithelium apically OCT2 and basally OCT1 operate. The bronchiolar H441 cell expresses OCT2 and OCTNs, but these transporters do not appear to be overly active, whilst basally localised OCT1 facilitates the bulk organic cation uptake. This hypothesis, however, does not consider the possible involvement of other ASP⁺ recognising transporters (e.g., MATE1) that have not been identified in these cells to date.

4.4 CONCLUSIONS

H441 are the first cell line of human distal lung descent with the ability to form electrically tight, polarised cell monolayers. Moreover, H441 express a number of key drug transporters very similarly to human alveolar epithelial cells in primary culture. P-gp function, as determined by rhodamine 123 transport, was comparable between the two systems. Within certain limitations, H441 are therefore a useful *in vitro* model for transport studies of distal lung epithelial barrier.

CHAPTER 5

Pathways of organic cation uptake across human respiratory epithelial barriers in vitro

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ABSTRACT

Organic cation/carnitine transporters (OCT/N; *SLC22*) are known to be involved in the translocation of cationic compounds across cell membranes. In Chapter 2, OCT/N expression in lung epithelial barriers was described. Here, we aimed to characterise and compare OCT/N function in bronchial, bronchiolar and alveolar epithelial cell lines *in vitro*. Cells were grown to confluent monolayers for 5 (A549), 8 (H441) and 12 (Calu-3) days, respectively. OCT/N mediated uptake was studied using [^{14}C]-tetraethylammonium (TEA) and [^3H]-acetylcarnitine (Ac-Car). The effects of OCT/N modulators such as amantadine, D- and L-carnitine, unlabelled TEA, verapamil and hemicholinium-3 (HC-3) were investigated. Furthermore, transepithelial permeation of TEA and Ac-Car was studied across Transwell-grown H441 and Calu-3 monolayers. TEA uptake was further studied in A549 cells after RNAi knockdown of OCT1, OCT2 or OCT3. In A549 cells, uptake of TEA and Ac-Car was temperature-sensitive and time- and concentration-dependent. Non-saturable as well as saturable transport for both OCT/N probes was noted. K_m values were calculated as $528.5 \pm 373.1 \mu\text{M}$ (TEA) and $16.1 \pm 10.9 \mu\text{M}$ (Ac-Car), respectively. MPP^+ , amantadine and verapamil markedly inhibited TEA uptake into A549 cells. In H441 and Calu-3 cells, TEA uptake was also time-dependent. Increased substrate concentrations, however, did not result in saturation. In the case of Ac-Car uptake into Calu-3 cells, a non-saturable as well as a saturable transporter site was observed with a K_m value of $21.9 \pm 52.1 \mu\text{M}$, whereas in H441 cells no saturation was obtained. TEA uptake into Calu-3 and H441 epithelial cells was decreased in the presence of corticosterone, MPP^+ and TEA. Ac-Car uptake was affected by D-carnitine and verapamil in H441 and Calu-3 cells. TEA transport studies revealed a P_{app} (a-to-b) of $3.4 \pm 0.3 \cdot 10^{-7} \text{ cm/s}$ for Calu-3 and P_{app} (a-to-b) of $2.8 \pm 0.2 \cdot 10^{-6} \text{ cm/s}$ for H441 cells. In the case of bidirectional transport of Ac-Car, P_{app}

values of $1.3 \pm 0.1 \cdot 10^{-7}$ cm/s (a-to-b) for Calu-3 and P_{app} of $3.6 \pm 0.2 \cdot 10^{-6}$ cm/s (a-to-b) for H441 cells were measured. Bronchial and alveolar epithelial cell lines functionally expressed OCT/Ns *in vitro*. Knockdown of OCT1 and OCT2 significantly inhibited [14 C]-TEA uptake to $52.5 \pm 3.2\%$ and $50.4 \pm 8.6\%$, respectively, whilst knockdown of OCT3 reduced TEA uptake only marginally. Alveolar epithelial cells generally showed higher uptake activity of organic cations; in particular OCTs appeared to play an important role. At the bronchial epithelial barrier, the main transporter for organic cations still remains to be identified.

5.1 INTRODUCTION

Whilst other organs such as the liver, gut and kidneys have largely been characterised with regards to their transporter expression and activity, the lung remains uncharted terrain. Only very recently reports were published describing transporter expression and activity in respiratory epithelial cells, but the picture emerging is far from complete. Data available in the public domain on OCT (*SLC22A1-3*) and OCTN (*SLC22A4-5*) expression in the respiratory epithelium *in situ* and *in vitro* is, in many cases, limited to PCR-based studies (Endter et al., 2009; Mukherjee et al., 2012; Bleasby et al., 2006, Horvath et al., 2007, Courcot et al., 2012), whilst little evidence is confirmed by immunohistochemistry or transporter function (Lips et al., 2005). In Chapter 2, we have confirmed the expression of OCT1, OCT2, OCT3, OCTN1 and OCTN2 on protein level in human respiratory epithelial cell lines. OCT/N protein abundance was found to be more or less equivalent for all transporters across the respiratory epithelial cell types, except for OCT2 (only detected in A549 cells) and OCTN2 (higher expression levels in the bronchial cells) (Salomon et al., 2012).

A variety of cell culture systems is available for the modelling of the respiratory epithelium and to study the transport mechanism of exogenous and endogenous xenobiotics. Amongst them is the commonly used alveolar epithelial model, A549. A549 cells have been used as a model for many biopharmaceutical investigations, although this cell line does not form tight monolayers (Forbes and Ehrhardt, 2005; Buckley et al., 2011). For permeability studies, however, this barrier function is essential. 16HBE14o- and Calu-3, both of bronchial epithelial origin, form tight junctions and generate high transepithelial electrical resistance (TEER). The bronchiolar epithelial cell line, H441, has not yet been widely used as a cell culture model in a

biopharmaceutical context. H441 cells possess hallmarks of both alveolar and bronchiolar epithelial phenotype and also have the ability to form tight monolayers (see Chapter 4). Although, freshly isolated cells in primary cultures most closely resemble the situation *in situ*, it is rather challenging to employ them for functional studies, due to the restricted access and the relatively high costs.

In Chapter 3 of this thesis, 4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (ASP⁺), a cationic fluorescent dye, was used for initial investigations of the uptake of organic cations in several epithelial cell types. In addition, other labs have widely used this compound in a similar context (Stachon et al., 1996; Horvath et al., 2007). We found concentration- and temperature-dependent net absorption across monolayers of human respiratory epithelial cells. However, in our studies, evidence emerged that ASP⁺ was a sub-optimal surrogate for radiolabelled OCT/N substrates, as it is a high affinity ligand of monoamine transporter such as NET, DAT and SERT. Reported K_m values for ASP⁺ in heterologous systems expressing monoamine transporter were about 1000-times lower than those found in our study. For example, a K_m of 834 nM for ASP⁺ was published for a NET-MDCK transfectant-based HTS assay (Haunsø and Buchanan, 2007). Therefore, the aim of this Chapter was to elucidate the functional expression of OCT/Ns using radiolabelled, more specific compounds, such as the OCT/N substrate, [¹⁴C]-TEA and the OCTN2 probe, [³H]-acetylcarnitine. Organic cation/carnitine transporters were studied likewise in different models of the lung epithelial barrier, using alveolar (A549), bronchiolar (H441) and bronchial (Calu-3) epithelial cell culture models as well as freshly isolated human alveolar epithelial cells in primary culture (hAEPc).

5.2 MATERIALS AND METHODS

5.2.1 Materials

[Ethyl-1- ^{14}C] tetraethylammonium chloride (^{14}C -TEA; 55 mCi/mmol) and [n-methyl- ^3H] acetyl-L-carnitine hydrochloride (^3H -acetylcarnitine; 85 Ci/mmol) were purchased from American Radiolabeled Chemicals (UK) Limited (Herts, UK). Unlabelled TEA, acetylcarnitine, amantadine, corticosterone, D-carnitine, 1,1-diethyl-2,2-cyanine (decynium-22), hemicholinium-3, 1-methyl-4-phenylpyridinium (MPP^+), lysine, norepinephrine, probenecid, pyrilamine, verapamil, and all cell culture media and supplements were obtained from Sigma-Aldrich (Dublin, Ireland or St. Louis, MO), unless otherwise stated. Adenosine, quinidine and ergothioneine were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and 4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide (ASP^+) from Biosciences (Dun Laoghaire, Ireland). All cell culture plastics were obtained from Greiner BioOne (Frickenhausen, Germany) with the exception of the Transwell Clear filters (12 mm in diameter, pore size $0.4\text{ }\mu\text{m}$; Corning, VWR, Dublin, Ireland). All other chemicals used were of highest commercially available grade.

5.2.2 Cell culture

A549 (American Type Culture Collection, ATCC CCL-185) cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK), NCI-H441 (ATCC HTB-174) cells and Calu-3 (ATCC HTB-55) cells were from LGC Promochem (Teddington, UK). Cells were cultured at a seeding density of $40,000\text{ cells/cm}^2$ (A549) and $75,000\text{ cells/cm}^2$ (H441, Calu-3), respectively. A549 cells were grown in

Dulbecco's modified Eagle's medium/Ham's F-12 (1:1 mix) (DMEM/F12) supplemented with 5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. H441 cells were maintained in GibcoRPMI-1640 medium (Biosciences, Dun Laoghaire, Ireland) supplemented with 5% FBS, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Calu-3 cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) FBS, 1% non-essential amino acids, 1% sodium pyruvate solution, 0.5% glucose solution and 100 U/ml penicillin and 100 µg/ml streptomycin. All cell types were maintained at 37°C in 5% CO₂ atmosphere and the medium was exchanged every 48 h until confluent monolayers were reached after 5 (A549), 8 (H441) and 12 (Calu-3) days, respectively.

5.2.3 Human alveolar epithelial cell isolation and culture

Human alveolar type II (ATII) epithelial cells were freshly isolated from non-tumour lung tissue obtained from patients undergoing lung resection. The use of human material was approved by Saarland State Medical Board, Germany. Isolation of ATII was performed according to a protocol modified from Demling *et al.* (Demling *et al.*, 2006). Purified ATII cells were seeded at a density of 600,000 cells/cm² on collagen/fibronectin-coated plastics using SAGM supplemented with penicillin, streptomycin and 1% FBS. ATII cells were either used after 24 h of culture or after transdifferentiation into monolayers of type I-like (ATI-like) phenotype, following 8 days of culture.

5.2.4 Uptake studies

Uptake experiments were carried out using A549 monolayers in extra-cellular fluid buffer (Toyama University, Toyama, Japan; ECF; composed of 122 mM NaCl, 3 mM KCl, 0.4 mM KHPO₄, 25 mM NaHCO₃, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 10 mM HEPES and 10 mM D-glucose, pH 7.4). Cell experiments with ATI-like, H441 and Calu-3 cell monolayers were performed in freshly prepared bicarbonated Krebs-Ringer buffer (KRB) composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, 0.81 mM MgSO₄, and 5.55 mM glucose, pH 7.4. Buffers were checked for biological analogy and found not be significantly different.

Prior to uptake experiments, cell monolayers were washed three-times with pre-equilibrated ECF or KRB solution. To initiate organic cation uptake, 200 µl buffer containing [¹⁴C]-TEA (10 µM) or [³H]-acetylcarnitine (5.5 nM) were added to each well. Uptake studies were terminated by removing the solution, followed by immersing the cells in ice-cold buffer. For time-course analyses, organic cation uptake was measured for 30 min (TEA and acetyl-carnitine). To study concentration dependency and (self)-inhibitory effects on organic cation uptake into cell monolayers, cells were incubated with TEA in the presence of various concentrations (0 to 20 mM) of the relevant unlabelled compound for 10 min (Calu-3, H441, ATI-like) and 30 min (A549), respectively. However, all uptake experiments using acetylcarnitine (range of 0-1000 µM) were performed for 20 min in all cell lines investigated. Uptake of [¹⁴C]-TEA and [³H]-acetylcarnitine was also performed in the presence of several inhibitors of transporter function (i.e., amantadine, choline, corticosterone, L-carnitine, decynium-22, ergothioneine, MPP⁺, pyrilamine and quinidine). To elucidate the effect of membrane

depolarisation, $[K^+]$ was increased to intracellular levels with a concurrent reduction of $[Na^+]$. The Na^+ -free buffer was prepared by replacing NaCl and $NaHCO_3$ with equimolar concentrations of KCl and $KHCO_3$, respectively. Uptake of $[^{14}C]$ -TEA and $[^3H]$ -acetylcarnitine was also tested for sodium dependency by isotonically exchanging Na^+ with N-methyl-D-glucamine. Finally, the influence of a proton gradient on organic cation uptake was analysed using buffers of varying pH values (i.e., 5.5, 6.5 and 8.5) by adjusting the normal transport buffer with HCl and NaOH to the desired pH.

At the indicated time points, the uptake was stopped by washing the cell monolayers three-times with ice-cold buffer and then 400 μ l of 1N NaOH was added to permeabilise the cells. Monolayers were left to lyse for at least 12 h, before 400 μ l of 1N HCl was added for neutralisation of the cell lysate. Five-hundred microlitres of lysate was used to measure the cell-associated radioactivity in a liquid scintillation counter (Tri Carb TR2100 Packard Scintillation Counter, Ireland). In parallel, the total cell protein content was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

5.2.5 Transport studies

Cells were grown on Transwell Clear filters for at least 8 (H441) or 14 (Calu-3) days (TEER > 350 Ω cm²) (Ehrhardt et al., 2002). To initiate transport studies, both sides of the cultured cell layers were washed twice with pre-warmed KRB solution, followed by a 60 min equilibration in KRB at 37°C. For the actual transport studies, the buffer was then replaced with 0.51 ml donor solution ($[^{14}C]$ -TEA or $[^3H]$ -acetylcarnitine) in the apical chamber. The basolateral chamber was filled with 1.5 ml of fresh pre-warmed

KRB. Furthermore, for basolateral-to-apical transport, the donor solution was placed in the basolateral compartment (1.51 ml) and 0.5 ml fresh pre-warmed KRB was placed in the apical compartment, respectively. The initial donor concentration was determined by taking a 10 µl sample directly after adding the donor solution. The cell layers were kept at 37°C during experiments and 200 µl samples were collected from the receiver compartment at the designated time points from the respective compartments (radioactivity of samples was determined with a Tri Carb TR2100 Packard Scintillation Counter, Ireland). To keep sink-conditions, an equal amount of fresh pre-warmed KRB solution was returned to the receiver compartments. At the end of transport studies, again a 10 µl sample was collected. Furthermore, cell monolayers were permeabilised in 400 µl 1N NaOH for at least 12 h, neutralised with 400 µl 1N HCl and intracellular accumulation of the organic cations was measured using a liquid scintillation counter (Tri Carb TR2100 Packard Scintillation Counter, Ireland). Each experiment was conducted at least in triplicates. TEER values were recorded before and after the flux studies, in order to assess the cell layer integrity.

The utilised equation to calculate the apparent permeability coefficient (P_{app}) is the following:

$$P_{app} = (\Delta Q / \Delta t) / (A \cdot C_0) \quad [\text{Eq.1}]$$

where ΔQ was the change in quantity of the compound over a designated period of time (Δt), A was the nominal surface area of the cell layers (1.13 cm²) and C_0 was the initial concentration of the drug in the donor fluid and used in this study.

5.2.6 RNAi studies

A549 cells were seeded in 24-well cell culture plates at a density of 40,000 cells/cm² in culture medium containing FBS and antibiotics and grown for 24 h at 37°C in 5% CO₂ atmosphere. Two microliters of HyperFect Reagent (Qiagen, West Sussex, UK), 50 µl of DMEM/ F12 and OCT1-, OCT2-, or OCT3-siRNA (75 nM; Santa Cruz Biotechnology, Heidelberg, Germany) or the control scrambled siRNA (AllStars, Qiagen) were mixed, incubated at room temperature for 5 -10 min and added drop wise to the relevant wells. Cells were then incubated at 37°C for 24 h, before the medium was changed back to the respective normal cell culture medium. After 24, 48 and 72 h, cell monolayers were lysed in cell extraction buffer containing protease inhibitors (Invitrogen Karlsruhe, Germany). Protein sample concentrations were determined using a standard protein concentration assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer’s instructions and used for Western blot analysis. For uptake studies, cells were used for analysis after 4 days (72 h after transfection).

5.2.7 Data analysis

The uptake of [¹⁴C]-TEA and [³H]-acetylcarnitine by human respiratory epithelial cells was expressed as the cell-to-medium (cell/medium) ratio calculated by the following equations:

Cell/medium ratio = [³H] dpm per mg cell protein / [³H] dpm per µl buffer [Eq. 2]

[¹⁴C] dpm per mg cell protein / [¹⁴C] dpm per ml buffer [Eq. 3]

To estimate the kinetic uptake parameters of [^{14}C]-TEA and [^3H]-acetylcarnitine, the initial uptake rates were fitted to Equation 3 by means of non-linear least-squares regression analysis using WinNonlin (Pharsight, Sunnyvale, CA)

$$v = V_{\max} \times s / K_m + s \quad [\text{Eq. 4}]$$

where v is the initial uptake rate of the substrate (nmol/min·mg protein), s is the substrate concentration in the medium (μM), K_m is the Michaelis-Menten constant (μM), and $V_{\max}$ is the maximum uptake rate (nmol/min·mg protein).

Results were expressed as mean $\pm$ SD. Specifically for inhibition experiments, results were expressed as mean $\pm$ SEM. The significance of differences between the mean values was determined by unpaired, two-tailed Student's t -test. Statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA), followed by the modified Fisher's least squares difference method. All experiments were carried out at least in triplicate.

5.3 RESULTS

5.3.1 Time course of organic cation uptake by human respiratory epithelial cell lines

The time course of uptake of TEA and acetylcarnitine into human respiratory epithelial cell monolayers is shown in Figure 1A and 1B, respectively.

TEA uptake was found to be linear over the whole 30 min of the experiment in A549 cells, reaching a cell/medium ratio of 8.4 $\mu\text{l}/\text{mg}$ protein after 30 min, whereas primary-cultured alveolar cells (ATI-like) showed decreased uptake of TEA after 10 min with a maximum cell/medium ratio of 6.1 $\mu\text{l}/\text{mg}$ protein after 30 min. In comparison, in Calu-3 and H441 cells, the cell medium ratio of TEA uptake was much lower at 1.0 (Calu-3) and 1.8 (H441) $\mu\text{l}/\text{mg}$ protein after 30 min. TEA uptake increased linearly up to 10 min. Based on these findings, subsequent studies were carried out for 10 (ATI-like, Calu-3, H441) or 30 (A549) min.

Uptake of [^3H]-acetylcarnitine in A549 cells increased linearly up to 30 min with a peak cell/medium ratio of 64.9 $\mu\text{l}/\text{mg}$ protein. Notably, ATI-like, Calu-3 and H441 cells resulted in significantly lower uptake values of 13.6 (ATI-like), 15.3 (Calu-3) and 9.7 (H441) $\mu\text{l}/\text{mg}$ protein after 30 min, respectively. In the case of [^3H]-acetylcarnitine uptake, subsequent studies were performed for 20 min in all used cell models.

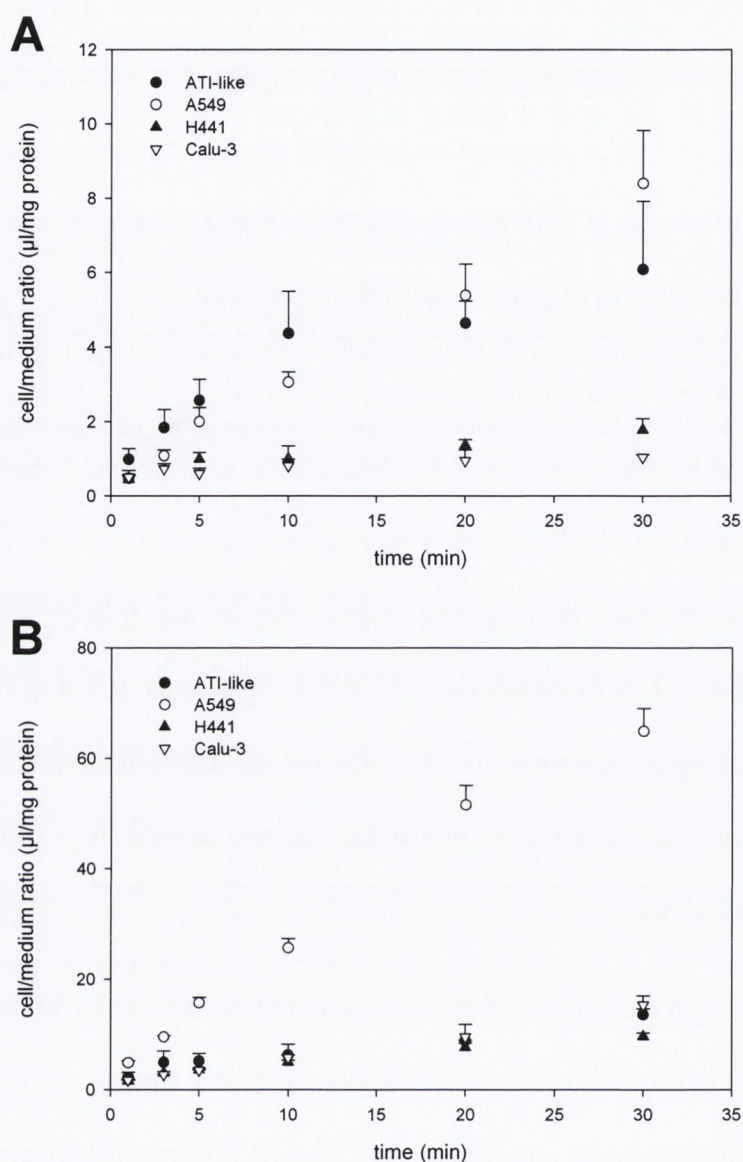


Figure 1. Time course of [^{14}C]-TEA (**A**) and [^3H]-acetylcarnitine (**B**) into human alveolar (ATI-like and A549), bronchiolar (H441) and bronchial (Calu-3) epithelial cell types. Cell uptake of [^{14}C]-TEA and [^3H]-acetylcarnitine was performed at 37°C for 30 min. Each point represents means $\pm$ SD ($n = 3$).

5.3.2 Kinetic analysis of organic cation uptake

Concentration dependence of TEA and acetylcarnitine uptake was also examined in human respiratory epithelial cell lines. The results were obtained by calculating the differences in TEA and acetylcarnitine uptake at 37°C and 4°C.

In A549 cell monolayers, the concentration dependence uptake profile of [^{14}C]-TEA showed a saturable uptake profile (Figure 2A). Eadie-Hofstee transformation (Figure 2B) indicated a substantial saturable component with a $K_m = 528.5 \pm 373.1 \mu\text{M}$ and $V_{\max} = 0.3 \pm 0.1 \text{ nmol/min/mg protein}$. The non-saturable uptake component had a K_d of $0.02 \mu\text{l/min/mg protein}$, suggesting a minor contribution to the uptake of [^{14}C]-TEA into A549 cells (Figure 2B). By contrast in H441 and Calu-3 epithelial monolayers, no saturation of [^{14}C]-TEA uptake was obtained in the concentration range studied (Figure 2C). Thus, no transformation of the uptake data was possible and no kinetic parameters were calculated. This suggests that the transporter activity varies highly between the cell culture models, which was also noted by much lower uptake activity in bronchiolar/bronchial compared to alveolar epithelial cells. Furthermore, the transporter involved might be different or might be localised to a different membrane; here, the formation of tight monolayers via tight junctional complexes might play an important role.

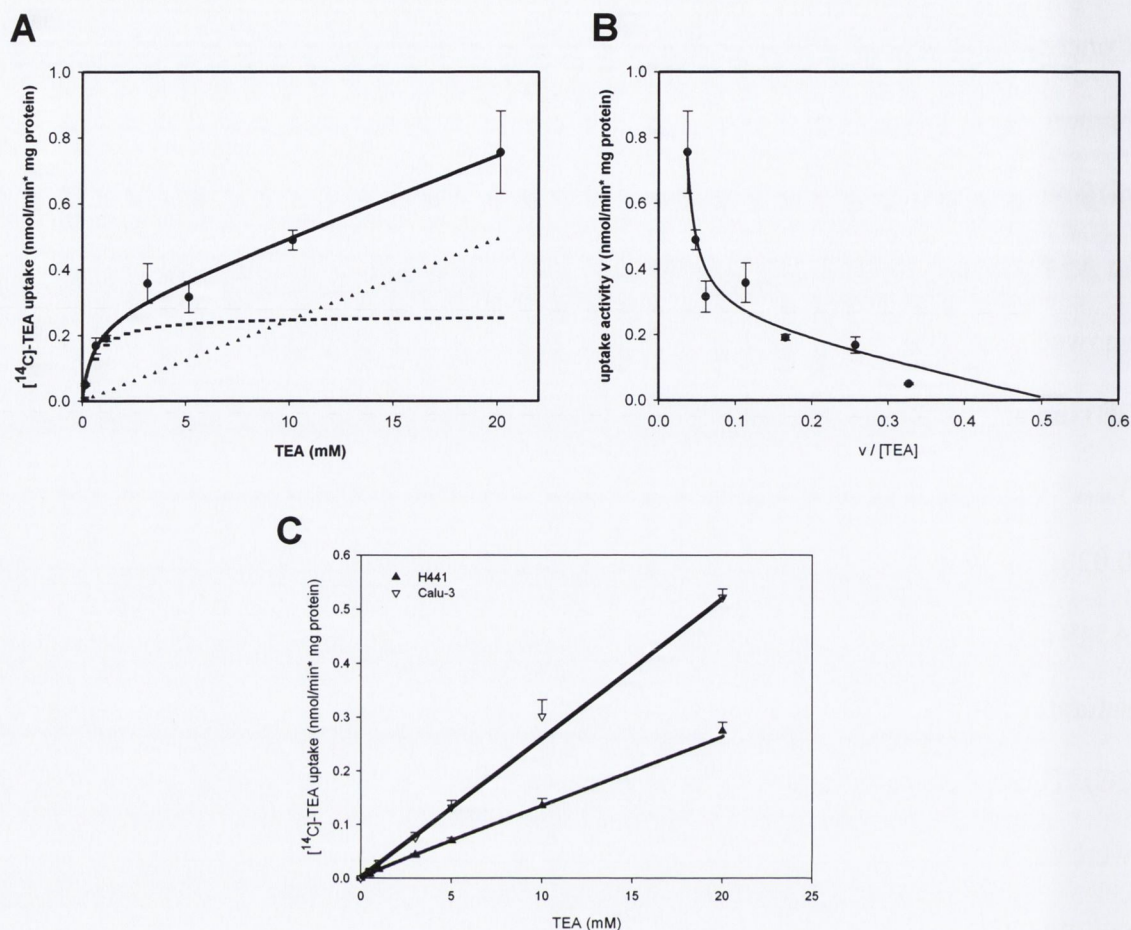


Figure 2. Concentration dependence of $[^{14}\text{C}]\text{-TEA}$ uptake by human respiratory epithelial cell monolayers. Alveolar (A549) cells were incubated for 30 min at concentrations ranging from 0.5 to 20 mM at pH 7.4 (**A**). Pharmacokinetic analysis of $[^{14}\text{C}]\text{-TEA}$ was performed: total uptake (solid line), saturable part (broken line) and non-saturable part (triangle) were calculated according to obtained K_m and V_{max} values. Furthermore, $[^{14}\text{C}]\text{-TEA}$ uptake data was analysed using Eadie-Hofstee transformation in A549 cells (**B**). In the case of bronchiolar (H441) and bronchial (Calu-3) epithelial cells, $[^{14}\text{C}]\text{-TEA}$ uptake was performed for 10 min at the before-mentioned concentration range (**C**). These results were obtained by subtraction of $[^{14}\text{C}]\text{-TEA}$ uptake at 4°C as the non-specific component from the total uptake. Each point represents means $\pm$ SD ($n = 3$).

Moreover, [^3H]-acetylcarnitine uptake into A549 monolayers was examined and a saturable uptake of [^3H]-acetylcarnitine was obtained (Figure 3A and 3B). Eadie-Hofstee transformation of the data (see insert Figure 3A) revealed a K_m value of $16.1 \pm 10.9 \mu\text{M}$ and $V_{\max}$ of $0.03 \pm 0.01 \text{ nmol/min/mg protein}$ with a passive diffusion constant (K_d) of $0.7 \mu\text{l/min/mg protein}$. In Calu-3 cell monolayers, [^3H]-acetylcarnitine uptake was studied at a concentration range up to $200 \mu\text{M}$ of acetylcarnitine and found to be saturable (Figure 3C). Uptake data was applied to Eadie-Hofstee equation and the kinetic parameters were estimated as follows: $K_m = 35.4 \pm 26.7 \mu\text{M}$ and $V_{\max} = 0.005 \pm 0.003 \text{ nmol/min/mg protein}$ with a K_d of $0.1 \mu\text{l/min/mg protein}$. Notably, the contribution of carrier-mediated transport (Figure 3D, marked line) and passive diffusion (Figure 3D, open triangle) to acetylcarnitine uptake into Calu-3 cells was kinetically equal compared to transporter-driven uptake in A549 cells (Figure 3B, marked line); K_m values of acetylcarnitine uptake were comparable in both cell lines, whereas $V_{\max}$ values varied. In the case of H441 cell monolayers, [^3H]-acetylcarnitine uptake was not saturable (Figure 3E). A linear increase of intracellular accumulated acetylcarnitine was seen over the chosen concentration range, suggesting that the transporter for the uptake had a very low affinity for acetylcarnitine or was not easily accessible so that saturated uptake processes could take place.

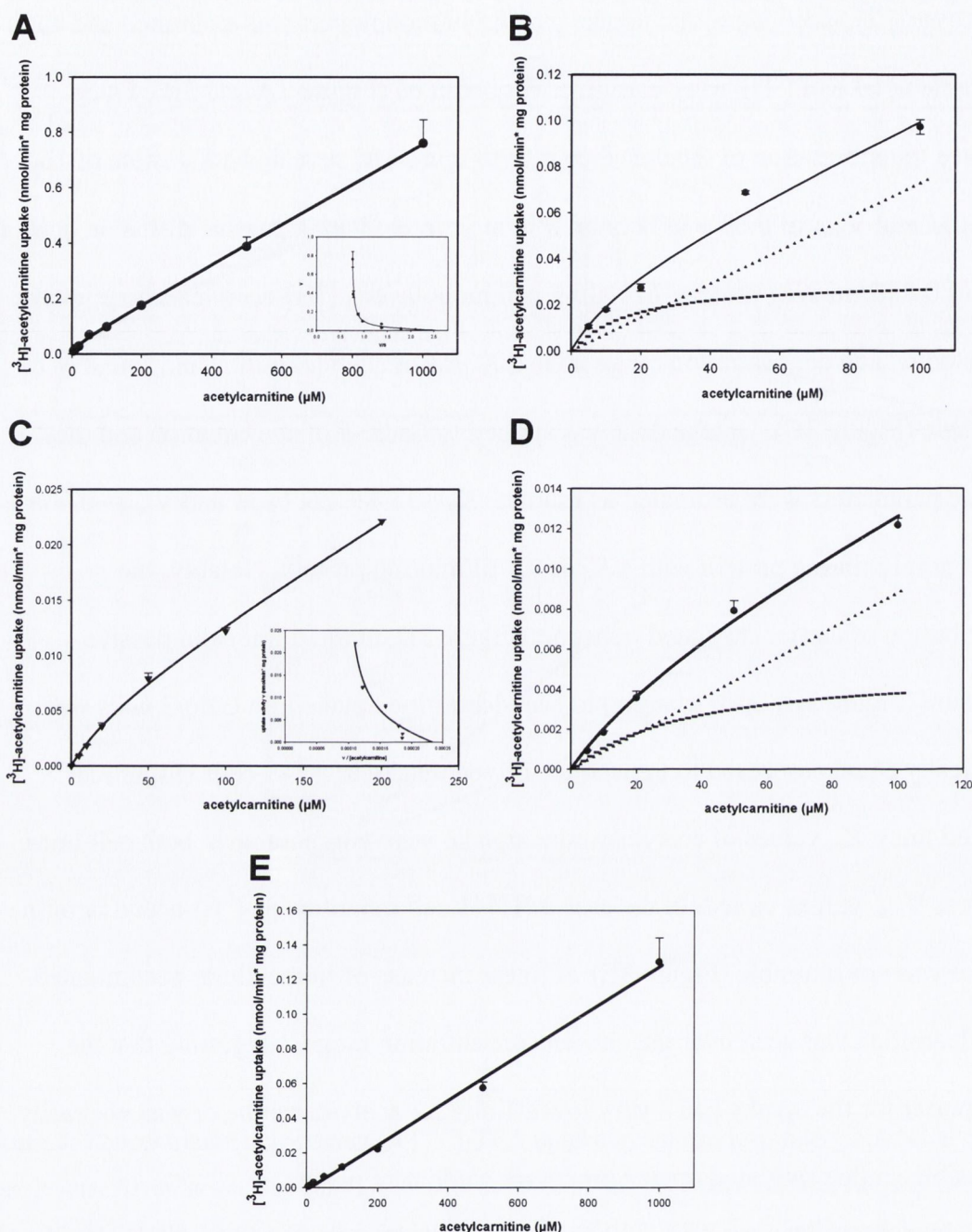


Figure 3. Concentration dependence of ^3H -acetylcarnitine uptake by A549 (A,B), Calu-3 (C, D) and H441 (E) monolayers over a concentration range up to 1000 μM for 20 min at pH 7.4. The apparent uptake of ^3H -acetylcarnitine can be seen in A (A549), C (Calu-3) and E (H441). Furthermore, ^3H -acetylcarnitine uptake was analysed by means of Eadie-Hofstee equation in the case of A549 (A, small insert) and Calu-3 cells (C, small insert), respectively. Total (solid line), saturable part (broken line) and non-saturable part (triangle) of ^3H -acetylcarnitine uptake is shown up to 100 μM calculated by using the kinetic parameters K_m , $V_{\max}$ and K_d for A549 (B) and Calu-3 (D) cells. These results were obtained by subtraction of ^3H -acetylcarnitine uptake at 4°C as the non-specific component from the total uptake. Data represent means $\pm$ SD ($n = 3$).

5.3.3 Effect of extracellular conditions on organic cation uptake by A549, H441 and Calu-3 cells

Next, the effects of extracellular Na^+ concentration, membrane potential- and pH-dependency on TEA and acetylcarnitine uptake by human respiratory epithelial cells were investigated.

Additionally to pH- and Na-dependency studies and inhibition experiments, uptake of TEA and acetylcarnitine was carried out at 4°C for a similar time period to assess the contribution of non-specific uptake and adsorption to the actual uptake process. In A549 cells, uptake of both compounds at 4°C was reduced to $7.4 \pm 0.5\%$ of control (TEA) and $7.6 \pm 0.2\%$ (acetylcarnitine) resulting in $\sim 92\%$ involvement of active, carrier-mediated processes (which represents the part that can actually be inhibited by specific inhibitors). The picture was different in human bronchial and bronchiolar cell lines, Calu-3 and H441: TEA uptake at 4°C represented a percentage of $49.7 \pm 1.7\%$ of control (Calu-3) and $75.7 \pm 5.4\%$ (H441), respectively. Acetylcarnitine uptake at 4°C accounted for $17.8 \pm 0.5\%$ in Calu-3 and $31.5 \pm 0.9\%$ of the uptake at 37°C in H441 cells, respectively yielding quite high involvement of non-specific processes and lower substrate specificity of the organic cation transport system. Hence, data in the following [^{14}C]-TEA and [^3H]-acetylcarnitine uptake experiments were always corrected by subtraction of values obtained in [^{14}C]-TEA and [^3H]-acetylcarnitine uptake studies at 4°C in A549, H441 and Calu-3 cells, respectively.

[^{14}C]-TEA uptake was significantly affected by decreased amount of extracellular Na^+ in A549 (to $29.5 \pm 1.5\%$ or $54.3 \pm 10.4\%$ of control by replacing Na^+ salts with NMG or K^+) and in H441 (to $1.1 \pm 1.6\%$ or $21.4 \pm 22.9\%$ of control in the presence of NMG or K^+ salts) cells, respectively; whereas no significant effect could be seen in the case of [^{14}C]-TEA uptake into Calu-3 (Figure 4A). Moreover, [^3H]-acetylcarnitine uptake was strongly decreased to $8.5 \pm 0.5\%$ of control by replacement of Na^+ with NMG in A549 cells, while the increase in K^+ concentration caused some reduction ($35.8 \pm 3.2\%$) of the [^3H]-acetylcarnitine uptake (Figure 4C). These results suggest that the uptake of both, [^{14}C]-TEA and [^3H]-acetylcarnitine, into A549 cells was affected by extracellular sodium concentration and membrane potential. However, [^3H]-acetylcarnitine uptake was reduced to $64.3 \pm 3.5\%$ and $50.4 \pm 3.7\%$ of control in Calu-3 and H441 cells, respectively, when Na^+ was replaced by NMG, indicating a sodium-dependent uptake activity. However, no effect was seen in the presence of high K^+ concentrations and concomitant low concentrations of Na^+ on [^3H]-acetylcarnitine uptake in Calu-3 and H441 cells.

We further examined the effect of pH on [^{14}C]-TEA and [^3H]-acetylcarnitine uptake into human respiratory cells (Figure 4B and D, respectively). The uptake of [^{14}C]-TEA into A549 cells was found to be sensitive to decreasing (pH 5.5) and increasing (pH 8.5) pH. However, the uptake of [^3H]-acetylcarnitine in A549 cells did not change in the range of pH 5.5 to pH 8.5. Characteristics of [^{14}C]-TEA and [^3H]-acetylcarnitine uptake into H441 cells at different extracellular pH were similar to effects observed in A549 cells, but less pronounced, except for a decreased uptake at basic pH in both cases. Interestingly, the apparent uptake of [^{14}C]-TEA and [^3H]-acetylcarnitine into Calu-3 cells increased at acidic pH, but did not change at basic pH.

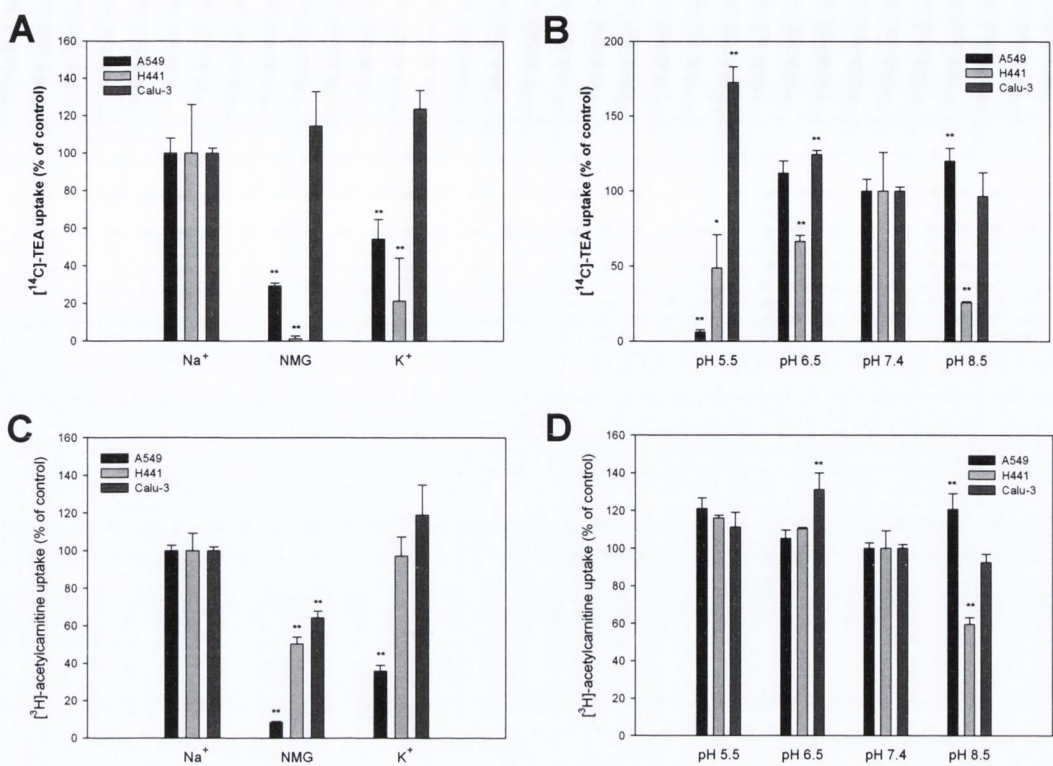


Figure 4. Effect of extracellular pH and sodium concentration on the uptake of [^{14}C]-TEA (**A**, **B**) and [^3H]-acetylcarnitine (**C**, **D**) into human respiratory epithelial cell monolayers. Uptake of [^{14}C]-TEA (**A**) and [^3H]-acetylcarnitine (**C**) into A549 (black columns), H441 (grey columns) and Calu-3 (dark grey columns) cells was measured at pH 7.4. Na^+ was replaced with N-methylglucamine (NMG) or equimolar K^+ salts. Furthermore, uptake of [^{14}C]-TEA (**B**) and [^3H]-acetylcarnitine (**D**) was measured at various pH values of the incubation buffer into A549 (black columns), H441 (grey columns) and Calu-3 (dark grey columns) cell monolayers. Cells were incubated for 10 (Calu-3, H441) and 30 (A549) min in the case of [^{14}C]-TEA uptake, respectively. In the case of acetylcarnitine, studies were carried out for 20 min in all cell lines. Each point represents means $\pm$ SD ($n = 3$). **Indicates a significant difference from the uptake at pH 7.4 ($P < 0.01$). These results were obtained by calculating the difference between uptake at 37°C and 4°C .

5.3.4 Effect of inhibitors of organic cation uptake into human respiratory and primary alveolar epithelial cells

The inhibitory potential of OCT/OCTN substrates and/or inhibitors on TEA and acetylcarnitine uptake by human respiratory epithelial cells and hAEPc were investigated. Concentrations of pharmacological substrates/inhibitors were chosen according to their known inhibitory potential (IC_{50} or K_i) published by Koepsell *et al.* (Koepsell *et al.*, 2007) and Nies *et al.* (Nies *et al.*, 2010). In these studies, all values are represented as mean $\pm$ SEM (see Figure 5 and Table 1).

The effects of several inhibitors on [^{14}C]-TEA uptake into human alveolar epithelial primary cells (ATI-like) cultured for 8 days were also studied. Cationic compounds, such as decynium-22, MPP⁺ and TEA strongly decreased [^{14}C]-TEA uptake to $18.6 \pm 9.9\%$, $33.9 \pm 14.8\%$ and $40.9 \pm 2.9\%$ of control, respectively. Amantadine showed no significant effect ($85.8 \pm 14.5\%$), whereas hemicholinium-3 reduced [^{14}C]-TEA uptake to $32.0 \pm 11.4\%$. The strongest inhibitions were exhibited by corticosterone ($19.3 \pm 10.2\%$), quinidine ($1.4 \pm 1.4\%$) and verapamil ($15.7 \pm 10.5\%$).

We also analysed the effects of inhibitors/modulators on [^{14}C]-TEA uptake in A549 epithelial cells. Cationic substances markedly or completely decreased [^{14}C]-TEA uptake into A549 cells; such as amantadine (1 mM: $24.6 \pm 1.8\%$ of control), ASP⁺ (100 μ M: $7.0 \pm 1.2\%$), corticosterone (500 μ M: $12.5 \pm 1.8\%$), decynium-22 (500 μ M: $0.0 \pm 0.8\%$), MPP⁺ (1 mM: $4.5 \pm 1.2\%$) and unlabeled TEA (20 mM: $28.4 \pm 2.8\%$), whilst carnitine, a specific OCTN substrate, did not significantly diminish the uptake into A549 cells (Figure 5A, Table 1). Ergothioneine (200 μ M), the physiological substrate of OCTN1 (Gründemann *et al.*, 2005), reduced [^{14}C]-TEA uptake to $69.9 \pm 9.0\%$,

indicating an involvement of OCTN1 in TEA uptake. Acetylcholine, hemicholinium-3, norepinephrine and adenosine showed noticeable effects and inhibited [¹⁴C]-TEA uptake into A549 cells to 47.9 ± 1.1%, 39.6 ± 3.6%, 20.5 ± 1.2% and 60.7 ± 3.2%, respectively. Choline and lysine (used in concentrations of 2 and 1 mM, respectively) did not show an effect. Interestingly, verapamil (200 μM) as well as quinidine (500 μM) decreased uptake entirely to 0.0 ± 0.4% and 0.0 ± 0.8% of control, respectively. Their potential to affect organic cation transport systems recently received more interest (Okura et al., 2008).

Table 1. Inhibitory effects of cationic and anionic substances on [¹⁴C]-TEA and [³H]-acetylcarnitine uptake were tested in A549 cell monolayers. Uptake of [¹⁴C]-TEA was measured after 30 min and [³H]-acetylcarnitine after 20 min at 37°C. Each value represents the means ± SEM (*n* = 3). Data were obtained by the subtraction of the [¹⁴C]-TEA and [³H]-acetylcarnitine uptake at 4°C * *P* < 0.05, ** *P* < 0.01 significantly different from control.

Inhibitor	Concentration (mM)	Relative Uptake (% of control)	
		[¹⁴ C]-TEA	[³ H]-acetylcarnitine
Acetylcholine	5	47.9±1.1**	n.d.
Adenosine	1	60.7±3.2**	78.5±2.4*
L-carnitine	0.2	88.8±1.0	n.d.
Choline	1	n.d.	117.9±6.3
	2	92.9±5.5	n.d.
Ergothioneine	0.2	69.9±9.0**	see figure 6C
Lysine	1	101.0±7.3	88.5±3.4
Norepinephrine	5	20.5±1.2**	n.d.
Probenecid	0.5	n.d.	115.4±2.9
TEA	1	see figure 6A	80.7±1.3*
	20		22.3±0.4**

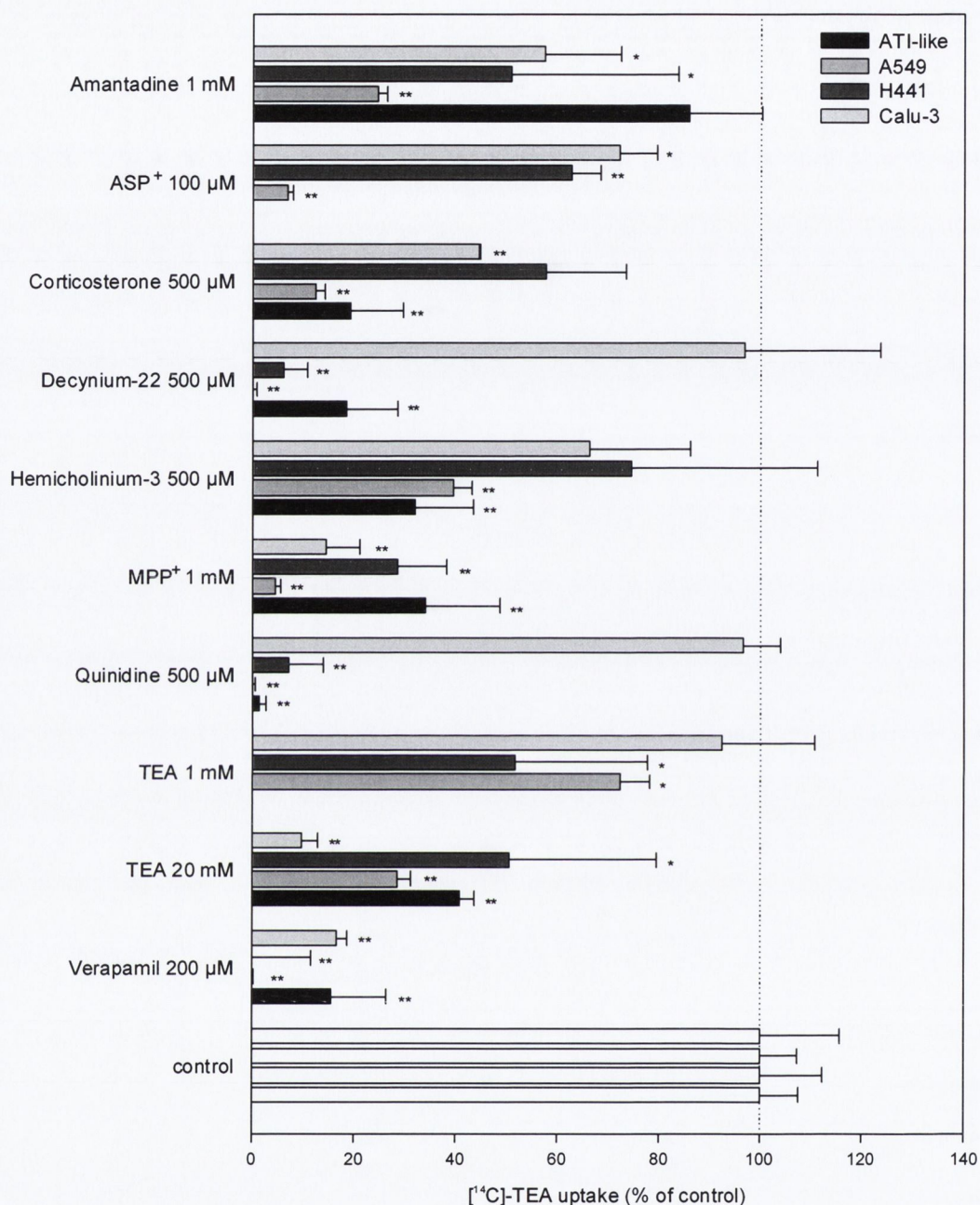


Figure 5. Inhibitory effects of organic cations on [¹⁴C]-TEA uptake by hAEPc, A549, H441 and Calu-3 cell monolayers. Uptake of was measured after 30 min (A549) and 10 min (hAEPc, Calu-3, H441) at 37°C and pH 7.4. [¹⁴C]-TEA uptake was measured in the presence and absence of organic cations in hAEPc (black columns), A549 (grey columns), Calu-3 (dark grey columns) and H441 (light grey columns) cells. Data represent means ± SEM (*n* = 6 - 9). ***P* < 0.01, **P* < 0.05 indicates a significant difference from the uptake at pH 7.4. Results were obtained by calculating the difference between uptake at 37°C and 4°C.

In general, effects of substrates/inhibitors were less pronounced in Calu-3 and H441 cells (Figure 5). [^{14}C]-TEA uptake was significantly decreased to similar levels in both cell models in the presence of cationic substrates such as amantadine (1 mM: $57.4 \pm 15.0\%$ in Calu-3, $50.8 \pm 32.8.0\%$ in H441 cells), ASP $^{+}$ (100 μM : $72.3 \pm 7.3\%$ (Calu-3) and $62.6 \pm 5.8\%$ (H441)), corticosterone (500 μM : $44.5 \pm 0.3\%$ (Calu-3) and $57.7 \pm 15.9\%$ (H441)), hemicholinium-3 (500 μM : $66.3 \pm 19.7\%$ (Calu-3) and $74.4 \pm 37.1\%$ (H441)) and MPP $^{+}$ (1 mM: $14.6 \pm 6.5\%$ (Calu-3) and $28.6 \pm 9.8\%$ (H441)). In Calu-3 cells, neither decynium-22, TEA (at a concentration of 1 mM) nor quinidine showed effects on [^{14}C]-TEA uptake, whilst TEA (at a concentration of 20 mM) was able to reduce the uptake to $9.9 \pm 3.0\%$ of control. Furthermore, verapamil (200 μM) decreased [^{14}C]-TEA uptake to $16.6 \pm 2.3\%$ of control. [^{14}C]-TEA uptake in H441 cells was not concentration-dependently reduced in the presence of 1 and 20 mM TEA (both concentrations showed similar effects of $51.7 \pm 26.1\%$ and $50.5 \pm 28.9\%$ of decrease, respectively). Interestingly, in the presence of decynium-22, quinidine and verapamil, [^{14}C]-TEA uptake was remarkably inhibited (decynium-22 (500 μM): $6.1 \pm 4.7\%$, quinidine (500 μM): $7.2 \pm 6.8\%$ and verapamil (200 μM): $0.0 \pm 11.8\%$) and comparable to those values obtained in A549 cells. The inhibitory effect of cationic compounds was compared between the individual pulmonary cell culture models (Figure 6). Of particular interest was to compare data obtained in continuously growing cell lines with human alveolar primary cells. We found that inhibitors were more effective in A549 cells than in ATI-like cells, except for hemicholinium-3 (Figure 6D), suggesting that the interactions with OCTs are not always comparable. In H441 cells, on the other hand, effects were very similar to the situation in primary cells. In Calu-3 cells, observed inhibition patterns were very dissimilar to ATI-like cells, most evidently in the case of decynium-22 or quinidine (Figure 6C and F).

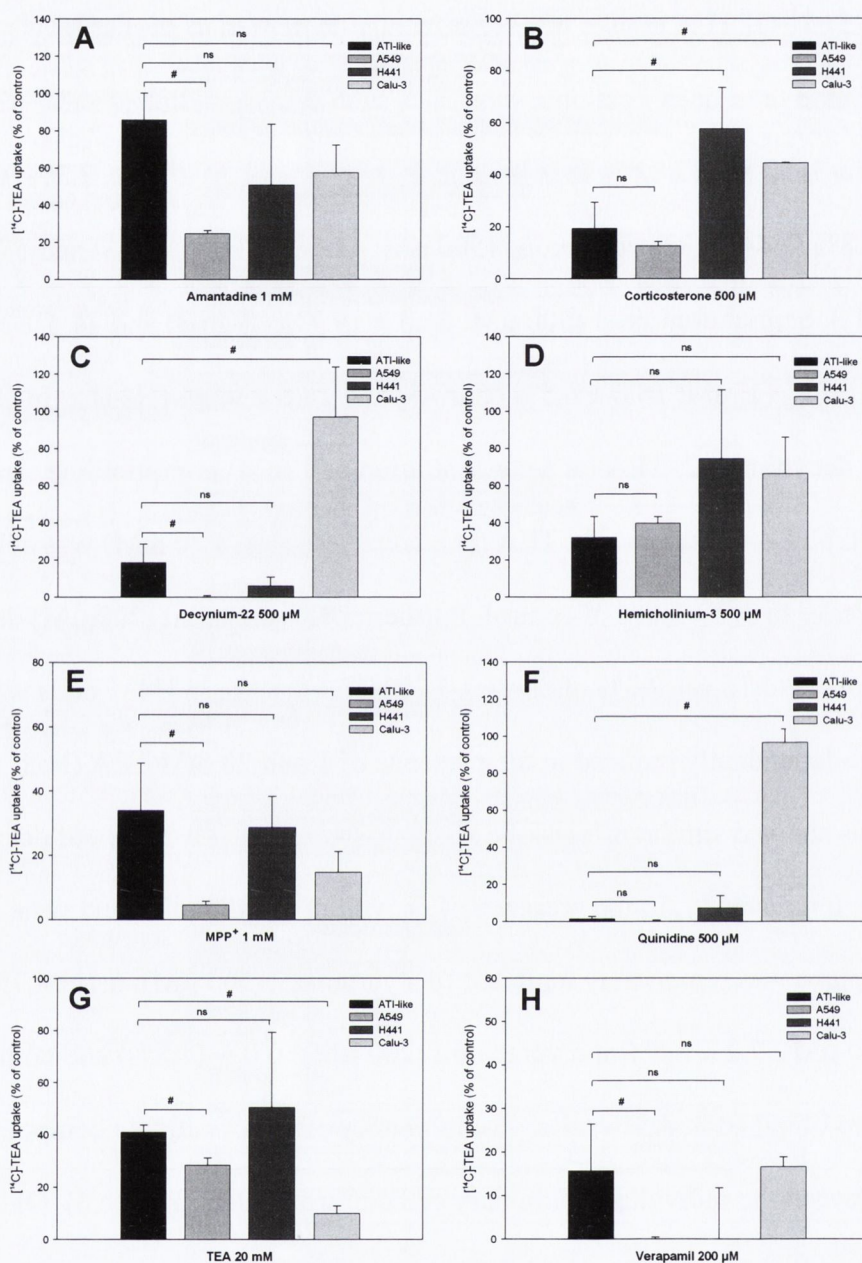


Figure 6. Comparative analysis of inhibitory effects of substrates on [14 C]-TEA uptake into ATI-like, A549, H441 and Calu-3 cell monolayers. Values were compared between human alveolar primary cells (ATI-like, black columns) and continuously grown A549 (grey columns), H441 (dark grey columns) and Calu-3 (light grey columns) cells. Data represent mean $\pm$ SEM ($n = 6 - 9$). # $P < 0.01$ indicates a significant difference from the uptake at pH 7.4 in ATI-like; ns = not significant.

Further insight into functionality of OCTN2 was provided by measuring [^3H]-acetylcarnitine uptake into human respiratory cell culture models and alveolar primary cells. Simultaneous incubation of [^3H]-acetylcarnitine with 200 μM ergothioneine, 500 μM D-carnitine and 200 μM verapamil led to distinct effect in ATI-like cells (Figure 7A). Ergothioneine did not affect [^3H]-acetylcarnitine uptake ($95.4 \pm 7.2\%$), whereas D-carnitine and verapamil reduced the uptake to $31.6 \pm 1.3\%$ and $28.2 \pm 1.6\%$, respectively. This confirmed that OCTN1 was not involved in the translocation of acetylcarnitine in human alveolar primary cells.

With regard to [^3H]-acetylcarnitine uptake into A549 cells (Figure 7B), D-carnitine (100 μM), ergothioneine (200 μM) and TEA (20 mM) exhibited strong inhibitory potential and reduced uptake to $25.8 \pm 1.3\%$, $61.4 \pm 3.7\%$ and $22.3 \pm 0.4\%$, respectively. Notably, the organic cation, TEA inhibited the uptake of TEA and acetylcarnitine in a similar manner. Other OCT substrates such as ASP^+ (100 μM : $80.4 \pm 2.8\%$), MPP^+ (1 mM: $103.3 \pm 6.8\%$), choline (1 mM: $117.9 \pm 6.3\%$) and adenosine (1 mM: $78.5 \pm 2.4\%$), lysine (1 mM: $88.5 \pm 3.4\%$) and probenecid (500 μM : $115.4 \pm 2.9\%$) did not show any significant decrease of [^3H]-acetylcarnitine uptake in A549 cells. However, corticosterone diminished [^3H]-acetylcarnitine uptake to $70.8 \pm 5.0\%$ at a concentration of 500 μM . Moreover, similar inhibitory potential of verapamil (200 μM) and quinidine (500 μM) was observed on [^3H]-acetylcarnitine uptake into A549 cells, a reduction to $20.7 \pm 0.5\%$ and $30.3 \pm 1.5\%$ was observed, respectively. In the case of [^3H]-acetylcarnitine uptake into Calu-3 and H441 cells, ergothioneine (200 μM) likewise reduced the uptake to $71.8 \pm 3.9\%$ (Calu-3) and $72.7 \pm 2.2\%$ (H441) of control, whilst D-carnitine (100 μM) decreased uptake to $63.4 \pm 3.0\%$ in Calu-3 cells and only moderately to $82.2 \pm 4.9\%$ of control in H441 cells.

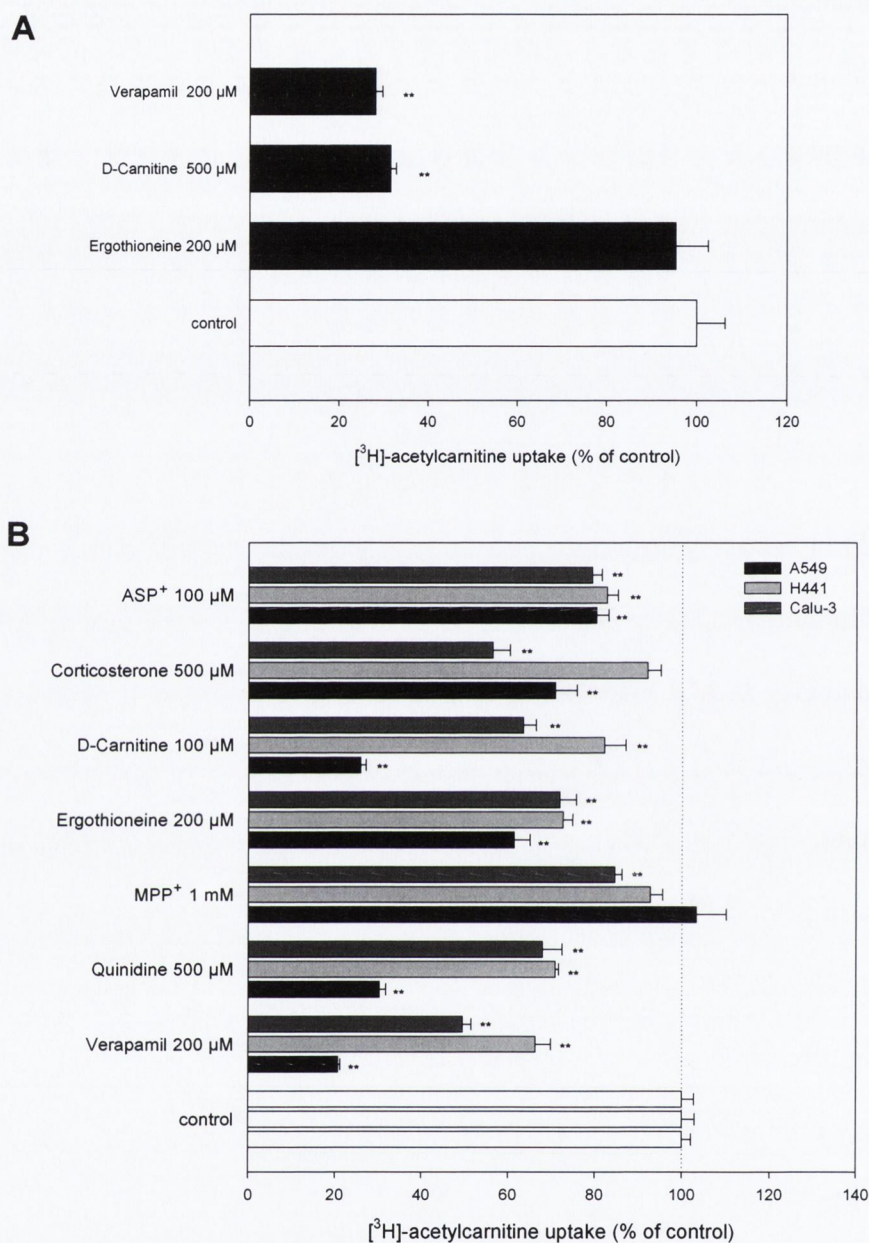


Figure 7. [³H]-acetylcarnitine uptake was studied in the presence and absence of OCT/N modulators in human respiratory epithelial cells. A limited set of inhibition studies was carried out in hAEPc (A). [³H]-acetylcarnitine uptake was studied in A549 (black columns), H441 (grey columns) and Calu-3 (dark grey columns) cells (B) for 20 min at 37°C. Data represent mean ± SEM (*n* = 6 - 9). ***P* < 0.01, **P* < 0.05 indicates a significant difference from the uptake at pH 7.4. Results were obtained by calculating the difference between uptake at 37°C and 4°C.

Furthermore, distinct inhibition effects were obtained with corticosterone (500 μ M): a decrease to $56.4 \pm 4.0\%$ in Calu-3 cells, but no significant effects on [3 H]-acetylcarnitine uptake into H441 cells ($92.2 \pm 3.0\%$). OCT inhibitors such as ASP $^+$ (100 μ M) and MPP $^+$ (1 mM) showed marginal inhibition in both cell models: ASP $^+$: $79.4 \pm 2.2\%$ (Calu-3) and $82.8 \pm 2.6\%$ (H441) and in the case of MPP $^+$: $84.6 \pm 1.6\%$ (Calu-3) and $92.8 \pm 2.7\%$ (H441). Moreover, quinidine and verapamil showed only modest inhibition on [3 H]-acetylcarnitine uptake into Calu-3 (quinidine: $68.0 \pm 4.6\%$ and verapamil: $49.5 \pm 2.0\%$) as well as in H441 cells (quinidine: $70.9 \pm 0.8\%$ and verapamil: $66.3 \pm 3.6\%$).

These data confirm OCTN2 functional activity in human respiratory epithelial cell culture models, including primary cell cultures. The inhibition effects of several compounds were comparable between the cell lines, e.g., ASP $^+$, corticosterone and MPP $^+$. D-carnitine (which inhibits both OCTN1 and OCTN2 but with a preference for OCTN2) exhibited stronger inhibitory effects in hAEpC and A549 cells. Of note, a higher concentration of D-carnitine was used in primary cells. Moreover, quinidine and verapamil (both substrates are reported to have inhibitory affinities on OCTN1 and OCTN2) significantly reduced acetylcarnitine uptake into A549 cells, but these effects were less pronounced in H441 and Calu-3 cells.

Effects of potential inhibitors were also tested on [14 C]-TEA uptake after pre-incubation for 10 min with the respective inhibitor in H441 cells. This study was carried out in order to investigate if a longer time period of the inhibitor binding to the transporter resulted in increased levels of inhibition of organic cation. Intriguingly, [14 C]-TEA uptake was extremely attenuated after pre-incubation with ASP $^+$ (500 μ M) to $10.2 \pm$

9.9%, when compared to the inhibition effect obtained without pre-incubation ($69.4 \pm 26.8\%$), which might be explained by a very efficient binding of ASP^+ to the transporter proteins in the absence of TEA, whereas in direct competition to the organic cation ASP^+ is less effective. On the other hand, pre-incubation with hemicholinium-3 (500 μM) and TEA (20 mM) showed no significant changes (Figure 8). Additionally, tight junctional complexes were loosened by incubating Calu-3 monolayers with EDTA (5 mM) for 10 min before $[^{14}\text{C}]$ -TEA uptake was studied. EDTA acts as a complexation agent of calcium ions, opening the tight junctions and thus, increasing the accessibility to transporter sites localised to the basolateral aspect. After the treatment with EDTA, $[^{14}\text{C}]$ -TEA uptake was significantly increased (Figure 8B).

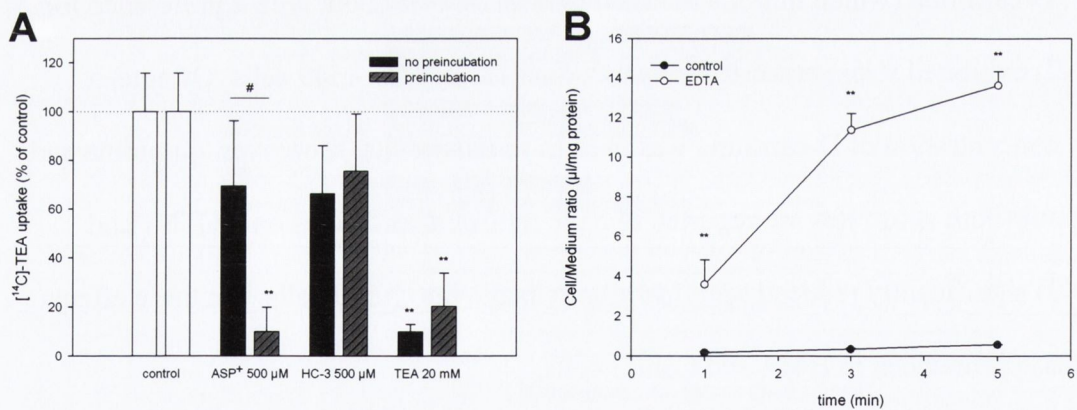


Figure 8. $[^{14}\text{C}]$ -TEA uptake was studied after pre-incubation with organic cations in H441 cells (A). Cells were incubated for 10 min with ASP^+ (500 μM), hemicholinium-3 (500 μM) and TEA (20 mM) at 37°C , before the actual uptake of $[^{14}\text{C}]$ -TEA uptake was measured for 10 min at 37°C . Values were obtained by subtraction of TEA uptake at 4°C . Moreover, $[^{14}\text{C}]$ -TEA uptake into Calu-3 monolayers was investigated in the presence of EDTA (5 mM) for 15 min in order to open the tight junctional complexes for up to 5 min at 37°C (B). Data represent mean $\pm$ SD ($n = 3$). $**P < 0.01$ indicates a significant difference from the uptake at pH 7.4 (control). $\#P < 0.01$ indicates a significant difference between preincubation and no preincubation.

5.3.5 Transport of organic cation uptake across human bronchial and bronchiolar epithelium

Bidirectional transport studies were performed across Transwell Clear-grown Calu-3 and H441 cell monolayers using TEA and acetylcarnitine (Ac-Car). The time course of TEER development can be seen in Figure 9.

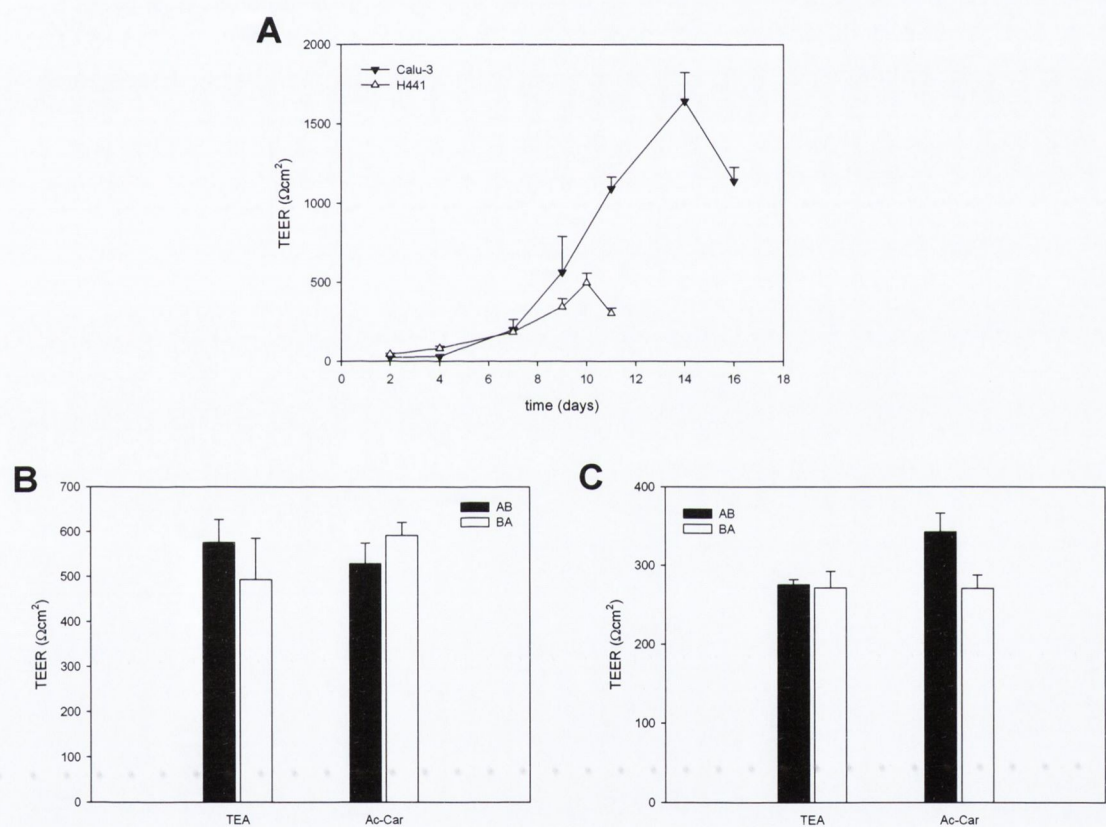


Figure 9. (A) Time course of TEER development in Calu-3 and H441 cell layers. Cells were seeded at a density of 8×10^4 (Calu-3) or 2.5×10^5 (H441) cells per cm^2 on Transwell Clear filter inserts. Cells were cultured under LCC conditions for at least 14 (Calu-3) or 10 (H441) days. TEER values were measured after the transport experiment using Calu-3 (B) and H441 (C) cell monolayers to assess the cell layer integrity. As depicted, TEER values remained at high levels after the flux studies in both cell lines. Each data point represents mean $\pm$ SD ($n=3-6$).

From transport studies, P_{app} values were calculated and are summarised in Table 2. [^{14}C]-TEA transport across H441 and Calu-3 cell monolayers showed slightly but not significant secretory (i.e., basolateral-to-apical, b-to-a) net direction, and calculated P_{app} values were approximately 5-fold higher across H441 (P_{app} (b-to-a) $3.8 \pm 0.8 \times 10^{-6}$ cm/s) than in Calu-3 (P_{app} (b-to-a) $6.1 \pm 2.3 \times 10^{-7}$ cm/s) cells. Apical-to-basolateral direction revealed a P_{app} (a-to-b) of $2.8 \pm 0.2 \times 10^{-6}$ cm/s for H441 and of $3.4 \pm 0.3 \times 10^{-7}$ cm/s for Calu-3 cells. However, transport of [^3H]-acetylcarnitine across Calu-3 monolayers did not show any net direction (P_{app} (a-to-b) $1.3 \pm 0.1 \times 10^{-6}$ cm/s and P_{app} (b-to-a) $1.3 \pm 0.1 \times 10^{-6}$ cm/s), whilst transport across H441 cells showed marginal higher basolateral-to-apical direction (P_{app} (a-to-b) $3.6 \pm 0.2 \times 10^{-6}$ cm/s and P_{app} (b-to-a) $4.3 \pm 0.3 \times 10^{-6}$ cm/s).

Table 2. Transport of organic cations, [^{14}C]-TEA and [^3H]-acetylcarnitine, was measured across Calu-3 and H441 cell monolayers at 37°C for 90 min under liquid-covered conditions. Values represent means $\pm$ SD ($n = 3 - 6$).

Cell line	[^{14}C]-TEA P_{app} ($\times 10^{-6}$ cm*s $^{-1}$)		[^3H]-acetylcarnitine P_{app} ($\times 10^{-6}$ cm*s $^{-1}$)	
	AB	BA	AB	BA
Calu-3	0.34 $\pm$ 0.03	0.61 $\pm$ 0.23	1.29 $\pm$ 0.09	1.28 $\pm$ 0.11
H441	2.84 $\pm$ 0.21	3.76 $\pm$ 0.77	3.56 $\pm$ 0.22	4.30 $\pm$ 0.3

Furthermore, the intracellular accumulation of TEA and acetylcarnitine was analysed after the bidirectional transport experiments by lysing the cell monolayers (Figure 10).

Interestingly, significantly higher uptake of TEA was determined in the apical-to-basolateral direction in both cell culture models (0.09 ± 0.01 in Calu-3 cells and 0.1 ± 0.01 nmol/mg protein in H441 cells). The concentrations obtained in b-to-a direction were as follows: 0.05 ± 0.01 in the case of Calu-3 and 0.4 ± 0.01 nmol/mg protein in the case of H441 cells. These results are in line with the obtained permeability coefficients which showed slightly higher secretory directionality, suggesting that a transporter with a higher affinity for TEA is localised to the apical side which is able to pump out organic cations from bronchial and bronchiolar cells. The analysis of cellular acetylcarnitine accumulation revealed a significantly increased value in b-to-a versus a-to-b direction (0.12 ± 0.01 and 0.34 ± 0.01 nmol/mg protein, respectively) in Calu-3 cells, whereas in H441 cells, the intracellular accumulation remained at similar levels in both directions. Of note, P_{app} values of acetylcarnitine transport were similar (see above) indicating that the proposed efflux transporter for TEA might not accept acetylcarnitine as a substrate, which in the case of Calu-3 cells is crucial.

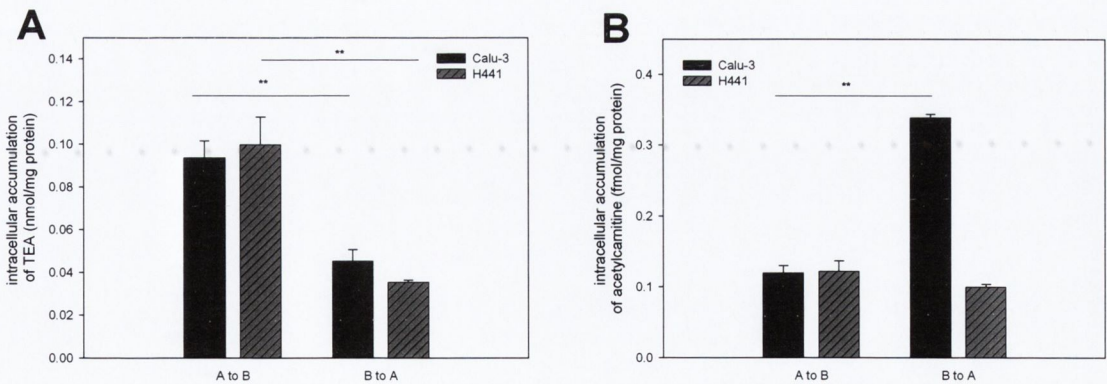


Figure 10. Respiratory epithelial cell monolayers were lysed after transport studies and the intracellular accumulation of [^{14}C]-TEA (A) and [^3H]-acetylcarnitine was determined (B). Transport was measured across Calu-3 and H441 cell monolayers at 37°C for 90 min. Each bar represents means $\pm$ SD ($n = 3$). ** $P < 0.01$ significantly different between directions.

5.3.6 Effect of RNAi on OCT protein expression and [¹⁴C]-TEA uptake in A549 cells

To investigate, whether knockdown of OCT1, OCT2 or OCT3 led to changes in [¹⁴C]-TEA uptake, A549 cells were transfected with OCT1-, OCT2- and OCT3-siRNA. Protein expression levels were determined 3 days after transfection and results are shown in Chapter 3. To assess if RNAi had an effect on transporter function, [¹⁴C]-TEA uptake studies were carried out under similar conditions as described above. All experiments were performed in non-treated, mock-transfected and OCT siRNA-transfected A549 cells. No significant differences were observed between non-treated and mock-transfected A549 cells. Nevertheless, all data obtained from OCT-siRNA transfected cells were compared to the relevant mock-transfected controls. Knockdown of OCT1 or OCT2 significantly inhibited [¹⁴C]-TEA uptake to $52.5 \pm 3.2\%$ and $50.4 \pm 8.6\%$, respectively, whilst knockdown of OCT3 did result in a lower decrease of $84.6 \pm 8.3\%$.

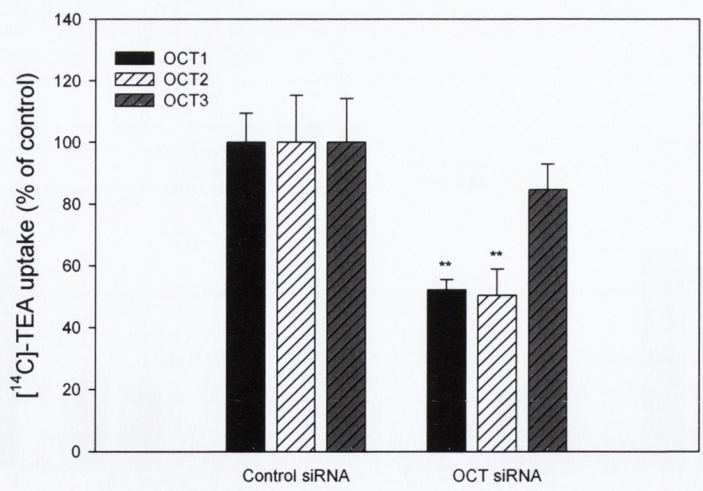


Figure 11. The effects of OCT1-3 RNAi was studied on [¹⁴C]-TEA uptake into A549 cells. A549 cells were transfected with 75 nM OCT1-, OCT2- and OCT3-siRNA. Uptake studies were performed 3 days after siRNA transfection at pH 7.4 and 37°C. Data represent means $\pm$ SD ($n = 3 - 6$). ** $P < 0.01$ indicates a significant difference from the uptake by mock transfected cells.

5.4 DISCUSSION

Membrane transporters set a complex framework regulating the uptake and efflux of substrates across the cell membrane. Given the fact that endogenous and exogenous substrates provide a large structural variety, a wide spectrum of drug transporters is required. Their substrate specificity and activity is of crucial importance for a compliant distribution between the inside and outside of the cell. Hence, to understand the network as a whole, it is essential to know how transporters connect and control pathways of compounds, e.g., organic cations. The functional characterisation of organic cation/carnitine transporters expressed in the lung epithelium is still in its infancy. Little information is available on the actual contribution of the transporter subtypes. So far, a transporter protein has not yet been identified which is mainly responsible for the uptake of organic cations such as, OCT1 in the liver or OCT2 in the kidney (International Transporter Consortium, 2010). Only a limited number of functional activity studies have been carried out in human pulmonary cells thus far (Horvarth et al., 2007; Mukherjee et al., 2012; Salomon et al., 2012). Here, we investigated the uptake pathways of the cationic substrates [^{14}C]-TEA and [^3H]-acetylcarnitine using respiratory epithelial cell models (A549, H441, Calu-3 and human alveolar primary cells), in order to differentiate between the contribution of OCT and OCTN transporters in various epithelial barriers of the lung.

Our results indicated that TEA increased in a time-dependent manner in all used cell models. Human alveolar epithelial cells in primary culture and A549 cell line exhibited a similar and considerable higher uptake than the bronchial and bronchiolar cell lines. Kinetic analysis of TEA uptake by A549 cells revealed the presence of a transport system with both sites: a saturable and a non-saturable transporter site. The estimated

K_m for the linear component of TEA uptake (529 μM) was in the same order of magnitude as those reported for hOCT1 (168 and 229 μM) in HeLa-cells (Zang et al., 1997; Bednarczyk et al., 2003), hOCT2 (76 and 431 μM) and hOCT3 (1372 μM) in *Xenopus laevis* oocytes (Gorboulev et al., 2007; Bourdet et al., 2005). A K_m of 436 μM was obtained in OCTN1-transfected HEK-293 cells by Tamai *et al.* (Tamai et al., 1997). This low-affinity uptake of TEA in human airway epithelium was significantly decreased by various specific OCT1-3 substrates as MPP⁺, decynium-22, norepinephrine or corticosterone. Carnitine (an endogenous OCTN substrate) did not show an effect on TEA uptake into A549 cells. Amantadine (OCT2 inhibitor) decreased the uptake by about 70% of the control value. Furthermore, hemicholinium-3 and acetylcholine (inhibitors of the CHT and CTL) did not significantly affect TEA uptake. Ergothioneine, a *bona fide* OCTN1 substrate, reduced uptake in A549 cells to about 70% of control. Accordingly, the uptake of TEA is most likely facilitated by OCT1 and/or OCT2. Further evidence was provided by RNAi studies in A549 cells. OCT1 and OCT2-siRNA transfection resulted in decreased TEA uptake activity; values were reduced to approximately 50%, whilst knockdown of OCT3 had no significant effect. However, when concentration-dependency of TEA was studied in H441 and Calu-3 cells, no saturation of the uptake process was observed. Nevertheless, inhibitory effects were evident in the presence of MPP⁺, decynium-22, TEA and also ASP⁺. Amantadine inhibited TEA uptake to a much lesser extent, and hemicholinium-3 did not affect the uptake in both cell lines. TEA uptake into human primary cells was also strongly decreased in the presence of classical OCT substrates (such as mentioned above). These results suggest that TEA uptake into ATI-like, H441 and Calu-3 cells was mediated by OCTs; however, functional activity was much lower than in A549 cells. The role of OCTN1 in this translocation needs further investigations.

Our investigations confirmed a time-dependent uptake of acetylcarnitine in bronchial and alveolar epithelial cell lines with uptake values in A549 being significantly higher compared to the other cell culture models. Concentration-dependent acetylcarnitine uptake into A549 and Calu-3 cells was calculated to exhibit a K_m of 17.7 μM and 35.4 μM , respectively, which was in good agreement with the K_m reported by Ohashi *et al.* (Ohashi *et al.*, 1999) for OCTN2 (8.5 μM). Tachikawa *et al.* confirmed a saturable acetylcarnitine uptake at the inner blood-retinal barrier and calculated a K_m of 26.1 μM (Tachikawa *et al.*, 2010). Moreover, acetylcarnitine uptake was most strongly inhibited by verapamil = quinidine = D-carnitine $\gg$ ergothioneine in A549 cells, whereas the classic OCT inhibitors such as MPP^+ and corticosterone did not affect the uptake. Verapamil and quinidine are both known to inhibit OCTN1 and OCTN2 (Koeppel *et al.*, 2007), whilst ergothioneine has been identified as the physiological substrate for OCTN1 (Gruendemann *et al.*, 2005). In ATI-like, we could also confirm a high inhibitory effect of verapamil and D-carnitine on the acetylcarnitine uptake transporter, compared to ergothioneine, which had no attenuating effect. Similarly, verapamil and D-carnitine inhibited acetylcarnitine uptake into Calu-3 cells to approximately 65% of control. MPP^+ and ASP^+ had almost no effect. In the case of H441 cells, several inhibitors could prove the involvement of OCTN2: D-carnitine and verapamil significantly inhibited acetylcarnitine uptake, however, a concentration-dependent uptake was not observed. These results suggest a major role of OCTN2 in the translocation of acetylcarnitine in A549 and Calu-3 epithelial cells which might conclusively be elucidated by RNAi studies.

Further investigations evaluated the influence of extracellular pH and sodium concentration on organic cation uptake into the respiratory epithelium. TEA uptake

exhibited a certain sodium- and pH-sensitivity in A549 cells. The uptake of acetylcarnitine by A549 cells also revealed sodium-dependency. Unlabelled TEA (at 20 mM) diminished both TEA and acetylcarnitine uptake into A549 cells to a similar extent, confirming that TEA is a substrate/inhibitor for all cloned OCT/N transporters. These findings have been already reported by Tamai and co-workers (Tamai et al., 1998; Tamai et al., 1999, Gründemann et al., 2005), who confirmed that TEA, verapamil and other drugs showed a remarkable inhibitory effect on hOCTN2-mediated carnitine transport. A pH-dependent transport of organic cations such as TEA via OCTN1 and OCTN2 was reported (Tamai et al., 2000) in stably transfected HEK-293 cells. However, we could not find a pronounced pH-dependency of acetylcarnitine uptake in A549 cells. In contrast to our findings, a number of studies confirmed that OCT function was independent from sodium and proton gradients (Koepsell et al., 2005; Ciarimboli, 2008; Nies et al., 2011). Interestingly, OCTN2 was reported to alternatively operate as a polyspecific and sodium-independent transporter (Koepsell et al. 2007). Most of these findings were obtained in HEK-293 cells.

A similarly complex picture was also evident in human bronchial and bronchiolar cell lines. We found a sodium-dependent and –sensitive uptake of TEA and acetylcarnitine into H441 cells. TEA uptake into these cells was pH-dependent, whilst no effect of extracellular pH could be seen on acetylcarnitine uptake. In Calu-3 cells, only acetylcarnitine uptake was sodium-dependent. By increasing the amount of protons in the extracellular fluid, the uptake of organic cations was not affected in Calu-3 cells.

Generally, we found a lower level of activity in H441, Calu-3 and ATI-like cells, which might be due to the fact that functional tight junctions govern the cation uptake rate of those cell monolayers (Forbes and Ehrhardt, 2005; Buckley et al., 2011). It is well

known that A549 cells are not capable of developing polarised monolayers (Foster et al., 1998). By opening the tight junctions via incubation with EDTA, we could demonstrate that organic cation transporters are localised to the basolateral membranes. In Chapter 3, our working model of organic cation transporter localisation was already discussed in detail. Briefly, Lips *et al.* found OCT1 and OCT2 were mainly located in apical membranes of ciliated cells and to a lesser extent also in basal cells (Lips et al., 2005). Immunoreactivity of OCT3 was localised to plasma membranes of basal cells, basolateral membranes of intermediate cells and apical membranes of ciliated cells. Currently, the role of organic cation transporter 3 remains unclear. OCT3 was equally strong expressed across human respiratory epithelial cell lines including H441 and human alveolar epithelial primary cells (Salomon et al., 2012). OCT3 siRNA transfection in A549 cells did not have an impact on TEA uptake. In contrast to our findings, Horvath *et al.* showed in human bronchial smooth muscle cells a reduced uptake of formoterol by OCT3-knockdown and hence, proposed that OCT3 might be a target for inhaled drugs (Horvath et al., 2011).

Our data suggest that organic cation transporters are predominant contributors to membrane transport of organic cations in the alveolar epithelium, in particular the high-affinity transporters OCT1 and OCT2. Due to the fact that OCT2 is exclusively expressed in alveolar epithelial cells, but not in Calu-3 cells (Chapter 2), it can be speculated that the significantly increased cellular uptake of TEA into ATI-like and A549 cells was the result of the presence of OCT2. It is possible that transport of TEA is mainly accounted for by OCT1 action. This transporter protein appears to be localised to the basal side of human bronchial epithelial cells (Calu-3).

Horvath *et al.* (Horvath et al., 2007) identified OCTN1 and OCTN2 at the apical surfaces of airway epithelial cells, in addition to alveolar type I and type II cells. Based

on our findings, it might be speculated that acetylcarnitine uptake is mainly mediated by lumenally localised OCTN2 throughout the lung.

No information is available on the localisation of OCT/Ns in the H441 cell culture model, due to the fact that it has received little attention in a biopharmaceutical context. Nevertheless, by providing alveolar and bronchiolar epithelial features, the H441 cell line might be a good candidate for drug absorption studies in the lower lung (Buckley et al., 2011). We could not find concentration-dependent uptake of TEA or acetylcarnitine, as passive diffusion exhibited relative high values. Uptake activity of both compounds was very low and inhibitors lacked efficacy in distinguishing organic cation pathways. Transport studies revealed a slight net secretion of both, TEA and acetylcarnitine, across the H441 cell monolayers. We therefore provide first evidence of functionally expressed OCT/Ns in this cell culture model. However, further studies are needed to fully understand the spectrum of transporters involved.

5.5 CONCLUSIONS

Data from this Chapter suggest that the absorption of organic cations by organic cation/carnitine transporters follows a complex pattern at the lung epithelium. An OCT-driven uptake of organic cations at the respiratory epithelium has been shown, mainly involving OCT1 and OCT2 at the alveolar epithelium. The actual contribution of OCTs at the bronchial epithelium still needs to be confirmed by knockdown of the individual transporter proteins. Secondly, the involvement of OCTNs, in particular OCTN1, in the uptake of TEA at the lung epithelium remains unexploited. In this context, also RNAi studies would provide further insight. OCTN2 most likely mediates acetylcarnitine uptake in alveolar and bronchial epithelial cells.

Currently, some characteristics of organic cation uptake at the lung epithelium cannot be fully elucidated, due to the fact that even more membrane transporters might be involved such as proteins encoded by *SLC5*, *SLC6*, *SLC7*, *SLC29*, *SLC44* and *SLC47* gene families, in particular *SLC47A1* (MATE1) (Salomon and Ehrhardt, 2012).

CHAPTER 6

Beta-2-agonists are specific inhibitors of organic cation transporters in the lung- salbutamol is not a substrate for these transporter proteins

ABSTRACT

In this work, we investigated the selectivity of β_2 -agonistic drugs such as formoterol and salbutamol (albuterol), which are cations at physiological pH, to inhibit organic cation uptake pathways at the respiratory epithelium. We studied if β_2 -agonists are substrates and/or inhibitors of putative organic cation transporters (OCTs). The inhibitory potential of β_2 -agonists (i.e., salbutamol, formoterol and salmeterol) on [^{14}C]-TEA and on [^3H]-acetylcarnitine uptake was investigated in A549, H441 and Calu-3 epithelial cells. In HEK-293 transfectants, the uptake of [^3H]-1-methyl-4-phenylpyridinium (MPP^+) was studied in the presence of β_2 -agonists. Furthermore, uptake of [^3H]-salbutamol into alveolar (A549) and bronchial (Calu-3) epithelial cells was assessed in the presence of OCT modulators. [^3H]-salbutamol transport studies were carried out in bronchiolar (H441) and bronchial (Calu-3) cells, respectively. [^{14}C]-TEA uptake into A549 cells was reduced in the presence of salbutamol (500 μM) to 32.6%, in the presence of formoterol (500 μM) to 8.8% and in the presence of salmeterol (100 μM) to 17.7%. Formoterol inhibited TEA uptake with a K_i of 92.3 μM , whereas a K_i value of 271 μM was obtained for salbutamol. In the case of salmeterol, a K_i of 16.6 μM was calculated. In H441 cells, [^{14}C]-TEA uptake was significantly attenuated in the presence of β_2 -agonists to 35.5% (salbutamol), to 19.3% (formoterol) and to 8.4% (salmeterol), respectively. [^{14}C]-TEA uptake into Calu-3 cells was less sensitive at 78.1% (500 μM salbutamol), 52.4% (500 μM formoterol) and 52.9% (100 μM salmeterol) of control. The most pronounced inhibition by aforementioned β_2 -agonists on [^3H]-1-methyl-4-phenylpyridinium (MPP^+) uptake was observed in OCT1-transfected HEK-293 cells. OCT3 expressing HEK-293 cells were also affected, whereas in OCT2 transfectants only marginal inhibition was measured. The rank order of OCT inhibition by β_2 -receptor agonists was formoterol>salmeterol >>salbutamol. IC_{50} values of 47.8 μM for

salmeterol and of 22.3 μM for formoterol were obtained. [^3H]-salbutamol uptake was not attenuated by *bona fide* OCT substrates in both cell lines and its transport across Calu-3 cell monolayers revealed net secretive P_{app} values of $6.9 \pm 0.9 \cdot 10^{-7}$ cm/s (A-to-B) and $6.2 \pm 1.4 \cdot 10^{-6}$ cm/s (B-to-A). P_{app} values of $1.2 \pm 0.2 \cdot 10^{-6}$ cm/s (A-to-B) and $4.8 \pm 2.3 \cdot 10^{-7}$ cm/s (B-to-A) were obtained from salbutamol transport across H441 cell monolayers.

These data suggest that β_2 -agonists are specific inhibitors of OCT1 in human respiratory epithelial cells. However, β_2 -agonists are not substrates of organic cation transporters. MATE1 might play a role in the translocation of salbutamol across the lung epithelium.

6.1 INTRODUCTION

The impact of drug transporter proteins on disposition of inhaled therapeutics is still poorly understood (Patton et al, 2007; Bosquillon, 2010; Gumbleton et al., 2011). Many drugs that are therapeutically inhaled as aerosols, such as β_2 -receptor agonists and muscarinergic antagonists, carry a net positive charge at physiologic pH values. This led us and others to the hypothesis that transporters associated with organic cation translocation in other organs, e.g., OCTs and OCTNs (*SLC22A1-A5*) might also be involved in absorption and clearance processes in the lung (Ehrhardt et al., 2005; Horvath et al., 2007; Salomon et al., 2012).

A number of studies have recently been reported investigating the interactions between β_2 -agonists and transporters in the lung. Initially, our laboratory reported that the β_2 -adrenoceptor agonist, salbutamol exhibited concentration- and temperature-dependent net absorption across monolayers of human respiratory epithelial cells (Ehrhardt et al., 2005). Subsequent studies provided evidence that β_2 -agonists inhibited OCT-mediated 4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (ASP⁺) uptake in respiratory epithelial and smooth muscle cells *in vitro* (Horvath et al., 2007; Salomon et al., 2012). Horvath and colleagues reported that inhaled corticosteroids did not interfere with the transport of organic cations in the lung, but also that formoterol was a substrate for OCT3 in airway smooth muscle cells (Horvath et al., 2011). In a similar study, Chiappori *et al.* suggested that salbutamol uptake into bronchial smooth muscle cells was also OCT3 mediated (Chiappori et al., 2012). It is of note that Gnadt and co-workers recently reported cation transporter interactions during pulmonary absorption of β_2 -agonists using an isolated perfused human lung lobe model (Gnadt et al., 2012). Whilst there are clinically relevant data emerging, the exact nature of β_2 -receptor

agonists-transporter interactions is far from being understood. Therefore, we do not know if mutations of organic cation transporter subtypes contribute to inter-individual variations in pulmonary drug disposition of this class of therapeutics. Sakata *et al.* investigated five SNPs in the OCT3 gene and found a link to reduced activity in three of these variants (Sakata et al., 2010). The conducting airways are the main target for most pharmaceuticals used as aerosols, however, a significant amount of these drugs reaches the distal lung, where absorption at the air-blood barrier causes systemic side effects (Morgan et al., 1990). Consequently, it is pivotal to analyse the absorption pathways of β_2 -agonists in different areas of the lung.

In the present study, we investigated whether β_2 -adrenergic drugs interfere with the uptake of the model substrates, [^{14}C]-TEA and [^3H]-acetylcarnitine into monolayers of human alveolar (A549), bronchiolar (H441) and bronchial (Calu-3) epithelial cells, in order to allow differentiation of OCT- and OCTN-mediated pathways. Additionally, we studied how [^3H]-MPP⁺ uptake into hOCT1, hOCT2 or hOCT3-over-expressing HEK-293 cells is affected by β_2 -receptor agonists. Lastly, [^3H]-salbutamol uptake and transport into and across respiratory epithelial cells was studied.

6.2 MATERIALS AND METHODS

6.2.1 Materials

[Ethyl-1- ^{14}C] tetraethylammonium chloride (^{14}C -TEA; 55 mCi/mmol, 10 μM), [n-methyl- ^3H] acetyl-L-carnitine hydrochloride (^3H -acetylcarnitine; 85 Ci/mmol, 7.5 nM) and salbutamol [ring- ^3H] (^3H -salbutamol; 20 Ci/mmol, 25 nM) were purchased from American Radiolabeled Chemicals (UK) Limited (Herts, UK). Unlabelled TEA, acetylcarnitine, amantadine, corticosterone, D-carnitine, 1,1-diethyl-2,2-cyanine (decynium-22), hemicholinium-3, 1-methyl-4-phenylpyridinium (MPP^+), lysine, norepinephrine, probenecid, pyrilamine, verapamil and all cell culture media and supplements were obtained from Sigma-Aldrich (Dublin, Ireland), unless otherwise stated. Adenosine, ergothioneine, formoterol fumarate, quinidine and salmeterol xinafoate were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and 4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide (ASP^+) from Biosciences (Dun Laoghaire, Ireland). Salbutamol base was purchased from Chemos GmbH (Regenstauf, Germany) and salbutamol sulphate from Sigma-Aldrich (Deisenhofen, Germany). Cell culture plastics were purchased from Greiner BioOne (Frickenhausen, Germany).

6.2.2 Cell culture

A549 (American Type Culture Collection, ATCC CCL-185) cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and Calu-3 (ATCC HTB-55) cells were purchased from LGC Promochem (Teddington, UK). Cells were cultured at a seeding density of 40,000 cells/ cm^2 (A549) or 75,000 cells/ cm^2 (Calu-3). A549 cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1 mix)

(DMEM/F12) supplemented with 5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Calu-3 cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) FBS, 1% non-essential amino acids, 1% sodium pyruvate solution, 0.5% glucose solution and 100 U/ml penicillin and 100 µg/ml streptomycin. All cell types were maintained at 37°C in 5% CO₂ atmosphere and the medium was exchanged every 48 h, until confluent monolayers were reached.

6.2.3 Organic cation uptake studies in the presence of β_2 -agonists

Uptake experiments were carried out in freshly prepared bicarbonated Krebs-Ringer buffer (KRB) composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, 0.81 mM MgSO₄ and 5.55 mM glucose; pH 7.4. Cells were grown to confluent monolayers for 5 (A549) or 12 (Calu-3) days. Prior to uptake experiments, cell monolayers were washed three-times with pre-warmed buffer solution.

The inhibitory potency of salbutamol (base and sulphate, 500 µM), formoterol fumarate (500 µM) and salmeterol xinafoate (100 µM) on [¹⁴C]-TEA uptake was measured for 10 (Calu-3) and 30 min (A549 cells), respectively. [³H]-acetylcarnitine uptake was studied in the presence of β_2 -agonists for 20 min in both cell types.

For kinetic analyses in A549 cells, salbutamol (1 mM), formoterol (500 µM) and salmeterol (100 µM) were applied simultaneously with [¹⁴C]-TEA and unlabelled TEA (in the concentration range of 0.25 to 2 mM), and uptake was measured after 30 min of parallel incubation. Moreover, in Calu-3 cells [³H]-acetylcarnitine uptake was measured in the presence of a constant concentration of 500 µM formoterol and unlabelled acetylcarnitine in the range of 50 to 200 µM for 20 min. At the indicated time points, the uptake was stopped by washing the cell monolayers three-times with ice-cold buffer and

then, 400 µl of 1N NaOH was added to permeabilise the cells. Monolayers were left to lyse for at least 12 h, before 400 µl of 1N HCl was added for neutralisation of the cell lysate. Five-hundred microlitres of lysate was used to measure the cell-associated radioactivity in a liquid scintillation counter (Tri Carb TR2100 Packard Scintillation Counter, Ireland). In parallel, the total cell protein content was quantified using a protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

6.2.4 [³H]-MPP⁺ uptake by OCT-over-expressing HEK-293 cells

These experiments were carried out by Dr. Yohannes Hagos (Georg-August-Universität, Göttingen, Germany).

HEK-293 cells stably expressing hOCT1, hOCT2 or hOCT3 were selected by hygromycine (10 µg/ml) and grown in tissue culture flasks in high-glucose DMEM medium (Invitrogen, Germany) supplemented with 10% foetal calf serum (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Deisenhofen, Germany). For uptake studies, HEK-293 cells were seeded in 24-well plates (2x10⁵ cells/well) and grown for three days until 90-100% confluence. Initially, cells were washed with 1 ml of pre-warmed mammalian Ringer solution (130 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 mM Mg₂SO₄, 1 mM NaH₂PO₄, 20 mM HEPES, and 20 mM D-glucose; pH 7.4). Cells were then incubated with prewarmed mammalian Ringer solution, containing radioactively labelled 1 µM MPP⁺ (composed of 20 nM [³H]-MPP⁺ plus 980 nM unlabelled MPP⁺) for 5 min. Uptake of MPP⁺ was measured in the presence and absence of salbutamol (base and sulphate), formoterol fumarate and salmeterol xinafoate (all compounds were used at concentrations of 100 and 500 µM). Moreover, in concentration-dependent inhibition studies, MPP⁺ uptake was performed in hOCT1-transfected HEK-293 cells in the presence of various concentrations of

formoterol fumarate and salmeterol xinafoate (i.e., 20, 40, 60, 80, 100 120, and 140 μM) as indicated in the results section. To stop the reaction, cells were washed with ice-cold PBS and lysed with 500 μl of 1 M NaOH solution for 20 min. The solution was then neutralised with 1N HCl, transferred to scintillation vials and 2.5 ml Lumasafe scintillation solution was added to each sample. Incorporated radioactivity was determined by using a scintillation counter (TriCarb 1500 Packard, Meriden, CT). For Dixon plot analysis, data were calculated by SigmaPlot 10 (SPSS Science, Chicago, IL) multiple regression. IC_{50} values were calculated by SigmaPlot 10 Non-linear Regression Equation: Hyperbola; Hyperbolic Decay, 3 Parameter [$f=y_0+(a*b)/(b+x)$].

6.2.5 [^3H]-salbutamol uptake studies

A549 and Calu-3 cell monolayers were grown on plastics for 5 and 12 days, respectively. Cells were washed twice with KRB solution, followed by incubation with [^3H]-salbutamol (20 nM). For time-course analyses salbutamol uptake was followed for 30 min in both cell culture models. In addition, A549 cells were incubated with [^3H]-salbutamol in the presence of various concentrations (0-1 mM) of salbutamol base for 5 min. In inhibition experiments, [^3H]-salbutamol uptake was performed in the presence of unlabelled salbutamol base (0-1 mM) and a constant concentration of 10 μM of cyclosporine A to inhibit P-glycoprotein function. Moreover, the sensitivity of [^3H]-salbutamol to various organic cations was tested, i.e., uptake was measured in the presence of acetylcholine (200 μM), adenosine (1 mM), D-carnitine (100 μM), decynium-22 (500 μM), ergothioneine (200 μM), hemicholinium-3 (500 μM), MPP^+ (500 μM to 1 mM), lysine (1 mM), quinidine (500 μM), TEA (1 to 20 mM) and verapamil (200 μM).

[³H]-salbutamol uptake was also studied at different extracellular pH values (i.e., 5.5, 6.5 and 8.5). The effect of membrane depolarisation and sodium depletion on salbutamol uptake was investigated as well. Sodium-free KRB medium was prepared by replacing NaCl, NaHCO₃ and NaH₂PO₄ with equimolar concentrations of KCl, KHCO₃ and KH₂PO₄ or with N-methyl-D-glucamine (NMG), respectively (i.e., Na⁺ concentration, 5.4 mM; K⁺ or NMG concentration, 142 mM). Salbutamol uptake was terminated by removing the tracer solution and washing the cells three times with ice-cold KRB. The amount of accumulated [³H]-salbutamol was determined by lysing the cells in 1N NaOH for at least 12 h, followed by neutralisation with 1N HCl and cell-associated radioactivity was then measured with a liquid scintillation counter (Tri Carb TR2100 Packard Scintillation Counter). In parallel, the total cell protein content was quantified using a DC protein assay kit (Bio-Rad) according to the manufacturer's instructions.

6.2.6 [³H]-salbutamol transport studies

Cells were grown on Transwell Clear filter inserts for at least 14 (Calu-3) days (TEER >350 Ohm/cm²) [13]. Briefly, transport studies were initiated by washing both sides of the cell monolayers twice with pre-warmed KRB solution, followed by a 60 min equilibration in KRB at 37°C. [³H]-salbutamol transport was studied under air-interfaced conditions, i.e., in the case of apical-to-basolateral transport only 5 µl of tracer solution was applied to the cell monolayer. This volume was calculated assuming physiological height of the lung lining fluid (5 µm). Seven-hundred microlitres of pre-warmed KRB solution were applied to the basolateral compartment. The cells were kept at 37°C during the experiment and 200-µl samples were collected from the receiver

compartment and replaced with an equal amount of fresh, pre-warmed KRB solution. Basolateral-to-apical transport was performed by adding 705 µl of [³H]-salbutamol solution to the basolateral (i.e., donor) compartment. A 5-µl sample was taken directly to assess the initial concentration. The transported amount of [³H]-salbutamol was collected in total, after the experiment by washing the surface of the cell monolayers six times with 100 µl of KRB solution. At the end of transport studies, another 5 µl sample was collected to calculate mass balance. Furthermore, cell monolayers were permeabilised in 400 µl 1N NaOH for at least 12 h, neutralised with 400 µl 1N HCl and intracellular accumulation of [³H]-salbutamol was measured. Each experiment was conducted at least in triplicates. Transepithelial electrical resistance (TEER) values were recorded before and after the flux studies, in order to confirm cell layer integrity. The radioactivity of samples was determined as described above with a Tri Carb TR2100 Packard Scintillation Counter.

The utilised equation to calculate the apparent permeability coefficient (P_{app}) is the following:

$$P_{app} = (\Delta Q / \Delta t) / (A * C_0) \quad [\text{Eq.1}]$$

where ΔQ was the change in quantity of the compound over a designated period of time (Δt), A was the nominal surface area of the cell layers (1.13 cm²) and C_0 was the initial concentration of the drug in the donor fluid and used in this study.

6.2.7 Data analysis

The uptake of [¹⁴C]-TEA, [³H]-acetylcarnitine and [³H]-salbutamol was expressed as the cell-to-medium ratio calculated by the following equations:

$$\text{Cell/medium ratio} = [\text{}^3\text{H}] \text{ dpm per cell protein} / [\text{}^3\text{H}] \text{ dpm per } \mu\text{l buffer} \quad [\text{Eq. 2}]$$

$$[\text{}^{14}\text{C}] \text{ dpm per cell protein} / [\text{}^{14}\text{C}] \text{ dpm per ml buffer} \quad [\text{Eq. 3}]$$

Results were expressed as mean $\pm$ SD. The significance of differences between means was determined by unpaired, two-tailed Student's *t*-test. Statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA), followed by the modified Fisher's least squares difference method. All experiments were carried out at least in triplicate. Representative K_i values were determined by using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

6.3 RESULTS

6.3.1 Effect of β_2 -agonists on organic cation uptake by human respiratory epithelial cells

We investigated, whether the β_2 -agonists, salbutamol, formoterol and salmeterol, inhibited OCT and/or OCTN function in A549, H441 and Calu-3 cells. As shown in Table 1A, all three compounds significantly reduced [14 C]-TEA uptake into A549 cells to $45.3 \pm 9.1\%$ (salbutamol base), $32.6 \pm 6.9\%$ (salbutamol sulphate), $8.8 \pm 2.1\%$ (formoterol fumarate) and $17.7 \pm 0.5\%$ (salmeterol xinafoate). The β_2 -agonists, however, did not have an effect on acetylcarnitine uptake in A549 cells, except for salmeterol ($P < 0.05$) (Table 1B). In Calu-3 cells, however, formoterol and salmeterol decreased uptake of [14 C]-TEA and [3 H]-acetylcarnitine to a similar extent to approximately 50-60%, whereas salbutamol only had a marginal effect. Moreover in H441 cells, all beta-agonists exhibited strong inhibition effects on [14 C]-TEA uptake. The uptake was reduced to $19.1 \pm 7.6\%$ (formoterol), to $27.0 \pm 7.6\%$ (salbutamol base), $35.5 \pm 7.5\%$ for (salbutamol sulphate) and $8.4 \pm 14.5\%$ (salmeterol). [3 H]-acetylcarnitine uptake into H441 cells was slightly more affected by beta-agonists than in A549 cells.

Table 1. Effects of salbutamol (500 µM both, base and sulphate), formoterol fumarate (500 µM) and salmeterol xinafoate (100 µM) on the uptake of [¹⁴C]-TEA (10 µM, **A**) and [³H]-acetylcarnitine (7.5 nM, **B**) by human respiratory epithelial cell layers (A549, H441 and Calu-3 cells). Uptake of [¹⁴C]-TEA was measured for 10 min (H441, Calu-3) and 30 min (A549), whilst uptake of [³H]-acetylcarnitine was studied over 20 min in all cell models in the presence of the β₂-agonists. Values were obtained by calculating the difference between uptake at 37°C and 4°C. Each value represents the mean ± SD (*n* = 3 - 6). ***P* < 0.01, **P* < 0.05 significantly different from control, n.d. = not determined.

A				
¹⁴ C]-TEA uptake	Uptake (% of control) in the presence of formoterol 500 µM	Uptake (% of control) in the presence of salbutamol base 500 µM	Uptake (% of control) in the presence of salbutamol sulphate 500 µM	Uptake (% of control) in the presence of salmeterol 100 µM
A549	8.8±2.1**	45.3± 9.1**	32.6± 6.9**	17.7±0.5**
Calu-3	52.4±14.5**	76.1±11.8*	78.1±34.2*	52.9±6.3**
H441	19.3±7.6**	27.0±10.9**	35.5±7.5**	8.4±14.5**
B				
³ H]-acetylcarnitine uptake	Uptake (% of control) in the presence of formoterol 500 µM	Uptake (% of control) in the presence of salbutamol base 500 µM	Uptake (% of control) in the presence of salbutamol sulphate 500 µM	Uptake (% of control) in the presence of salmeterol 100 µM
A549	107.6±14.2	n.d.	100.0± 12.4	85.0±4.8*
Calu-3	52.3±2.8**	96.5±6.9	103.8±1.8	60.0±3.0**
H441	73.3±3.7*	n.d.	129.8±9.0*	72.3±7.2*

6.3.2 Kinetic analysis of inhibitory effects on organic cation uptake in A549 and Calu-3 cells

The concentration dependence of TEA and acetylcarnitine uptake into human respiratory epithelial cell lines was investigated in Chapter 5. Based on these findings; the following studies were carried in the presence of β_2 -agonists in A549 and Calu-3 cells only. It should be noted that kinetic studies were performed when a saturation of the compound itself could be reached, e.g. TEA uptake into A549 cells, and acetylcarnitine uptake into Calu-3 cells.

The kinetics of the β_2 -agonists, formoterol, salbutamol and salmeterol on [^{14}C]-TEA and [^3H]-acetylcarnitine uptake were calculated for alveolar and bronchial epithelial cell models (Figure 1 and 2). In A549 cells, [^{14}C]-TEA uptake was significantly decreased in the presence of 1 mM salbutamol (Figure 1A), 500 μM formoterol (Figure 1C) and 100 μM salmeterol (Figure 1E). Eadie-Hofstee analysis, shown in Figure 1B (salbutamol), 1D (formoterol) and 1F (salmeterol) suggested competitive inhibitory kinetics for all three compounds. The evaluated K_m and $V_{\max}$ for TEA uptake were $1310.9 \pm 380.0 \mu\text{M}$, whereas with salbutamol present, the apparent K_m increased approximately 3.5-fold. $V_{\max}$ did not change ($0.42 \pm 0.06 \text{ nmol/min/mg protein}$ versus $0.35 \pm 0.02 \text{ nmol/min/mg protein}$ in the absence and presence of salbutamol, respectively). A K_i of $276.6 \pm 90.4 \mu\text{M}$ was calculated for salbutamol. The Eadie-Hofstee plot of TEA uptake in the absence of formoterol showed similar kinetics with a K_m value of $848.2 \pm 71.9 \mu\text{M}$, which increased in the presence of formoterol, 5-fold to $4.33 \pm 3.22 \text{ mM}$. Again, no significant change in $V_{\max}$ was observed ($0.28 \pm 0.01 \text{ nmol/min/mg protein}$ vs. $0.20 \pm 0.14 \text{ nmol/min/mg protein}$ in the absence and presence of formoterol, respectively). The K_i of formoterol for TEA uptake was calculated as

92.3 ± 20.3 μM. A K_m value of 2.02 ± 0.10 mM in the absence and 12.07 ± 1.33 mM in the presence of salmeterol was found.

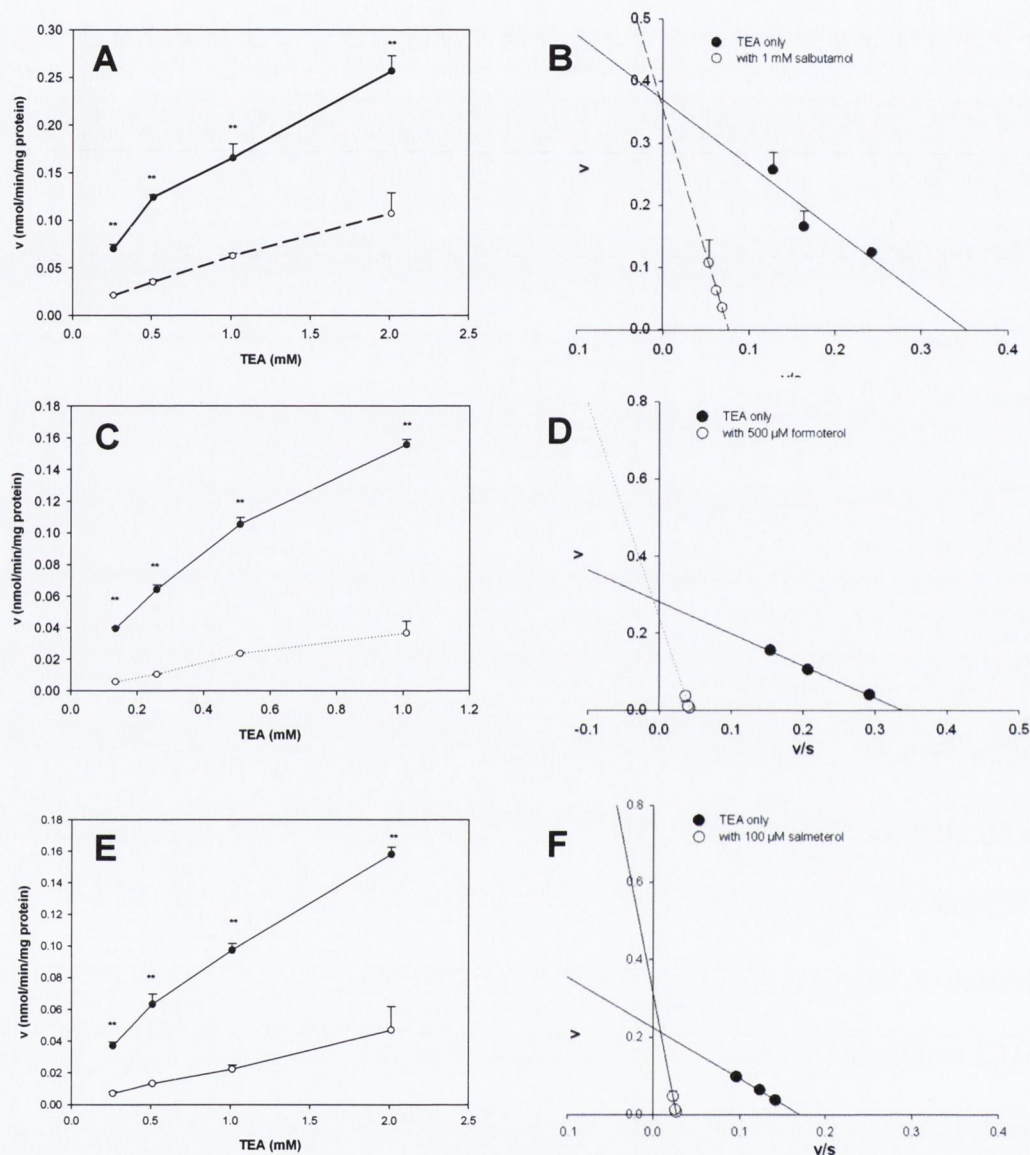


Figure 1. Organic cation uptake into A549 cells in the presence (○) or absence (●) of β_2 -agonists. [14 C]-TEA uptake was measured for 30 min at constant concentrations of salbutamol (A; 1 mM), formoterol (C, 500 μM) and salmeterol (E, 100 μM) in A549 cells. The results of inhibitory effects of β_2 -agonists on [14 C]-TEA uptake into A549 cell monolayers were analysed by Eadie-Hofstee transformation (B: salbutamol; D: formoterol, F: salmeterol). Each data point represents mean ± SD ($n = 3$). ** $P < 0.01$ significantly different from control.

In line with the other observations, $V_{\max}$ remained at constant levels (i.e., 0.32 ± 0.01 nmol/min/mg protein in the absence and of 0.34 ± 0.03 nmol/min/mg protein in the presence of the inhibitor). A K_i value of $16.6 \pm 0.5 \mu\text{M}$ was obtained for salmeterol. The K_i values of all compounds were significantly lower than the respective K_m values of TEA.

When the effect of formoterol on acetylcarnitine uptake in Calu-3 cells was studied, the kinetic analyses revealed a different inhibition pattern. A constant concentration of 500 μM formoterol significantly reduced acetylcarnitine uptake (Figure 2A), however, Eadie-Hofstee transformation showed uncompetitive inhibition characteristics (Figure 2B). In this case, K_m and $V_{\max}$ were both decreased: K_m values of $614.3 \pm 108.5 \mu\text{M}$ and $441.2 \pm 175.2 \mu\text{M}$ in the absence and presence of formoterol were found, respectively; $V_{\max}$ was reduced to 0.06 ± 0.01 nmol/min/mg protein in the presence of formoterol compared to $V_{\max} = 0.10 \pm 0.01$ nmol/min/mg protein in the absence of the compound.

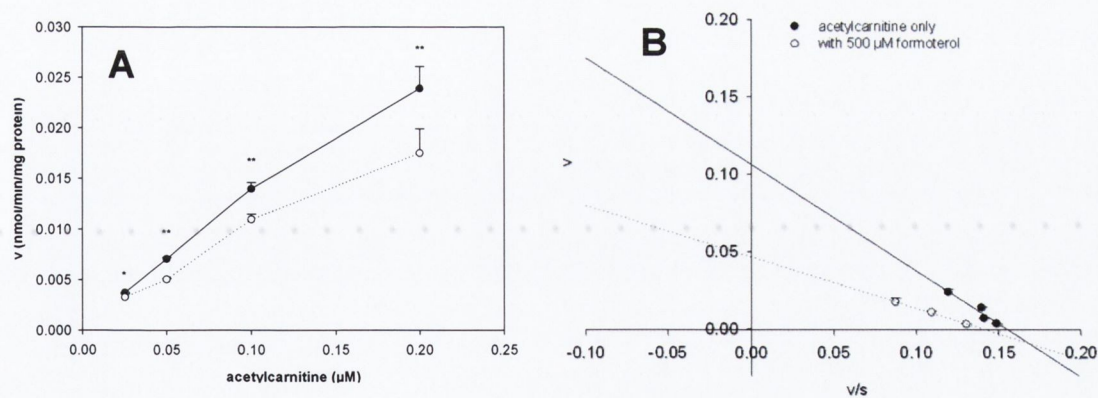


Figure 2. $[^3\text{H}]$ -acetylcarnitine uptake into Calu-3 cells was studied in the presence ($\circ$) or absence ($\bullet$) of a constant concentration of formoterol (A, 500 μM) for 20 min. Results were analysed by Eadie-Hofstee transformation (B). Each data point represents means $\pm$ SD ($n = 3$). ** $P < 0.01$, * $P < 0.05$ significantly different from control.

6.3.3 Beta-2-agonists inhibition by OCT subtype

The specificity of β_2 -agonist interaction with OCTs was studied by measuring [^3H]-MPP $^+$ uptake into HEK-293 cells, stably over-expressing hOCT1, hOCT2 or hOCT3 in the presence of the bronchodilators. OCT-transfected HEK-293 cells exhibited an at least 10-times higher [^3H]-MPP $^+$ uptake when compared to mock-transfected cells. As shown in Figure 3, MPP $^+$ and decynium-22 were used as positive controls for successful inhibition of OCTs.

Salbutamol showed the weakest inhibitory potential of the three drugs tested. No decrease in MPP $^+$ uptake was observed with 100 μM salbutamol (base and salt) in any of the OCT-clones (Figure 3A). When used at 500 μM , uptake of [^3H]-MPP $^+$ into OCT1-over-expressing cells was reduced to $75.2 \pm 5.4\%$ (salbutamol base) and $58.1 \pm 6.8\%$ (salbutamol sulphate), respectively. At 500 μM still no effects were obtained in OCT2-transfected cells, whereas a slight attenuation of OCT3 function was seen.

When formoterol was tested, 100 μM of the compound reduced MPP $^+$ uptake to $22.9 \pm 3.0\%$ in hOCT1-transfected cells (Figure 3B). In hOCT2-transfected cells, formoterol used at 100 μM did not cause OCT inhibition; however, when used at 500 μM , MPP $^+$ uptake was reduced to $77.1 \pm 5.3\%$. In hOCT3-HEK-293 formoterol showed inhibition to $85.1 \pm 1.4\%$ (100 μM) and $28.3 \pm 1.4\%$ (500 μM), respectively.

Consistent with what was observed with formoterol, salmeterol showed the highest inhibition levels of MPP $^+$ uptake in OCT1-transfected cells ($28.0 \pm 3.1\%$ of control at 100 μM , Figure 3C). MPP $^+$ uptake was also significantly decreased in OCT2-HEK-293 cells, i.e., $83.1 \pm 2.2\%$ (100 μM) and $62.4 \pm 0.4\%$ (500 μM). In hOCT3 clones respective values of $77.1 \pm 2.8\%$ (100 μM) and $19.4 \pm 0.1\%$ (500 μM) were measured. As hOCT1 was much more sensitive to inhibition by β_2 -agonists than the other transporters, in a follow-up study, IC_{50} values of formoterol and salbutamol were

established. Formoterol fumarate inhibited hOCT1 function with an IC_{50} of $22.3 \pm 1.6 \mu M$ and salmeterol had an IC_{50} value of $47.8 \pm 7.8 \mu M$ (Figure 4A and B).

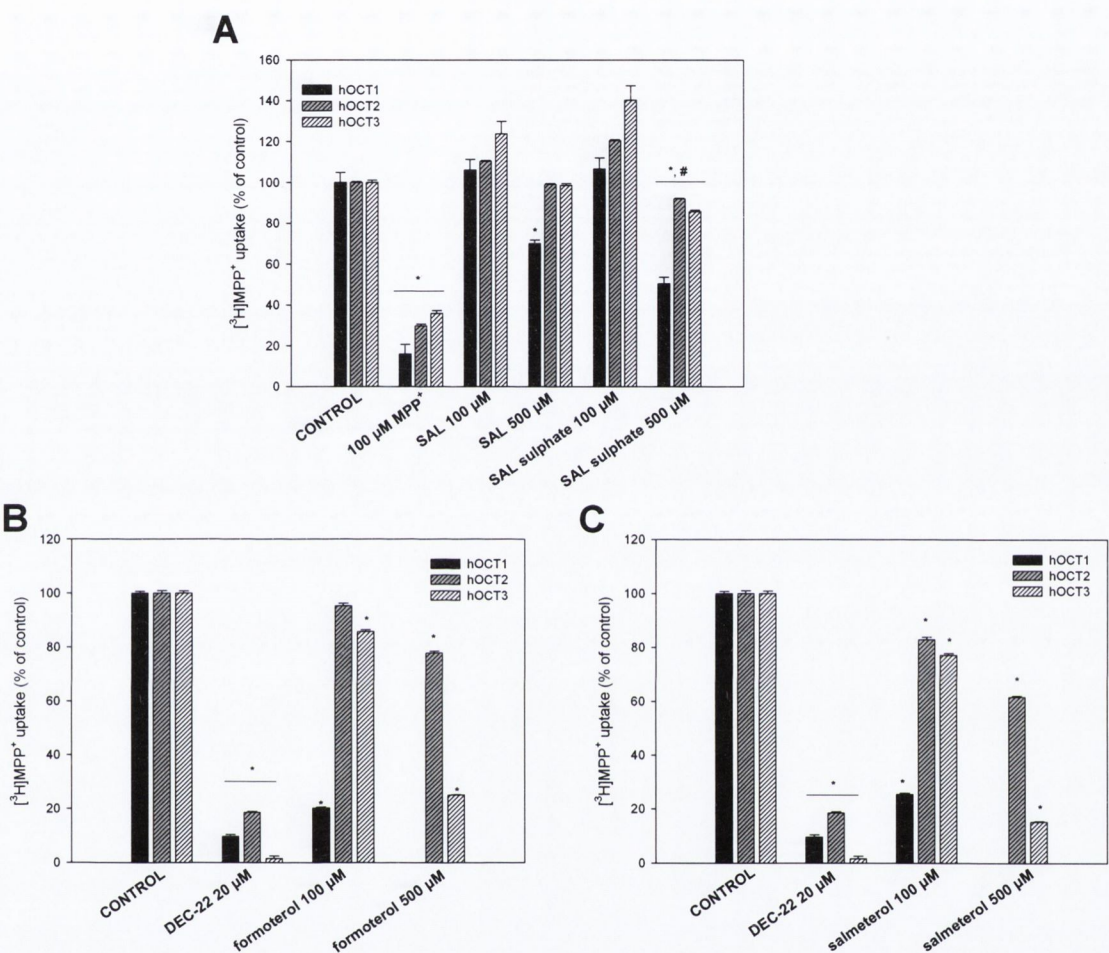


Figure 3. Inhibition of $[^3H]$ -MPP⁺ uptake by β_2 -agonists in hOCT-over-expressing HEK-293 cells. Uptake of $[^3H]$ -MPP⁺ was measured for 5 min at 37°C in the presence and absence of 100 μM MPP⁺ or salbutamol (SAL, base and sulfate salt, both at 100 and 500 μM) in hOCT1-, hOCT2- and hOCT3-transfected HEK-293 cells (A). Uptake of $[^3H]$ -MPP⁺ was performed for 5 min at 37°C in the presence and absence of 20 μM decynium-22, formoterol (B) and salmeterol (C) (both at concentrations of 100 and 500 μM) in hOCT-transfected HEK-293 cells. Data are presented as percentage of $[^3H]$ -MPP⁺ uptake corrected for uptake into mock-transfected cells. Each data point represents means $\pm$ SD ($n = 6$). * $P < 0.01$ significantly different from control, # significantly different from salbutamol base (500 μM).

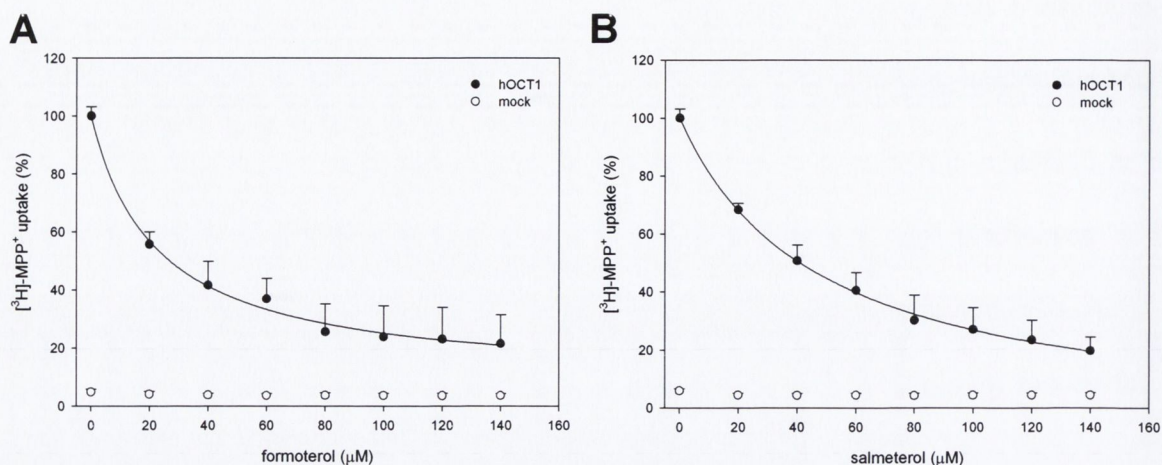


Figure 4. Inhibition of [^3H]-MPP $^+$ uptake by formoterol (A) and salmeterol (B) in hOCT-over-expressing HEK-293 cells. [^3H]-MPP $^+$ uptake was measured in the presence of increasing concentrations (0-140 μM) of β_2 -agonists for 5 min at 37°C. Uptake into mock-transfected cells ($\circ$) is also shown. Each data point represents mean $\pm$ SD ($n = 6$).

6.3.4 Salbutamol uptake by A549 and Calu-3 cell monolayers

To determine, if β_2 -agonists are both inhibitors and substrates for organic cation transporters, [^3H]-salbutamol uptake was studied in human bronchial (Calu-3) and alveolar (A549) epithelial cell lines.

The time course of salbutamol uptake is shown in Figure 5A. Uptake reached a plateau in A549 cells after 20 min and a peak value of $88.1 \pm 14.6 \mu\text{l/mg protein}$ was obtained after 30 min. In Calu-3 cell monolayers, salbutamol uptake increased linearly over the duration of the experiment, reaching a cell/medium ratio of $54.6 \pm 0.1 \mu\text{l/mg protein}$ after 30 min. When concentration dependence of salbutamol uptake was studied, no saturation was apparent in A549 cells up to 1000 μM (Figure 5B).

To determine if this effect was caused by the action of an efflux pump, the study was repeated in the presence of 10 μ M cyclosporine A, an inhibitor of P-glycoprotein, MRP1 and CYP3A4. Although uptake was significantly enhanced, still no saturation could be obtained. Uptake of salbutamol was also only marginally affected by replacing Na^+ by K^+ ($90.9 \pm 4.7\%$ of control) or NMG ($81.1 \pm 2.3\%$ of control) (Figure 6A). Acidic pH values led to a significant decrease of salbutamol uptake, i.e., to $19.2 \pm 2.7\%$ at pH 5.5 and $49.2 \pm 1.3\%$ at pH 6.5 (Figure 6B). At pH 8.5, uptake was increased to $156.0 \pm 10.3\%$ of control (i.e., pH 7.4).

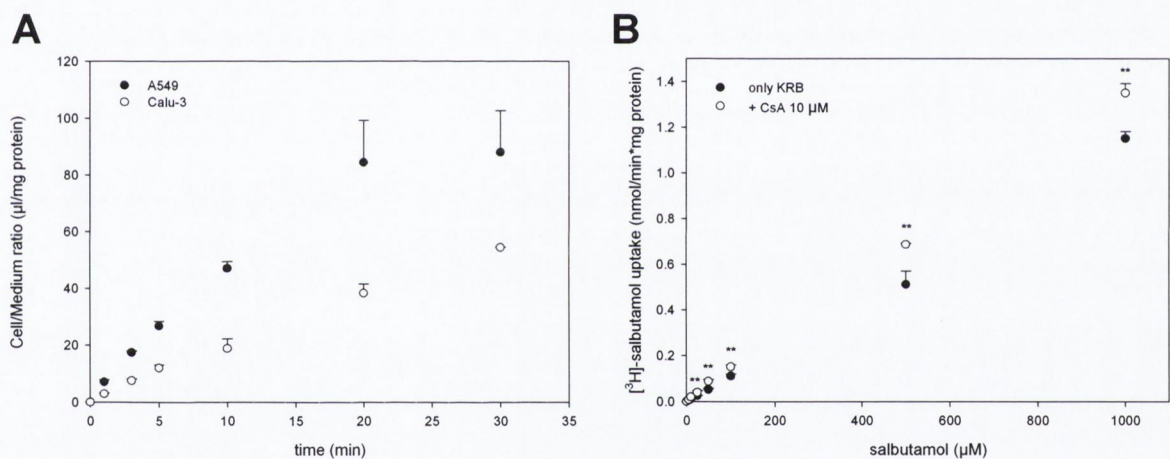


Figure 5. Salbutamol uptake into alveolar (A549) and bronchial (Calu-3) epithelial cells. Time course of $[^3\text{H}]$ -salbutamol uptake into A549 and Calu-3 cell monolayers (A). Uptake of $[^3\text{H}]$ -salbutamol was studied at 37°C for 30 min. Each point represents means $\pm$ SD ($n = 3 - 6$). Concentration-dependence of $[^3\text{H}]$ -salbutamol uptake into A549 alveolar epithelial cells at a concentration range of 0-1000 μM (B). $[^3\text{H}]$ -salbutamol uptake was also studied in the presence of 10 μM cyclosporine A to inhibit the impact of efflux pumps. Data represent mean $\pm$ SD ($n = 3$). $**P < 0.01$, $*P < 0.05$ significantly different from control.

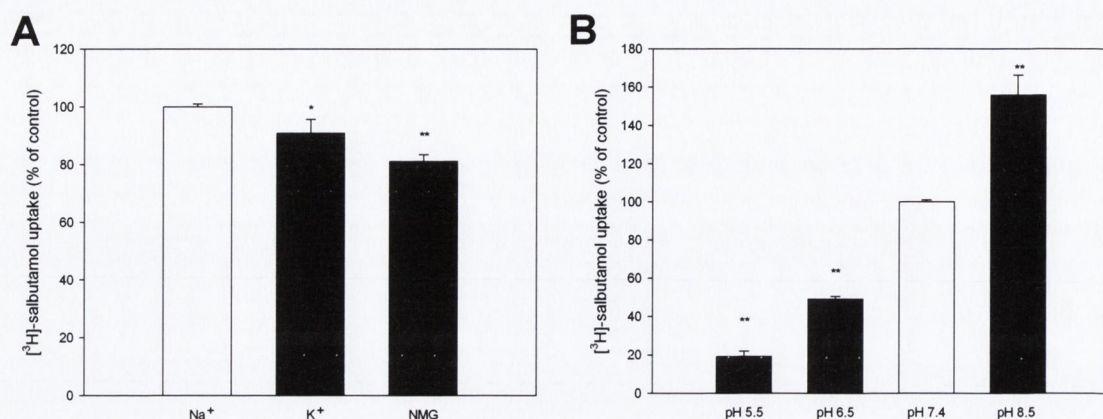


Figure 6. The impact of extracellular sodium on [³H]-salbutamol uptake into A549 cells was studied. Cells were incubated for 5 min at pH 7.4 in sodium-free buffer (**A**). Na⁺ (open column) was replaced by equimolar concentrations of potassium salts or N-methylglucamine (NMG). [³H]-salbutamol uptake was performed for 5 min at pH 5.5, pH 6.5, pH 7.4 (control, open column) and pH 8.5 in A549 cells (**B**). Results in C and D were obtained by subtraction of [³H]-salbutamol uptake at 4°C. Data represent means ± SD (*n* = 3). ***P* < 0.01, **P* < 0.05 significantly different from control.

As illustrated by Figure 7A, none of the tested OCT/N inhibitors (e.g., decynium-22, MPP⁺, D-carnitine, ergothioneine and TEA) inhibited with [³H]-salbutamol uptake into A549 cells. To the contrary, D-carnitine and ergothioneine (both OCTN inhibitors) significantly stimulated salbutamol uptake to 125.0 ± 1.8% (100 μM D-carnitine) and 142.8 ± 7.8% (200 μM ergothioneine), respectively. Furthermore, cellular uptake into A549 cells was not attenuated by the cationic amino acid, lysine, the nucleoside, adenosine, the neurotransmitter, acetylcholine or the specific choline transporter inhibitor, hemicholinium-3. Only verapamil and quinidine were able to significantly reduce salbutamol uptake to 86.6 ± 20.5% and 73.2 ± 2.3%, respectively. A limited set of the aforementioned inhibitors was used in studies involving Calu-3 cells (Figure 7B).

Again, ergothioneine, lysine MPP⁺ and formoterol did not inhibit salbutamol uptake. Of note, salbutamol uptake into Calu-3 cells was significantly decreased in the presence of 20 mM TEA (81.0 ± 3.8%).

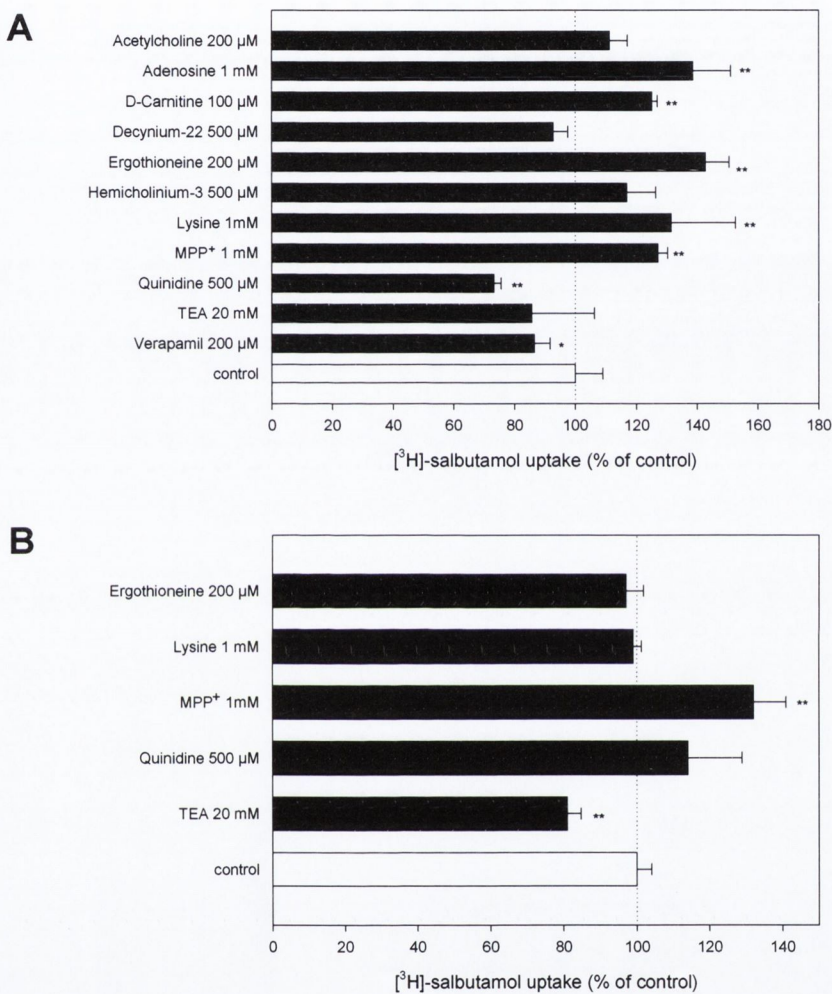


Figure 7. Inhibitory effects of organic cations on [³H]-salbutamol uptake by A549 (**A**) and Calu-3 (**B**) cell monolayers. [³H]-salbutamol uptake was measured in the presence of acetylcholine (200 μM), adenosine (1 mM), D-carnitine (100 μM), decynium-22 (500 μM), ergothioneine (200 μM), hemicholinium-3 (500 μM), MPP⁺ (1 mM), lysine (1 mM), quinidine (500 μM), TEA (20 mM) and verapamil (200 μM) in A549 and cells. A limited set of compounds, i.e., ergothioneine (200 μM), MPP⁺ (1 mM), lysine (1 mM), quinidine (500 μM) and TEA (20 mM) were tested in Calu-3 cells. Uptake of [³H]-salbutamol was measured for 5 min at 37°C. Results were obtained by subtraction of [³H]-salbutamol uptake at 4°C. Each bar represents mean ± SD (*n* = 3 - 6). * *P* < 0.05, ** *P* < 0.01 significantly different from control.

6.3.5 Salbutamol transport across H441 and Calu-3 cell monolayers

The bidirectional transepithelial permeability of salbutamol across H441 bronchiolar and Calu-3 bronchial epithelial cell monolayers was measured under air-interfaced and liquid-covered conditions. Air-interfaced conditions, *id est*, to not interfere with physiological conditions, only a very small volume of apical donor solution was used. The time course of TEER development can be seen in Figure 8.

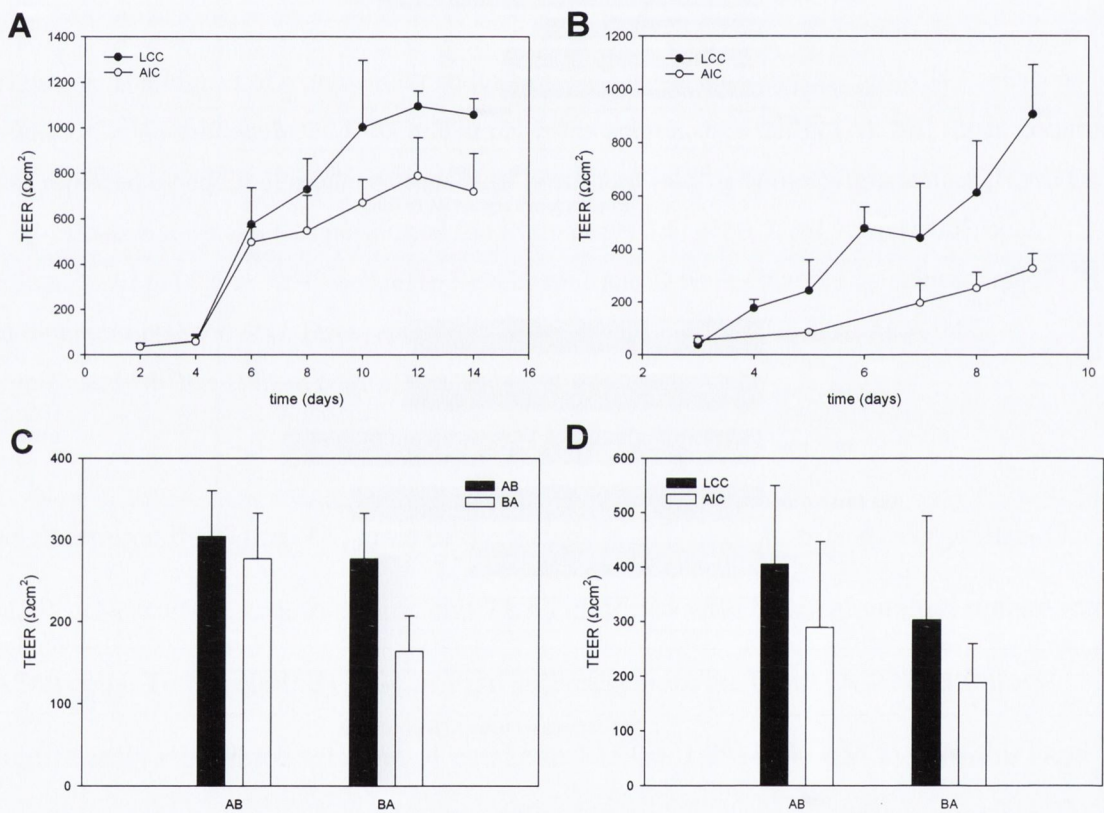


Figure 8. Time course of TEER development in Calu-3 (A) and H441 (B) cell layers. Cells were seeded at a density of 8×10^4 (Calu-3) or 2.5×10^5 (H441) cells per cm^2 on Transwell Clear filter inserts. Cells were cultured under LCC and AIC conditions for at least 14 (Calu-3) or 9 (H441) days. TEER values were measured after the transport experiment using Calu-3 (C) and H441 (D) cell monolayers to assess the cell layer integrity. As depicted, TEER values remained at moderate levels after the flux studies in both cell lines. Each data point represents mean $\pm$ SD ($n=3 - 6$).

Table 2. [³H]-salbutamol transport was measured across Calu-3 and H441 cell monolayers at 37°C for 90 min. Calculated P_{app} values after the transport studies are shown. Cells were seeded on Transwell Clear inserts and grown under AIC (**A**) and LCC (**B**) conditions for at least 12 days, ** $P < 0.01$ significantly different from a-to-b direction (AB).

A

Cell line	P_{app} ($\times 10^{-6}$ cm·s ⁻¹)			
	AIC		AIC (in the presence of 50 μ M verapamil)	
	AB	BA	AB	BA
Calu-3	0.69±0.09	6.17±1.37**	0.80±0.10	5.88±2.18**
H441	1.20±0.23	0.48±0.23**	0.50±0.04	0.48±0.22

B

Cell line	P_{app} ($\times 10^{-6}$ cm·s ⁻¹)			
	LCC		LCC (in the presence of 50 μ M verapamil)	
	AB	BA	AB	BA
Calu-3	4.33±0.29	4.63±0.50	4.76±0.20	4.93±0.23
H441	6.30±0.53	7.19±1.80	6.95±0.58	6.26±0.36

Transport of salbutamol across AIC-grown Calu-3 monolayers exhibited a strong net secretive direction, i.e., the apical-to basolateral permeability coefficient was $6.8 \pm 0.1 \times 10^{-7}$ cm/s, whereas a significantly higher P_{app} ($6.2 \pm 1.4 \times 10^{-6}$ cm/s) was observed in the secretory direction (Table 2A). Neither flux was affected by the presence of 50 μ M

verapamil in the transport medium. In H441 cells, a pronounced absorption (i.e. apical-to-basolateral) was obtained exhibiting a P_{app} value of $1.2 \pm 0.2 \times 10^{-6}$ cm/s vs. a P_{app} value of $0.5 \pm 0.2 \times 10^{-6}$ cm/s (basolateral-to-apical direction). When flux experiments were studied in the presence of 50 μ M verapamil, the b-to-a transport significantly decreased to $0.5 \pm 0.0 \times 10^{-6}$ cm/s, whereas no changes were observed in the a-to-b direction.

Furthermore, transport experiments were carried out using Transwell-grown Calu-3 and H441 cell monolayers under liquid-covered conditions (Table 2B). Overall, no distinctive observations could be made between directions in both, Calu-3 (P_{app} value were about 4.5×10^{-6} cm/s) and slightly higher in H441 (P_{app} value were about 6.7×10^{-6} cm/s) cells. However, in the presence of verapamil P_{app} values remained the same.

Additionally, the intracellular accumulation of salbutamol in Calu-3 and H441 cells was determined by lysing the cells after the flux experiment (Figure 9). The intracellular salbutamol concentration in Calu-3 cells was 0.05 ± 0.0 fmol/mg protein in a-to-b direction, whilst b-to-a transport exhibited values of 0.8 ± 0.1 fmol/mg protein (Figure 9A). When Calu-3 cells were grown under LCC, intracellular concentrations of salbutamol were calculated as 1.7 ± 0.2 (a-to-b) and 0.8 ± 0.1 (b-to-a) fmol/mg protein. Furthermore, transport of salbutamol was studied in the presence of verapamil resulting in significantly decreased intracellular uptake of salbutamol in all cases. These data suggest verapamil inhibition of an uptake transporter of salbutamol into Calu-3 cells without affecting the efflux mechanism.

In H441 cells, intracellular accumulation levels were comparable to those obtained in Calu-3 cells (Figure 9B). Salbutamol a-to-b uptake revealed values of 1.7 ± 0.1 fmol/mg

protein (LCC) and 0.04 ± 0.0 fmol/mg protein (AIC). However, uptake in b-to-a direction exhibited concentrations of 0.9 ± 0.1 (LCC) and 0.5 ± 0.1 (AIC) fmol/mg protein, respectively.

In the presence of P-gp inhibitor verapamil, the amount of intracellular salbutamol increased in both directions to 0.1 ± 0.0 (AB) and 0.9 ± 0.1 (BA) fmol/mg protein under air-interfaced conditions. However, a-to-b uptake was significantly reduced (1.3 ± 0.1 fmol/mg protein), but not as highly as in Calu-3 cells, and b-to-a uptake levels remained the same (1.0 ± 0.1 fmol/mg protein) when studied in liquid-covered conditions. By contrast to Calu-3 cells, the effects in the presence of verapamil strongly differed and might suggest that P-glycoprotein is rather involved in the efflux of salbutamol.

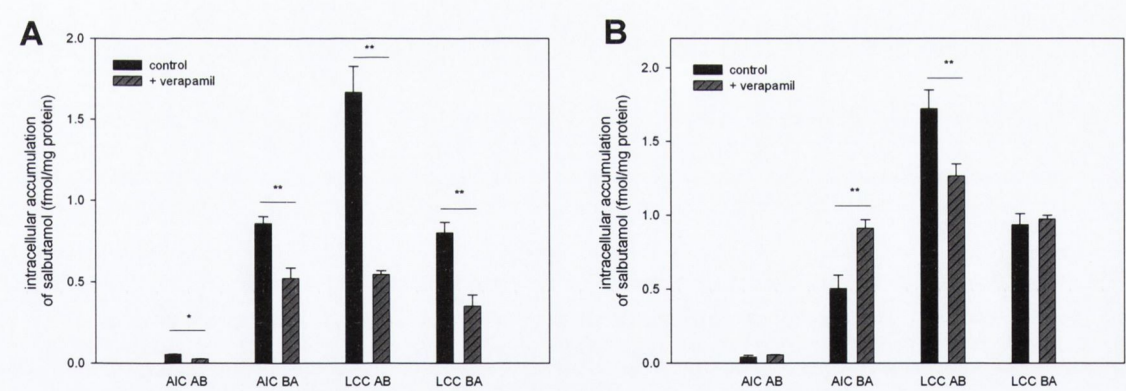


Figure 9. Respiratory epithelial cell monolayers were lysed after transport studies and the intracellular accumulation of [3 H]-salbutamol was determined in Calu-3 (A) and H441 (B) cells. Each bar represents mean $\pm$ SD ($n = 3$). ** $P < 0.01$ significantly different from control.

6.4 DISCUSSION

Salbutamol, formoterol, and salmeterol are well established drugs in the treatment of asthma and COPD, in single and combination therapy. In this work, we investigated how these β_2 -agonists interact with organic cation transporters at the respiratory epithelium. We provided evidence that all three β_2 -receptor agonists competitively inhibit uptake of TEA, but not of acetylcarnitine. Moreover, we demonstrated in hOCT-over-expressing HEK-293 cells that β_2 -agonists exhibited selective inhibition effects on hOCT1-mediated uptake with IC_{50} values of 22.3 μ M for formoterol fumarate and of 47.8 μ M for salmeterol xinafoate. A time- and pH-dependent uptake mechanism was found for salbutamol, and the compound showed net secretive transport across bronchial epithelial monolayers *in vitro*. The molecule responsible for β_2 -receptor-agonist transport intriguingly remains elusive, as few of the typical OCT/N inhibitors modulated salbutamol uptake.

Beta-2-adrenoceptor agonists are cations at physiological pH in the lung. Therefore, the question arose, if the class of drugs is absorbed by passive diffusion or by a transporter-facilitated process. A number of candidate transporters involved in organic cation processing have been identified in the pulmonary epithelium (Salomon and Ehrhardt, 2012), but molecular evidence of β_2 -agonist transport is still scarce. Several reports, however, have provided indirect data of, e.g., a concentration-dependent inhibitory effect on the uptake of the organic cation, ASP⁺ by β_2 -agonists (Horvath et al., 2007; Salomon et al., 2012). Here, we show in alveolar A549 cells that β_2 -agonists significantly decreased [¹⁴C]-TEA uptake, whereas no comparable inhibitory effects on [³H]-acetylcarnitine uptake was observed. These data strongly suggest that β_2 -agonists

inhibit OCT, but not OCTN transporters in the lower lung. These experiments were confirmed in OCT-transfected HEK-293 cells, revealing that hOCT1 was mainly inhibited by β_2 -agonists followed by hOCT3, whilst hOCT2 was only marginally affected. Interestingly, in bronchial epithelial Calu-3 cells, this preference for the OCT system was not apparent. Beta-2-agonists inhibited both pathways, OCT and OCTN, in a comparable manner. However, the inhibition kinetics showed that formoterol non-competitively inhibited the uptake process of [^3H]-acetylcarnitine, as both K_m and $V_{\max}$ values were decreased in the presence of the β_2 -agonist by approximately 30%.

When kinetically investigating the uptake of [^{14}C]-TEA into A549 cells in the absence and presence of formoterol, salbutamol and salmeterol, we found competitive inhibition. The K_i values of salmeterol (17 μM), formoterol (92 μM) and salbutamol (277 μM) were about 5 to 9-times lower than the K_m value of TEA itself. The inhibitory potential of the β_2 -agonists in the organotypic cells followed the compounds' lipophilicity, an observation that was different from what was found in the heterologous expression systems where IC_{50} values, were found to be in line with the compounds' binding affinity for the β_2 -adrenoceptor (Anderson et al., 1994). Based on these findings it can be speculated that the binding site for β_2 -agonists is located at the same transporter and shared with TEA. Organic cation transporters have previously been identified to be polyspecific and bidirectional (Ciarimboli, 2010; Koepsell et al, 2007; Nies et al., 2011). They have a large binding pocket containing partially overlapping binding domains for different substrates (Ciarimboli, 2010; Biermann et al., 2006).

Furthermore, we sought to clarify if β_2 -agonists are both, inhibitors and substrates for the OCT/N transport system. Prior to the work presented here, it was shown that HEK-293/OCTN1 and HEK-293/OCTN2 were both involved in the transport of ipratropium

(Nakamura et al., 2010). Unfortunately, the molecular identity of the uptake system in organotypic BEAS-2B cells could not be clarified. A possible contribution of OCTs was discussed, but not further investigated. In a more recent study, salbutamol uptake was investigated in bronchial smooth muscle cells by Chiappori *et al.* (Chiappori et al., 2012). They suggested a transporter-independent uptake mechanism for salbutamol. In line with these findings, Umwallah and co-workers reported that salbutamol was majorly taken up by paracellular mechanisms in airway smooth muscle cells (Umwallah et al., 2012). In the same study, the cellular uptake of salbutamol was found to be saturable and inhibitable by lysine and histidine. The uptake and transport of [^3H]-formoterol into Calu-3 cells was previously investigated by Mukherjee *et al.* (Mukherjee et al., 2012). A net secretive direction of formoterol transport was demonstrated across Calu-3 cell monolayers. It was concluded that the higher proportion of neutral formoterol at pH 7.4 was most likely the reason for this effect. The involvement of an efflux transporter was not considered, although a study by Valenzuela and colleagues (2006) had proposed an active efflux mechanism for β_2 -agonists in rat intestine (Valenzuela et al., 2006).

In our work, studies in alveolar and bronchial epithelial cells demonstrated that classical organic cation modulators (e.g., decynium-22, MPP⁺ and TEA) did not interfere with the uptake of salbutamol. Other organic cations such as hemicholinium-3 (choline transporter inhibitor), lysine (amino acid), adenosine (nucleoside), ergothioneine and carnitine (organic carnitine transporter substrates) were not able to exert significant effects. Only verapamil and quinidine showed significant inhibition of salbutamol uptake into A549 cells. The process was also found to be pH-dependent and sodium-independent. It can therefore be ruled out that organic cation/carnitine transporters are

involved in salbutamol uptake. Transport studies across Calu-3 and H441 cells were carried out under air-interface conditions to represent physiologically relevant conditions (Forbes and Ehrhardt, 2005; Sporty et al., 2008). In Calu-3 cells, these studies revealed a net secretion of salbutamol, which was not affected by the presence of verapamil. Whereas a net absorption of salbutamol across H441 cell monolayers was found, this was susceptible to the presence of verapamil. Our lab has previously reported net absorption of salbutamol across Calu-3 and 16HBE14o- bronchial epithelial cells *in vitro* (Ehrhardt et al., 2005). It can be speculated that the different experimental set-ups (i.e., air interface *versus* submersed cells) contributed to this effect. On the other hand, our data are in agreement with the net secretion of formoterol observed by Mukherjee and colleagues (Mukherjee et al., 2012). Although they also used submersed cells in their transport assay, the higher affinity of formoterol for the transporter has to be taken into account.

A number of different transporters have the ability to facilitate organic cation permeation such as proteins encoded by *SLC5*, *SLC6*, *SLC7*, *SLC29*, *SLC44* and *SLC47* gene families (Salomon and Ehrhardt, 2012). Many of the observed characteristics of salbutamol uptake and transport, i.e., proton-dependency, sodium independency and inhibition by quinidine and verapamil, suggested an involvement of *SLC47A1* (MATE1) (Otsuka et al., 2005; Astorga et al., 2012). MATE1 mRNA was very recently shown to be expressed in A549 and BEAS-2B cells by Courcot *et al.* (Courcot et al., 2012). In Chapter 2, we presented the first data on MATE1 protein expression in respiratory epithelial cell lines by immunoblot and immunocytochemistry. If MATE1 is indeed the elusive transporter responsible for β_2 -agonist transport in the bronchial epithelium is the subject of ongoing studies.

6.5 CONCLUSIONS

Polyspecific organic cation transporters (OCT), particularly hOCT1, are targets for inhibition by β_2 -agonists. And although salbutamol was actively taken up into the alveolar and bronchial epithelial cells, this translocation was not mediated by the five cloned organic cation transporters. Transport of salbutamol was found to be net-secretive across Calu-3 cell monolayers, due to an elusive efflux transporter. However, expression of MATE1 was confirmed in respiratory epithelial cells, which might prove to be the responsible molecule for the uptake and efflux of salbutamol.

CHAPTER 7

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

This chapter has been submitted as:

Salomon JJ, Ehrhardt C, Hosoya KI (2012) The verapamil transporter expressed in human alveolar epithelial cells (A549) does not interact with β_2 -receptor agonists. *Drug Metab Dispos*, under Revision

ABSTRACT

A transporter recognising verapamil as substrate has previously been identified in human retinal pigment epithelial (RPE) and in rat retinal capillary endothelial (TR-iBRB2) cells. This transporter is distinct from any of the cloned organic cation transporters. A recent study in healthy volunteers showed an increase in systemic exposure of the inhaled bronchodilators, GSK642444 (vilanterol) and GSK573719 (umeclidinium bromide) in the presence of verapamil. Therefore, we hypothesised that the verapamil transporter is also functionally expressed in the human respiratory mucosa, and that it interacts with pulmonary administered cationic drugs such as β_2 -agonists. The uptake of [^3H]-verapamil was studied in A549 human alveolar epithelial cell monolayers at different times and concentrations. Moreover, the influence of extracellular proton and sodium concentration and various organic cations on verapamil uptake was determined. Verapamil uptake into A549 cells was time- and concentration-dependent, sensitive to pH and had a K_m value of $39.8 \pm 8.2 \mu\text{M}$. Verapamil uptake was also sensitive to inhibition by amantadine, quinidine and pyrilamine, but insensitive to extracellular sodium concentration and other typical modulators of organic cation and choline transporters. Whilst we could demonstrate functional activity of the elusive verapamil transporter at the human respiratory epithelial barrier, our data suggest that this transporter does not interact with β_2 -agonists at therapeutic concentrations.

7.1 INTRODUCTION

The alveolar epithelium serves as an important physiological barrier, regulating ion transport and fluid absorption (Folkeson and Matthay, 2006). Intriguingly, whilst extensive research has been carried out identifying the underlying mechanisms of water and solute transport in the lung, uptake and secretion of organic molecules has largely been remained unstudied. In recent years, however, it is increasingly being recognised that the actions of membrane transporter proteins are pivotal in lung physiology as well as in pulmonary drug disposition (Gumbleton et al., 2010; Salomon and Ehrhardt, 2012). The functional expression of several members of the ATP binding cassette (ABC) and solute-linked carrier (SLC) transporter super families has previously been confirmed in the epithelia of proximal and distal lung (Endter et al., 2007; Endter et al., 2009; Salomon et al., 2012, van der Deen et al., 2005). Of these transporters, the organic cation transporters (OCT/Ns; encoded by the *SLC22A1-5* genes) have been suggested to interact with inhaled medicines such as β_2 -receptor agonists (Salomon et al., 2012b; Mukherjee et al., 2012), muscarinic antagonists (Nakamura et al., 2010) and corticosteroids (Horvath et al., 2011). Complementary to evidence emanating from *in vitro* studies, Gnadt and colleagues recently reported cation transporter interactions during the pulmonary absorption of β_2 -agonists using an isolated perfused human lung lobe model (Gnadt et al., 2012). Moreover, increases in $AUC_{(0-\tau)}$ after co-administration of verapamil with the novel long-acting muscarinic receptor antagonist, GSK573719 (umeclidinium bromide) and the long-acting β_2 -agonist, GSK642444 (vilanterol) in healthy subjects were 38% and 36%, respectively (Mehta et al., 2012; GSK Study Report 113950). A transporter for organic cations has been previously described in human retinal pigment epithelial and in rat retinal capillary endothelial (TR-iBRB2) cells, which recognises the calcium channel inhibitor, verapamil, as its substrate (Han et

al., 2001; Hosoya et al. 2011). Verapamil is also a substrate and inhibitor of several transporters including P-glycoprotein (Meier et al., 2007) and organic cation transporters (Koepsell et al., 2007). Albeit the verapamil transporter has not yet been cloned, we hypothesised that this elusive transporter is functionally expressed in the human respiratory mucosa, and that it might be involved in the drug disposition of inhaled β_2 -agonists.

7.2 MATERIALS AND METHODS

7.2.1 Materials

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

7.2.2 Cell culture

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

7.2.3 Uptake studies

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

effects on uptake were assessed by incubation of A549 monolayers for 3 min with the radioisotope in the presence of increasing concentrations of the unlabelled compound. Sodium-dependence of [^3H]-verapamil uptake was determined by exchanging all sodium salts in the buffer with equimolar concentrations of the relevant potassium salts. Furthermore, pH-dependence of [^3H]-verapamil translocation was studied by performing uptake studies at various pH values (i.e., 5.5, 6.5 and 8.5). Uptake of [^3H]-verapamil was also studied in the presence of modulators of cation transporter function (i.e., amantadine, L-carnitine, choline, decynium 22, 1-methyl-4-phenylpyridinium (MPP^+), pyrilamine, quinidine and tetraethylammonium (TEA)). In addition, the inhibitory potential of two β_2 -agonists, salbutamol sulphate and formoterol fumarate on [^3H]-verapamil uptake was determined. Uptake experiments were stopped by washing A549 cell monolayers three times with ice-cold ECF, followed by 12 h of incubation with 400 μl of 1N NaOH to lyse the cells, before 400 μl of 1N HCl were added for neutralisation. Five-hundred microlitres of lysate were used to measure the cell-associated radioactivity in a liquid scintillation counter (LSC5200, Aloka, Tokyo, Japan). In parallel, the total cell protein content was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

7.2.4 Data analysis

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

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

To determine the kinetics of verapamil uptake, including saturable and non-saturable compartments, the initial uptake rates were fitted to Michaelis-Menten kinetics by means of non-linear least-squares regression analysis using WinNonlin (Pharsight,

Sunnyvale, CA). Results were expressed as means $\pm$ SD. The significance of differences between the mean values was determined by unpaired, two-tailed Student's *t*-test. Statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA), followed by the modified Fisher's least squares difference method. $P < 0.05$ was considered significant. All experiments were carried out at least in triplicate.

7.3 RESULTS AND DISCUSSION

7.3.1 Time course

Verapamil uptake into A549 cell monolayers rapidly increased over time (Figure 1). The cell-medium ratio reached 94.4 $\mu\text{l}/\text{mg}$ protein at 30 s and increased to 307.5 $\mu\text{l}/\text{mg}$ protein after 5 min. By extrapolating the uptake data to time-point zero, a surface binding of 66.2 $\mu\text{l}/\text{mg}$ protein was calculated. When verapamil uptake was studied at 4°C, the cell-medium ratio was significantly reduced and only reached values of 29.7 $\mu\text{l}/\text{mg}$ protein at 30 s and 111.3 $\mu\text{l}/\text{mg}$ protein at 5 min. Hence, the extensive accumulation of verapamil in A549 cells can on the one hand be attributed to high levels of passive diffusion due to the drug's lipophilicity ($\log P = 3.45$), on the other hand, to involvement of a transporter, similar to the one reported by Han *et al.* in RPE cells (Han *et al.*, 2001) and Hosoya *et al.* in TR-iBRB2 cells (Hosoya *et al.* 2011).

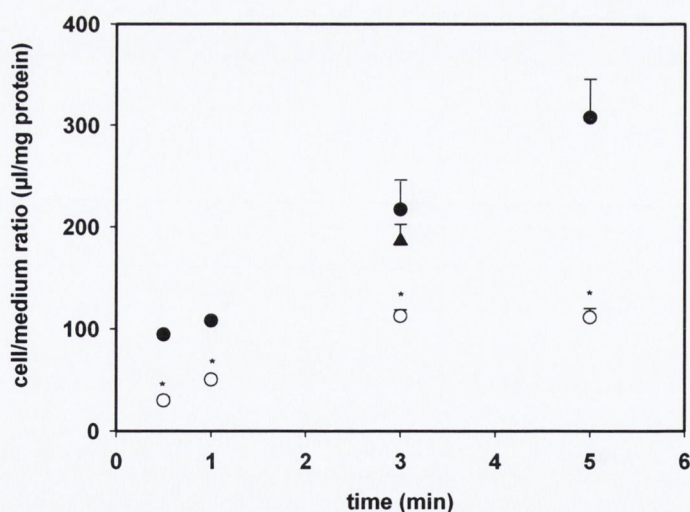


Figure 1. Time course of [^3H]-verapamil uptake into A549 cells at 37°C (●) and 4°C (○). Uptake of [^3H]-verapamil was measured for 5 min. The impact of low extracellular sodium concentration on [^3H]-verapamil uptake was studied after 3 min at 37°C (▲). Each point represents means $\pm$ SD ($n = 3$).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

7.3.2 Concentration dependence

As shown in Figure 2, uptake of verapamil reached saturation over the concentration range studied (i.e., 0-200 μM), alluding to a carrier-mediated process. Eadie-Hofstee transformation exhibited one dominant transporter site with an apparent K_m of $39.8 \pm 8.2 \mu\text{M}$ and a V_{max} of $2.2 \pm 0.32 \text{ nmol/min/mg}$ of protein. The passive diffusion constant K_d was $2.7 \pm 1.1 \mu\text{l/min/mg}$ of protein. Although these kinetic parameters suggest a higher active component compared to those published by others, data is by large quite similar, when lab-to-lab variability and differences in cell type are considered.

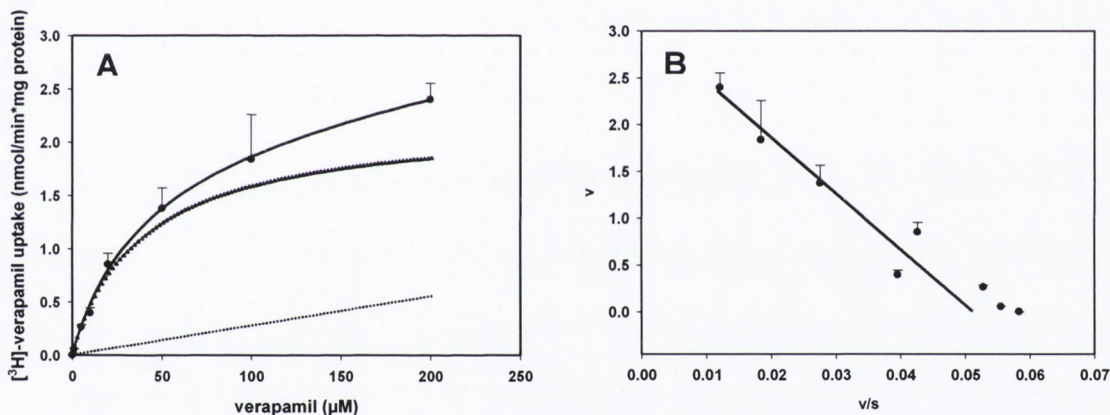


Figure 2. Concentration dependence of $[^3\text{H}]$ -verapamil uptake by A549 cell monolayers. Cells were incubated for 3 min at concentrations ranging from 1 μM to 200 μM (A). Total uptake (●) was analysed by Michaelis-Menten kinetics, transporter-mediated uptake (▲) and passive diffusion (dotted line) were calculated and represent the best fit. Eadie-Hofstee transformation is shown in (B) indicating a single transporter site: v (uptake rate; nmol/min/mg protein), v/s ($\mu\text{l/min/mg protein}$), s (concentration of unlabelled verapamil). Each point represents means $\pm$ SD ($n = 3$).

Furthermore, verapamil uptake was observed to not be sodium-dependent (Figure 1). Decreasing extracellular pH (i.e., pH 5.5), however, lead to a significant decrease of verapamil uptake, whereas at pH 6.5 and pH 8.5 no significant differences were

obtained (Figure 3). Again, these characteristics are consistent with the observations in retinal epithelial and endothelial cells (Han et al., 2001; Hosoya et al. 2011).

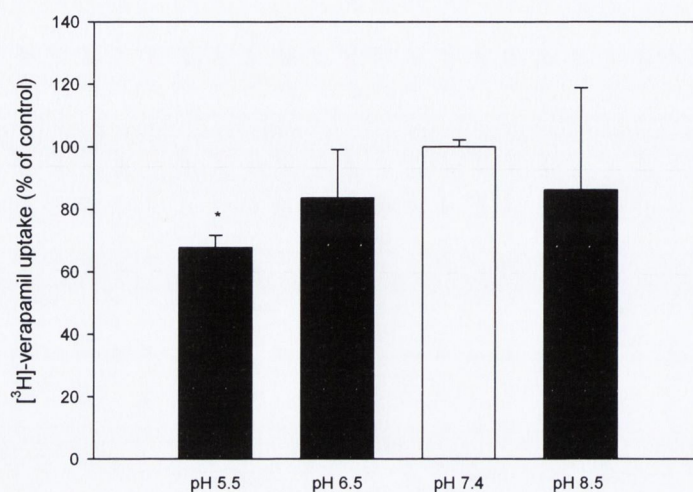


Figure 3. Proton concentration-dependence of [³H]-verapamil uptake by A549 cell monolayers. Cells were incubated for 3 min at pH 5.5, pH 6.5 and pH 8.5. Each point represents means $\pm$ SD ($n = 3$). * $P < 0.01$ significantly different from control.

7.3.3 Pharmacological inhibition

In order to investigate if the uptake of the organic cation, verapamil, by A549 cell monolayers was specific, the inhibitory potential of a number of compounds was evaluated that have been reported to interact with organic cation transporter function (Table 1). Generally, our data suggest similar specificity of the verapamil transporter in alveolar cells, when compared with the ones in RPE cells (Han et al., 2001) and at the inner blood-retinal barrier (Hosoya et al. 2011). Amantadine, pyrilamine and quinidine significantly ($P < 0.01$) inhibited the uptake of verapamil into A549 cells, whereas typical substrates of the cloned organic cation transporters (i.e., TEA, MPP⁺, decynium 22 and L-carnitine) only marginally interfered with the uptake of verapamil. Choline did not show an effect as well, ruling out an involvement of choline transporter (CHT) or choline transporter-like proteins (CTL). Amantadine, pyrilamine and quinidine are all of

very different chemical structure, but all three are quite lipophilic with respective log *P* values of 2.53, 2.89 and 2.6. Pyrilamine, quinidine and verapamil are transported by OCTN1 (Yabuuchi et al., 1999).

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

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

The *bona fide* OCTN inhibitor, L-carnitine at 500 µM, however, did not attenuate verapamil uptake. Therefore, it is unlikely that any of the 5 cloned OCT/Ns was involved in verapamil uptake into A549 cells. Pyrilamine has also been suggested to be translocated by a polyspecific organic cation-sensitive transporter, different from the

known OCT/Ns or multidrug and toxin extrusion transporter (MATE1), in TR-BBB13 cells (Okura et al., 2008), whereas quinidine is known to be an inhibitor of OCT1, OCT2 and for P-gp (Koepsell et al., 2007). Whilst P-gp is expressed in many biological barriers including the alveolar epithelium (Endter et al., 2007, Salomon and Ehrhardt, 2011), an involvement of this efflux pump in drug uptake is unlikely.

In order to determine a role for the verapamil transporter in the disposition of inhaled bronchodilators, we investigated if the two β_2 -agonists, formoterol and salbutamol, interacted with verapamil uptake at the respiratory epithelial barrier. Formoterol decreased verapamil uptake slightly but significantly to $71.7 \pm 10.2\%$ at $500 \mu\text{M}$, whereas salbutamol had even inhibition potential ($85.5 \pm 3.1\%$). Thus, it is unlikely that this inhibition is responsible for drug-drug interactions as those observed in the case of the two GlaxoSmithKline compounds (Mehta et al. 2012; GSK Study Report 133950), as the clinically relevant concentrations of drug reaching the pulmonary epithelium are considerably lower. A recently published study from our lab showed that formoterol reduced uptake the cationic fluorophore, 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+ ; $500 \mu\text{M}$) into A549 cells by over 90%, suggesting a common binding site for β_2 -agonists and organic cations at the alveolar epithelium (see Chapter 3), whilst in the case of verapamil uptake the binding site seems to be distinct. In conclusion, additional studies are needed to molecularly identify this elusive transporter that mediates verapamil uptake at the alveolar epithelial barrier. Whilst we can only speculate about possible clinical implications of this transporter in the lung, it is unlikely that it is the molecule responsible for drug-drug interactions involving bronchodilators observed previously (Mehta et al. 2012; Gnadt et al. 2012).

7.4 CONCLUSION

In this chapter, we could confirm the functional activity of the elusive verapamil transporter at the human alveolar epithelial barrier. Further studies are needed to molecularly identify this elusive transporter that mediates verapamil uptake at the alveolar epithelial barrier. Our data suggest that this transporter does not interact with classic organic cations and β_2 -agonists at therapeutic concentrations.

CHAPTER 8

New fluorescent bile acids: synthesis, chemical characterisation, and disastereoselective uptake by A549 and Caco-2 cells of 3-deoxy 3-NBD-amino deoxycholic and ursodeoxycholic acid

Parts of this chapter have been published in:

Májer F, Salomon JJ, Sharma R, Etzbach SV, Najib MN, Keaveny R, Long A, Wang J, Ehrhardt C, Gilmer JF. (2012) New fluorescent bile acids: synthesis, chemical characterisation, and disastereoselective uptake by Caco-2 cells of 3-deoxy 3-NBD-amino deoxycholic and ursodeoxycholic acid. *Bioorg Med Chem.* 20(5):1767-78

ABSTRACT

Deoxycholic acid (DCA), a secondary bile acid (BA), and ursodeoxycholic acid (UDCA), a tertiary BA, cause opposing effects *in vivo* and in cell suspensions. Fluorescent analogues of DCA and UDCA could help investigate important questions about their cellular interactions and distribution. We have prepared a set of isomeric 3 α - and 3 β -amino analogues of UDCA and DCA and derivatised these with the discrete fluorophore, 4-nitrobenzo-2-oxa-1,3-diazol (NBD), forming the corresponding four fluorescent adducts. These absorb in the range 465–470 nm and fluoresce at approximately 535 nm. Appropriate control fluorophore adducts were generated from cyclohexylamine and from 24-descarboxyamino UDCA. In order to determine the ability of the new flu-BAs to mimic the parents, their uptake was studied using monolayers of Caco-2 cells, which are known to express multiple organic anion-transporting peptides (OATP). Furthermore, to measure transport activity in the lung, functional uptake of BA was performed in human alveolar (A549) epithelial cells. Cellular uptake was monitored over time at 4°C and 37°C to distinguish between passive and active transport. All four BA analogues were taken up but in a strikingly stereo- and structure-specific manner, suggesting highly discriminatory interactions with transporter protein(s). In Caco-2 cells, the α -analogues of DCA and to a lesser extent UDCA were actively transported, whereas the β -analogues were not. The active transport process was saturable, with Michaelis- Menten constants for 3 α -NBD DCA being $K_m = 28.2 \pm 7.5 \mu\text{M}$ and for 3 α -NBD UDCA $K_m = 42.3 \pm 13.0 \mu\text{M}$. Whereas in A549 cells, both DCA-analogues were highly taken up in a saturable manner with estimated K_m values of $47.6 \pm 8.9 \mu\text{M}$ for 3 α -NBD DCA and a K_m of $45.7 \pm 3.5 \mu\text{M}$ for 3 α -NBD DCA, respectively.

The UDCA-analogues seemed to have less potential in A549 cells. These fluorescent bile acids are promising agents for investigating questions of bile acid biology and for detection of bile acids and related organic anion transport processes in both, the liver and the lung.

8.1 INTRODUCTION

The secondary bile acid, deoxycholic acid (DCA, **1** Figure 1) is a putative promoter of intestinal carcinogenesis. DCA causes DNA fragmentation, oxidative stress, Golgi fragmentation and apoptosis (Payne et al., 2011). Conversely, the tertiary bile acid, ursodeoxycholic acid (UDCA, **2** Figure 1) is cytoprotective, immunomodulatory, anti-inflammatory and anti-apoptotic (Zhou et al., 2009). In general, UDCA's effects oppose those of DCA (Amaral et al., 2009; Rodrigues et al., 2001; Sola et al., 2007). For example, pre-treatment with UDCA can prevent DCA induced apoptosis and Golgi fragmentation in colon cancer cells (Byrne et al., 2010).

The divergent cellular effects of DCA and UDCA have been widely investigated and there has been progress in characterising some of the molecular mechanisms involved (Hofmann 1999; Hofmann et al., 2008; Sharma et al., 2010; Sharma et al., 2011).

However, new tools and strategies are required to investigate direct cellular interactions of the compounds, their uptake and distribution. Fluorescent steroid analogues can prove useful for this kind of problem. Such compounds may also be used to probe interacting proteins and related cellular transport mechanisms.

The 4-nitrobenzo-2-oxa-1,3-diazole (NBD) group is a particularly useful reporter group in this context because of its relatively low steric impact, its sensitivity to its environment, satisfactory quantum yield and stability (Ghosh et al., 1968; Schramm et al., 1991). The synthesis and chemical characterisation of 3-, 7- and 12- α - and β NBD analogues of cholic acid (CA) and its tauro conjugate have been reported, followed by comprehensive studies of their uptake and hepatic metabolism *in vitro* and in rats (Schneider et al., 1991; Schramm et al., 1991; Schramm et al., 1993). Recently, the 3- and 7- amino NBD analogues of CA were studied as uptake probes for flow cytometry in isolated rat hepatocytes (Rohacova et al., 2009). NBD labelled 24-lysyl conjugates of

UDCA and DCA have been studied as substrates for hepatic (Yamaguchi et al., 2006) and intestinal transport proteins (Holzinger et al., 1997). Other investigations into (tauro)-UDCA employed the 7-NBD analogue (Sola et al., 2004; Sola et al., 2005; Sola et al., 2007).

Astonishingly, the 3-desoxy 3-NBD-amino derivatives of DCA and UDCA have not been reported to date. Placement of the fluorescent reporter group at C-3 appears to us to be optimal for exploring the disposition of UDCA and DCA because this preserves the 7- and 12-OH groups that account for the differing chemical and biological properties of the parent compounds. Importantly, C-3 modification also preserves the characteristic free bile acid side chain. Structural studies probing the interaction of UDCA with cognate proteins would also support incorporation of a reporter group at the BA C3. In the 3.0 Å crystal structure of human type III 3 α -hydroxysteroid dehydrogenase (HSD)/bile acid binding protein (AKR1C2) complexed with NADP(+) and UDCA anion, the carboxylate group of UDCA was modelled in the oxyanion hole, deep in the protein with the 3-OH solvent exposed. The liver cytosolic bile acid binding protein (BABP) binds BAs in a similar fashion, which led to the design of diagnostic compounds elaborated at C-3 with gadolinium binding EDTA groups (Assfalg et al., 2007). Additionally, various SAR surveys and QSAR models of the human apical sodium-dependent bile acid transporter (ASBT, SLC10A2) suggest that the C3-OH is tolerant of modification (Zheng et al., 2009; Hu et al., 2011). Finally, various bile acid carrier prodrug conjugates have proven that intestinal bile acid transporters can tolerate payload incorporation at C-3 (Balakrishnan et al., 2006).

Isomeric 3-desoxy 3-NBD-amino derivatives of UDCA and DCA (Figure 1) were synthesised as probes for studying the distribution of these important steroids. A control compound lacking the steroidal nucleus (Figure 1B, 7) was also prepared to detect non-

specific effects. In order to assess the suitability of the new analogues for investigating the disposition of the parent BAs, we have studied their uptake into polarised monolayers of intestinal epithelial Caco-2 cells. The Caco-2 cell line exhibits many of the features of the normal intestinal epithelium including expression of various BA transporter proteins, such as the sodium-dependent bile acid transporter (ASBT) and various members of the organic anion-transporting peptide (OATP) superfamily (Endter et al., 2009; Alrefai et al., 2005). Caco-2 cells are therefore routinely used for assessing bile acid uptake and transport (Geyer et al. 2006) and they are considered a suitable model for preliminary assessment of the NBD analogues as surrogates of DCA and UDCA. Additionally, BA transport was studied in human alveolar (A549) epithelial cells in order to functionally characterise involved drug transporter proteins at the lung epithelial barrier and compare transport activity between the tissues. Only little is known about expression, localisation and activity of for example OATPs in the lung, however, several studies previously confirmed mRNA expression of various members of the OATP family (Endter et al., 2009; Seki et al., 2009; Tamai et al., 2001).

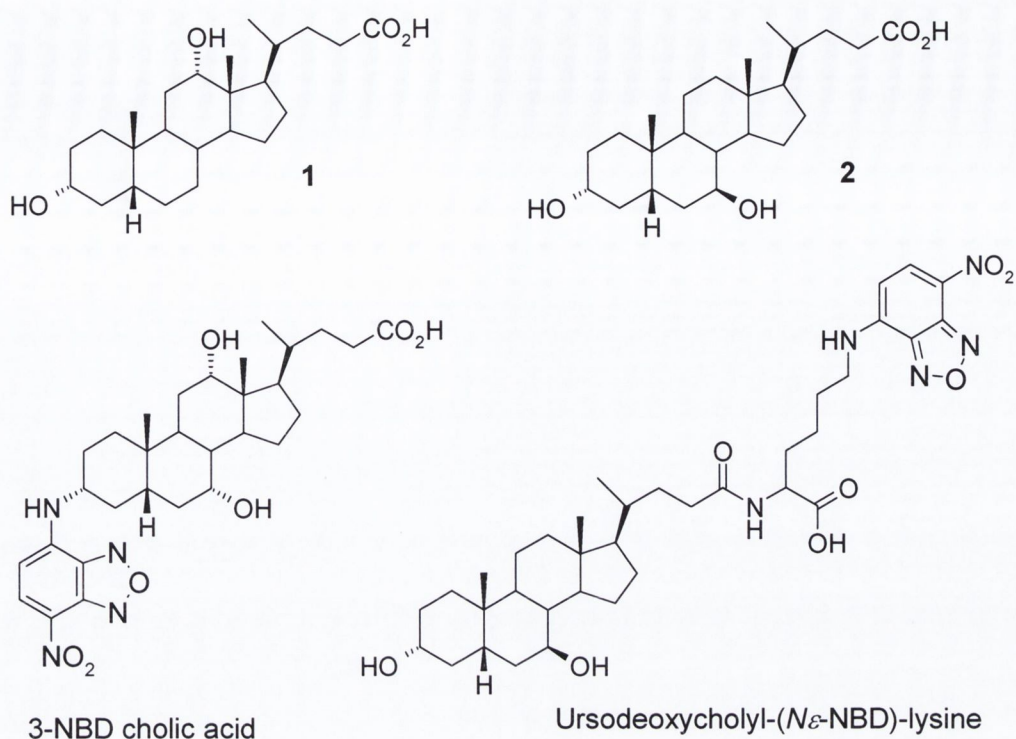


Figure 1A. Structures of bile acids DCA (1) and UDCA (2) along with fluorescent analogues of cholic acid and UDCA that have been studied previously.

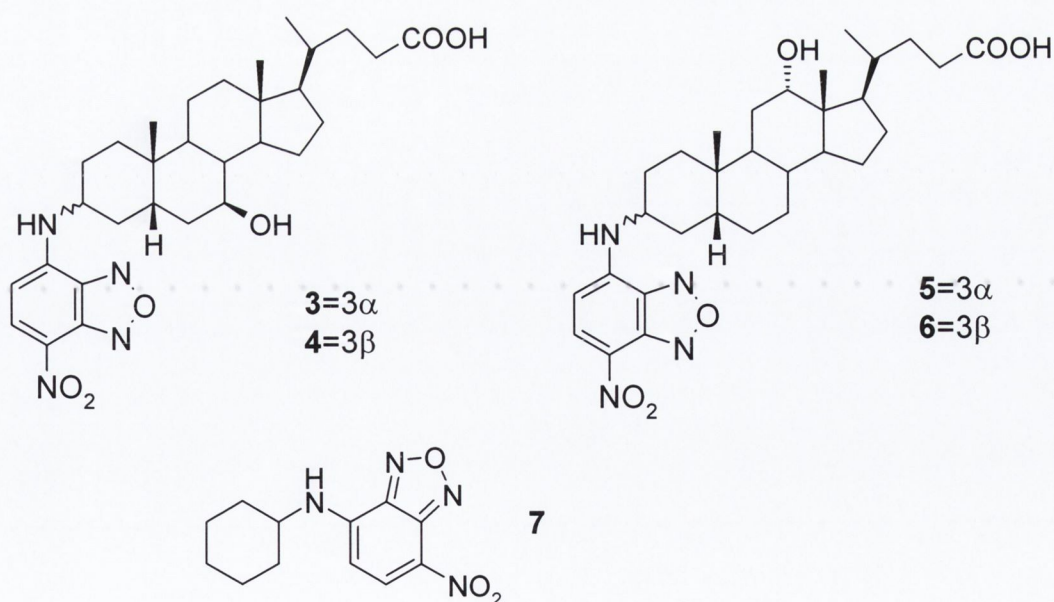


Figure 1B. New NBD analogues of UDCA and DCA 3-6; cyclohexylamine-NBD 7 was produced as a non-bile acid fluorescent control.

8.2 MATERIALS AND METHODS

8.2.1 Cell culture

A549 (American Type Culture Collection CL-185; LGC Promochem, Teddington Middlesex, UK) cells (passage 65 to 84) were seeded at a density of 40,000 cells/cm². Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM: F-12) supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The human colon carcinoma Caco-2 cell line was also purchased from ATCC (Manassas, VA, USA). In this case, cells (p.23 to 41) were seeded at a density of 66,000 cells/cm² in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 1% penicillin and streptomycin, 1% non essential amino acids and 1% sodium pyruvate. All cell types were cultured at 37°C in 5% CO₂ atmosphere and culture medium was exchanged every 48h. Cells were grown to confluent monolayers for 5 (A549) and 21 (Caco-2) days for all experiments.

8.2.2 Uptake studies

Functional cellular uptake experiments of bile acids were performed by Mohd Nadzri Mohd Najib (for A549 cells) and Simona Etzbach (in the case of Caco-2 cells).

Uptake studies were performed in bicarbonated Krebs-Ringer buffer (KRB: 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, 0.81 mM MgSO₄ and 5.55 mM glucose; pH 7.4). Uptake of the fluorescent bile acid compounds (**3-6**) was studied at different time points, concentrations and temperatures. Bile acid compounds were dissolved in KRB containing 0.1% DMSO at all times.

Time-dependence of bile acid uptake including the control fluorophore substances was studied by incubating cell monolayers (at 10 μ M) for up to 90 min at either 37°C or 4°C. After 5, 10, 15, 30, 45, 60 and 90 min, monolayers were washed twice with ice-cold KRB, solubilised in 1% (w/v) Triton X-100 solution and bile acid activity was measured in the cell. Concentration-dependence of bile acid uptake was studied at a compound concentration range of 1-100 μ M for 20 min at 37°C and 4°C. Active uptake of bile acid compounds was calculated as the difference between the fluorescence, after exposure to **3-6** at 37°C and 4°C.

The fluorescence activity of the bile acids compounds was analysed in 24-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively. The samples were diluted with KRB, where appropriate. For standardisation, the total protein amount of cell layers was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, USA).

8.2.3 Uptake kinetics

The saturable transport of the bile acid compounds was analysed by assuming Michaelis-Menten type carrier-mediated transport represented by the equation:

$v = V_{\max} \times (S) / [(K_m + (S))]$, where (S) was [bile acid compound].

Half-saturation constant (K_m) and maximum uptake rates ($V_{\max}$) of the bile acid compounds was calculated by this equation to the experimental profile of the uptake rate (v) versus the substrate concentration (S) using a non-linear least squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA, USA).

8.2.4 Cell viability

The viability of A549 and Caco-2 cells was determined by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded at a density of 30,000 cells/cm² and 20,000 cells/cm² in 96-well plates, respectively and left for 24 h to attach. In the case of time dependency, cells were exposed to 10 μM bile acid solutions for 5, 10, 15, 20, 30, 60, 90 minutes. In the case of concentration dependency, cells were incubated with different concentrations ranging from 1 to 100 μM for 20 min. After the designated incubation times in the presence of bile acid compounds, cell layers were washed twice with KRB, before 100 μl of fresh KRB and 10 μl of MTT solution (5 mg/ml in KRB) were added to each well. After further incubation at 37°C for 3 h, the supernatant was removed and cells were dispersed in 50 μl DMSO. Absorbance was measured at 570 nm by using a microplate reader (FLUOstar Optima). The samples were normalised to control buffer (KRB, pH 7.4), containing 0.1% DMSO as DMSO was used as a co-solvent in all experiments.

8.2.5 Statistics

All uptake studies were performed in triplicates. Results were expressed as mean ± SD, were compared using one-way analysis of variance (ANOVA), and followed by the Student–Newman–Keuls post hoc test. $P < 0.05$ was considered as significant.

8.3 RESULTS

Synthesis, physical and chemical characterisation of the bile acid analogues were performed by Fèrenc Majer (Trinity College Dublin).

Photo/physical characterisation

The UV/Vis absorption spectra of 3–6 measured in ethanol solution exhibited maxima at 228, 335 and 470 nm. The β -compounds were red-shifted relative to the α -compounds by approximately 5 nm (Figure 2). The fluorescence emission spectra for 3–6 exhibited maxima at λ_{Em} 537 nm with a 2–3 nm blue shift for the α -isomers (λ_{Em} was recorded using an λ_{abs} of 470 nm). These values were not affected by oxygen exposure. They are broadly in agreement with previously reported fluorescence values for cholic acid NBD derivatives (Schneider et al., 1991, Rohacova et al., 2009).

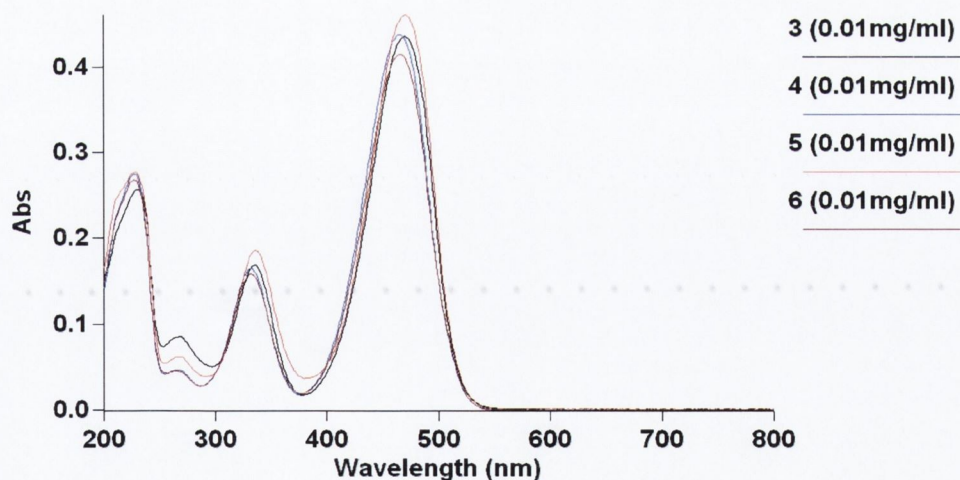


Figure 2. UV-Vis spectra of 3–6 aquired in EtOH at 0.01 mg/ml overlaid.

Molecular modelling

The structures of DCA and its 3- α and - β NBD analogues were generated by *in silico* modification in MOE (Chemical Computing Group, Montreal, Canada) of cholic acid extracted from pdb code 2QO437. The bile acid models were energy minimised using Merck Molecular Force Field 94x (MMFF94x) interfaced to MOE with gradient 0.5. Then structures were further manipulated in MOE and the picture in Figure 3 generated using the programme PyMOL.

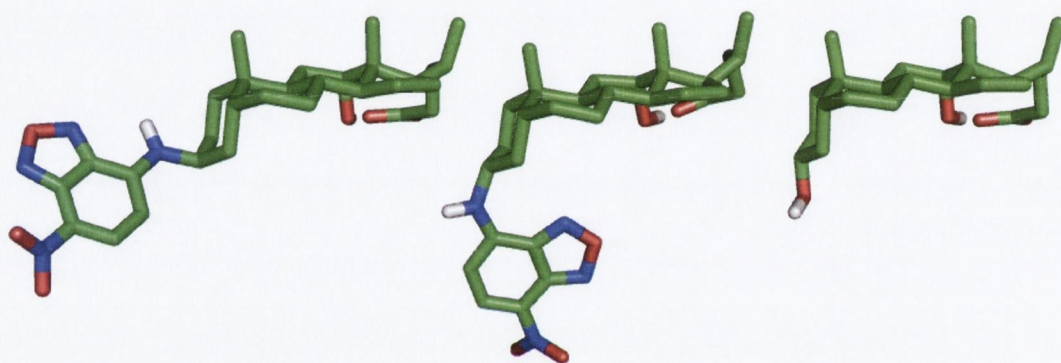


Figure 3. Minimum energy conformations for DCA-3- α and β analogs **5** (middle) and **6** (left) and DCA (right). These were generated by modifying the model of cholic acid (pdb code 2QO4) in MOE and energy minimizing with MMFF94x. The models each show a H-bond between the acid chain and 12 α -OH. The picture shows the different orientations available to the 3-NH in the two orientations. In the α -orientation, the NH may mimic the OH group of the parent bile acid in H-bond donation capacity.

8.3.1 Cell viability

Bile acids can cause apoptosis through intrinsic and extrinsic pathways and necrosis through detergent effects (Hofmann et al., 2008). The effect of derivatising the bile acids is not predictable though toxicity may be related to physicochemical properties such as hydrophobicity (Sharma et al., 2010). Since the NBD-bile acids were designed

to investigate the disposition and uptake of the parent compounds, it was important to characterise their effect on cell viability.

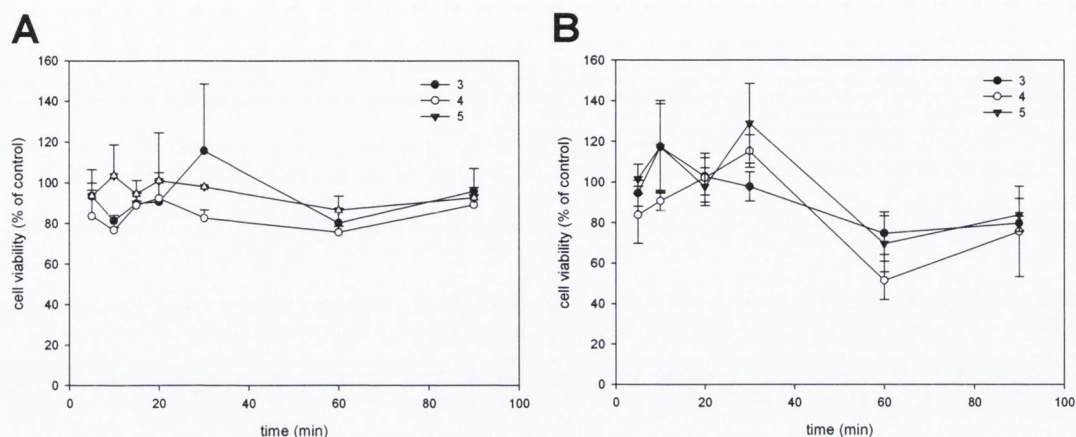


Figure 4. Cytotoxicity of NBD-bile acid analogues in (A) A549 and (B) Caco-2 cells was determined by using MTT assay, normalised to control buffer (0.1% DMSO). Both cell lines were exposed to **3–5** in a time dependent manner at pH 7.4. Results are expressed as mean $\pm$ SD (n = 6).

The cell viability effects of isomeric NBD-BAs (1–100 μ M) was therefore measured using the MTT assay after incubating A549 and Caco-2 monolayers with the compounds for up to 100 min. As depicted in Figure 4, neither of the three compounds tested seemed to damage the cells in A549, whereas a slightly higher cytotoxicity of **4** was obtained.

8.3.2 Functional uptake studies in Caco-2 cells

The time-course of uptake of **3–6** (10 μ M) into Caco-2 cell monolayers was studied at 37°C (which included contributions from active and passive uptake) and at 4°C (which reflects only passive diffusion) (Figure 5). In general, uptake increased in a time-dependent manner for up to 60 min. Differences in uptake of the β compounds (**4** and **6**)

between 37°C and 4°C were not significant ($P = 0.12$ and $P = 0.25$ for 4 and 6, respectively) (Figure 5B and D). The β compounds were therefore taken up exclusively through passive diffusion at 37°C. In contrast, uptake of the α -compounds 3 and 5 at 37°C was significantly higher ($P < 0.01$) to that at 4°C (Figure 5A and C). The passive component did not exceed 30% of the total uptake in either case. The active transport of 3 and 5 was then kinetically characterised. Cells were incubated for 20 min with the compounds at concentrations ranging from 1 to 100 μM at 37°C and 4°C. The obtained values at 4°C were subtracted from total uptake at 37°C, before performing kinetic analysis. The concentration-dependent uptake of the bile acid compounds, 3 and 5 was clearly saturable (Figure 6A and B). An Eadie-Hofstee transformation (i.e., a graphical representation of transport kinetics in which reaction velocity is plotted as a function of the velocity (v) versus substrate concentration ratio ($v/[S]$), resulted in a single straight line for both substrates suggesting that the uptake of 3 and 5 was mediated by a single transporter site in Caco-2 cells (Figure 6C). The kinetic parameters were estimated to be $K_m = 42.3 \pm 13.0 \mu\text{M}$ and $V_{\max} = 2.8 \pm 0.4 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for 3 and $K_m = 28.2 \pm 7.5 \mu\text{M}$ and $V_{\max} = 1.8 \pm 0.2 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for 5, respectively.

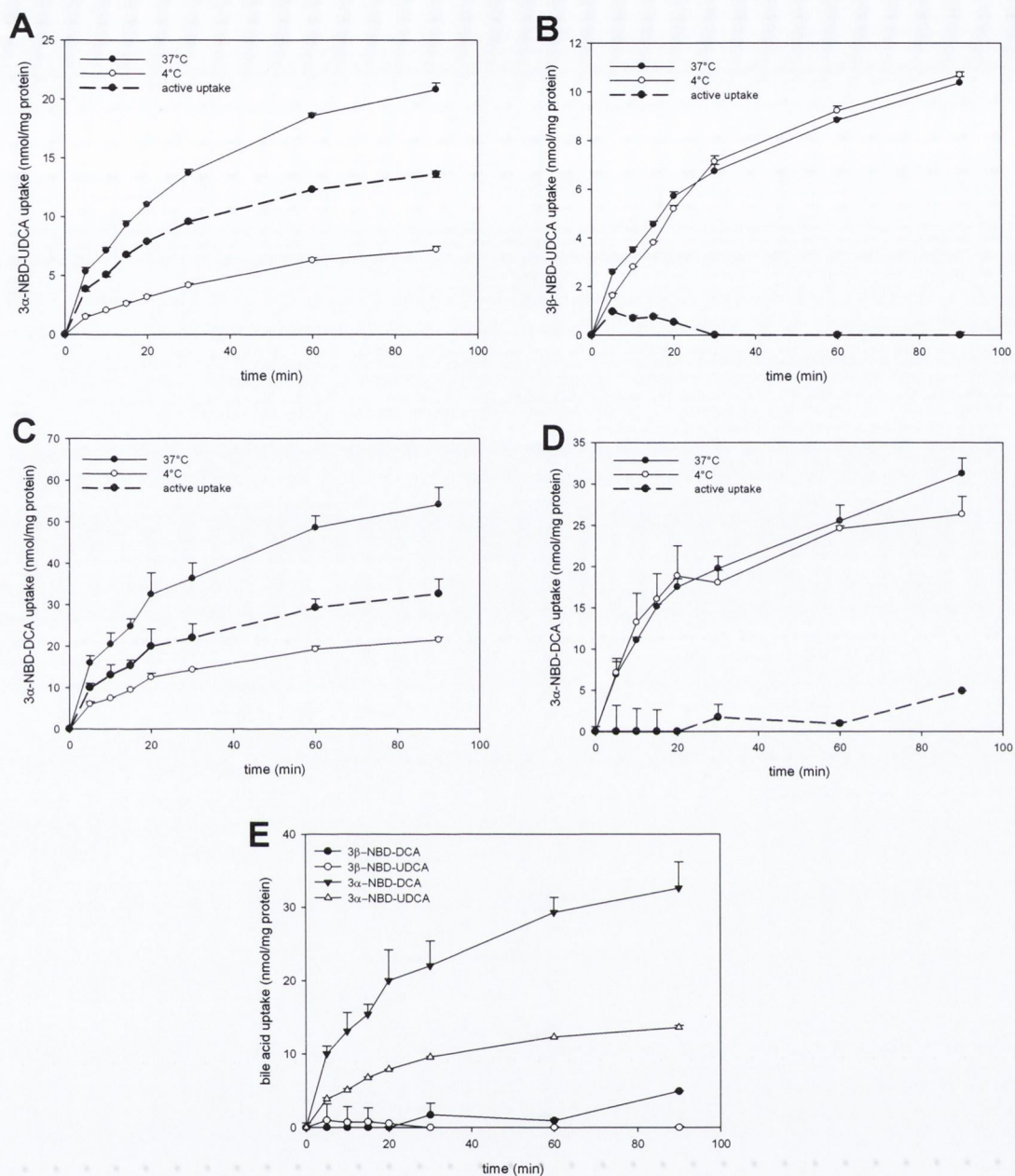


Figure 5. Time course of bile acid (**3**, **4**, **5** and **6**) uptake into Caco-2 cell monolayers. Uptake of **3** (**A**), **4** (**B**), **5** (**C**) and **6** (**D**) was studied for 90 min at 37°C (●) and 4°C (○). The active uptake of bile acid compounds (10 μM) was calculated as the difference between the fluorescence after exposure to **3**, **4**, **5** and **6** at 37°C and 4°C and is summarised in (**E**). Data represent mean ± SD ($n = 3$).

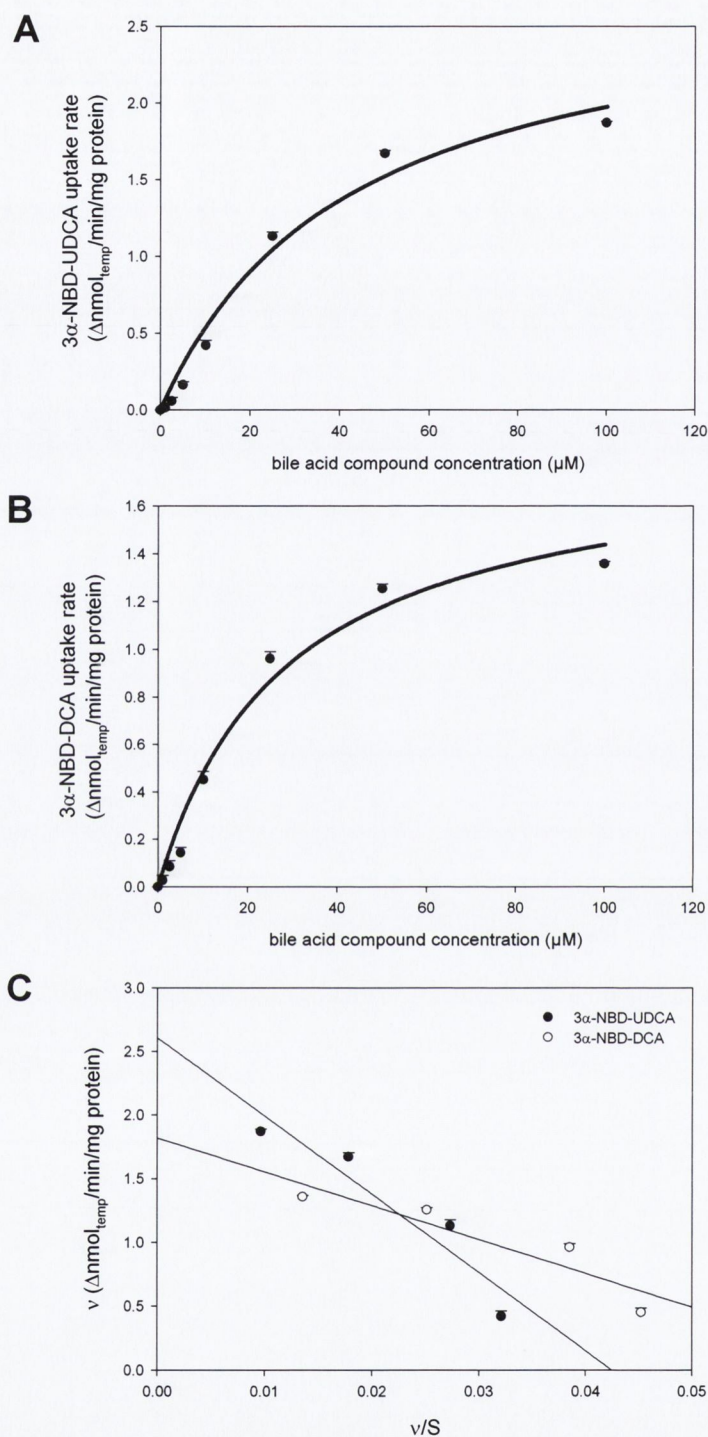


Figure 6. Concentration dependence of **3** and **5** uptake by Caco-2 cell monolayers. Uptake of bile acid compounds **3** (A) and **5** (B) was measured for 20 min at 37°C and 4°C. Actual uptake of bile acid compounds was calculated as the difference between the fluorescence after exposure to **3** and **5** at 37°C and 4°C. Data represent mean $\pm$ SD ($n = 3$). Eadie-Hofstee transformations of the data (C): v , uptake rate; S , bile acid compound concentration. In Caco-2 monolayers, one single transporter site was identified for the uptake of both NBD bile acid compounds. Data are represented as mean $\pm$ SD, ($n = 3$).

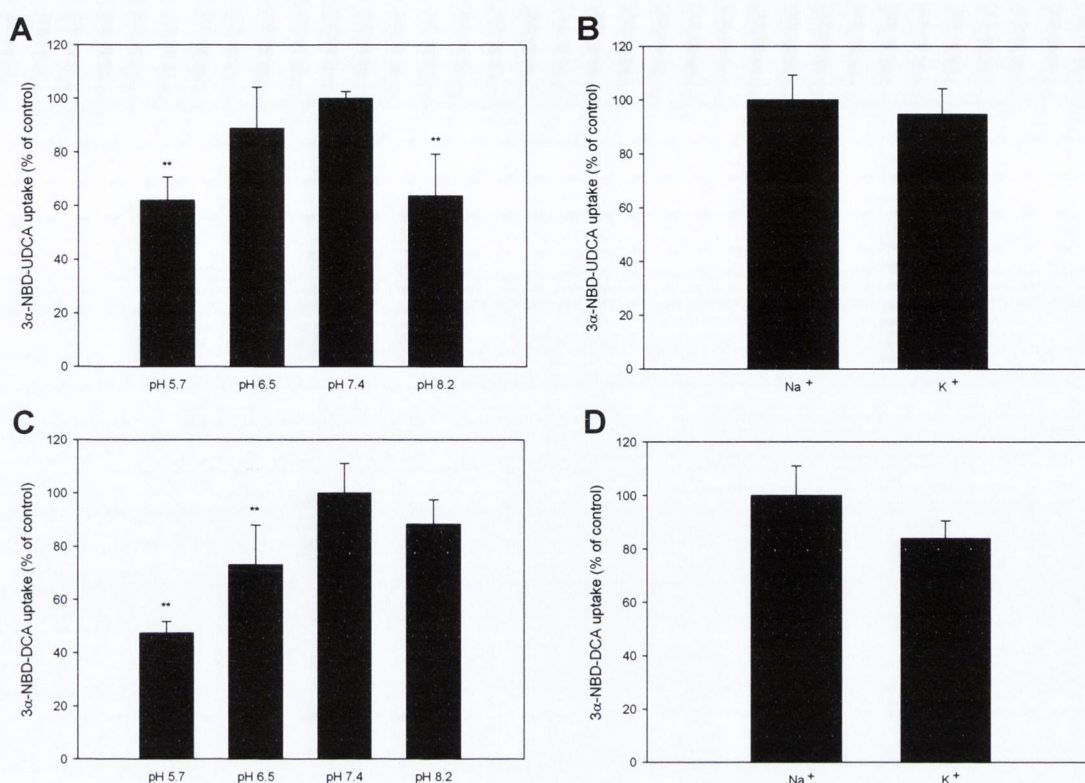


Figure 7. pH- and sodium dependency of NBD-bile acid uptake by Caco-2 cell monolayers.

Uptake of bile acid compounds **3** (A,B) and **5** (C, D) was measured at different pH for 20 min at 37°C. Furthermore, uptake was studied in the presence and absence of sodium (sodium salts were exchanged with potassium salts). Data are represented as mean $\pm$ SD, ($n = 3$).

Additionally, the effect of extracellular pH on the initial uptake of bile acid compounds **3** and **5** was examined by changing the extracellular pH over the range of 5.7 to 8.2 (Figure 7A and C). In both cases, uptake was decreased with decreased pH (pH 5.7) to $62.0 \pm 8.7\%$ in the case of compound **3** and to $62.0 \pm 8.7\%$ in the case of compound **5**, respectively. The replacement of sodium with potassium salts did not cause any significant changes in the uptake of **3** and **5** into Caco-2 cell monolayers (Figure 7B and D).

We also characterised the uptake of the cyclohexyl-NBD adduct (**7**), a substance used previously as a control for nonspecific fluorescent effects in a similar context.

Compound **7** established rapid equilibrium with the Caco-2 cells (Figure 8); however, there was no difference in uptake whether the experiments were carried out at 37°C or 4°C, indicating that only passive diffusion contributed to cellular uptake.

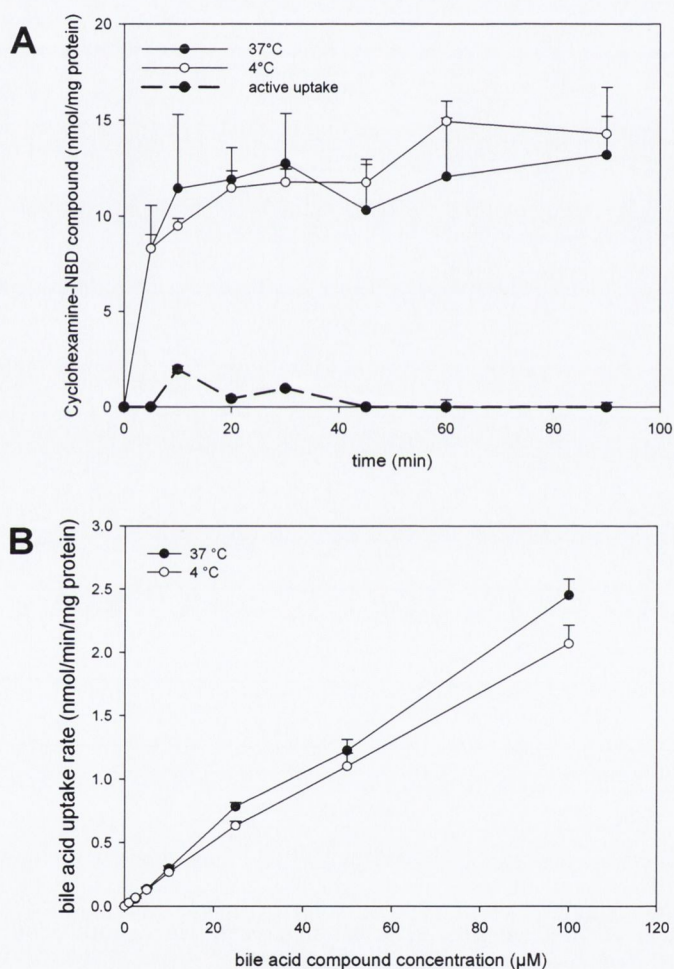


Figure 8. Uptake profile of the cyclohexamine NBD adduct (**7**) into Caco-2 cell monolayers was studied for 90 min at 37°C (●) and 4°C (○). The active component was estimated from the difference between the fluorescence levels after exposure at 37°C and 4°C. Panel B shows concentration-dependent uptake of **7** by Caco-2 cell monolayers. Uptake was measured for 20 min at 37°C and 4°C. Data are represented as mean ± SD, (*n* = 3).

8.3.3 Uptake studies in A549 cells

The molecules of the OATP/SLCO family transport a broad variety of exogenous and endogenous substrates, among them bile acids such as taurocholate (Hagenbuch et al., 2008; Tamai et al., 2000). In this work, we studied if the new bile acid derivatives could be further used as non-radioactive tools for SLCO function analysis in the lung. As well in A549 cells, uptake of 3–6 (10 μ M) was studied at 37° and at 4°C for up to 90 min. The difference between the uptake at 37°C and at 4°C was calculated and the active uptake of all four compounds can be seen in Figure 9. Generally, bile acid uptake increased time-dependently at 37°C and was significantly different from 4°C in all cases, except for 3 β -NBD-UDCA (4). Both DCA-analogues exhibited higher uptake activity in A549 cells in comparison to the UDCA-analogues, whilst 3 β -NBD-DCA reached a significant high value of 180.0 $\pm$ 9.5 nmol/mg protein. Differences could be seen between the DCA and UDCA analogues; however, distinctions between α - and β -compounds were not evident in all cases.

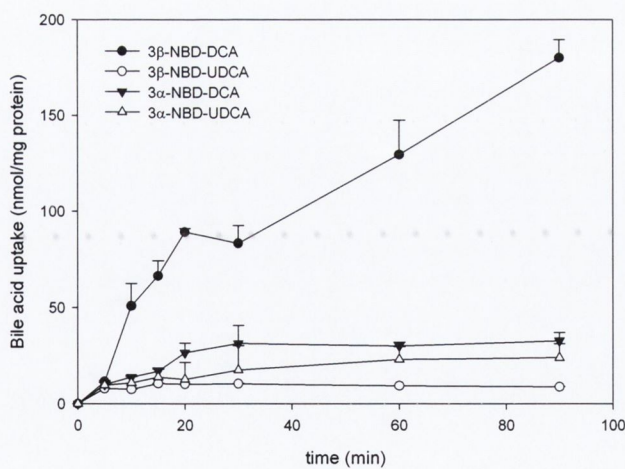


Figure 9. Time course of bile acid (3, 4, 5 and 6) uptake into A549 cell monolayers. Uptake was studied for 90 min at 37°C and 4°C. The active uptake of bile acid compounds (10 μ M) was calculated as the difference between the fluorescence after exposure to 3, 4, 5 and 6 at 37°C and 4°C and is shown here. Data represent mean $\pm$ SD ($n = 3$).

Furthermore, cells were incubated for 20 min with the compounds **3**, **5** and **6** at concentrations ranging from 1 to 100 μM at 37°C and 4°C. The obtained values at 4°C were again subtracted from total uptake at 37°C before pharmacokinetic analysis was performed. The concentration-dependent uptake of the bile acid compounds **5** and **6** was saturable (Figure 10), whilst uptake activity of compound **3** was significantly lower than **5** and **6**, furthermore no saturation could be obtained. Pharmacokinetic analysis of the data revealed a single straight line for compounds **5** and **6** suggesting that the uptake was also mediated by one single transporter site in A549 cells (Figure 3). The kinetic parameters were estimated to be $K_m = 47.6 \pm 8.9 \mu\text{M}$ and $V_{\max} = 3.8 \pm 0.3 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for **5** and $K_m = 45.7 \pm 3.5 \mu\text{M}$ and $V_{\max} = 6.1 \pm 0.2 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for **6**, respectively. This data suggest a preference of DCA-analogues for the recognition during uptake at the transporter and a similar binding affinity.

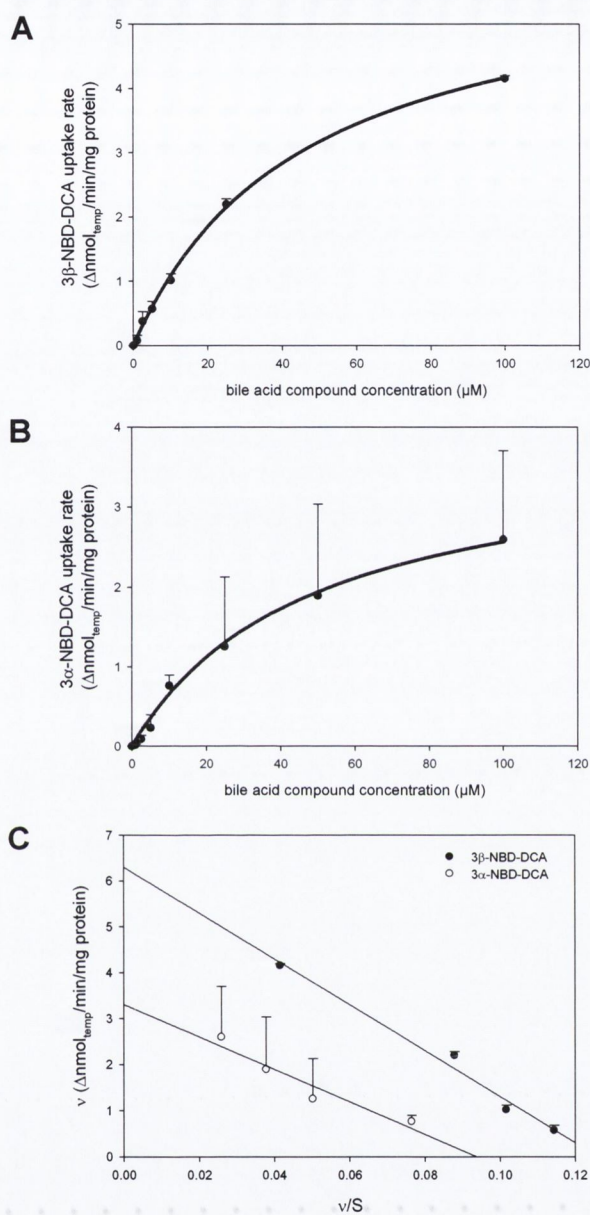


Figure 10. Concentration dependence of **5** and **6** uptake by A549 cell monolayers. Uptake of bile acid compounds **5** (A) and **6** (B) was measured for 20 min at 37°C and 4°C. Eadie-Hofstee transformations of the data (C): v , uptake rate; S , bile acid compound concentration, One single transporter site was identified for the uptake of both NBD bile acid compounds in A549 cells. Data are represented as mean $\pm$ SD, ($n = 3$).

8.4 DISCUSSION

The Caco-2 cell line has been used to assess the permeability of various bile acid pro-drug conjugates and it exhibits active uptake of sterically bulky 24-side chain peptide conjugates (Swaan et al., 1997). In Caco-2 cells, time-dependent uptake studies allowed ranking the uptake efficiency of the NBD compounds in the order 3α -NBD-DCA (**5**) $> 3\alpha$ -NBD-UDCA (**3**) $> 3\beta$ -NBD-DCA (**6**) $> 3\beta$ -NBD-UDCA (**4**). The DCA analogues in each pair were more effectively taken up than the corresponding UDCA compound, possibly due to their greater hydrophobicity.

Remarkably, the β -isomers in each case were taken up exclusively through passive diffusion, whereas both α -compounds were mostly actively transported. This peculiar diastereoselective exclusion of a fluorescent BA from active transport has not been noted in the bile acid literature as far as we are aware. Possible reasons for the observed diastereoselectivity include: (i) a steric clash between the β -group and the boundary of the pocket accommodating the steroidal A ring or (ii) a high dependence on the equatorial H-bond donor in the natural bile acid mimicked by the α -NH in the corresponding NBD analogue. This seems less likely since a loss of a H-bond opportunity would reduce binding enthalpy as reflected in the pseudo K_m value, rather than exclusion as observed. Representative low energy conformers for the DCA isomers appear along with a model of DCA. Whatever its cause, the exclusion of 3β -analogues is relevant to the design of bile acid pro-drug conjugates as well as inhibitors and substrates for intestinal bile acid transporters.

In contrast, the uptake efficiency of the NBD compounds showed the following ranking in A549 cells: 3β -NBD-DCA (**6**) $> 3\alpha$ -NBD-DCA (**5**) $> 3\alpha$ -NBD-UDCA (**3**) $> 3\beta$ -NBD-

UDCA (4). The importance of the α -position of the NBD-analogues in order to be taken up most actively could not be shown in A549 epithelial cells. However, it could be confirmed that DCA analogues are more efficiently transported than their respective UDCA analogues. Eadie-Hofstee analysis revealed a single transporter to be involved in the uptake of both DCA analogues with estimated K_m values of $47.6 \pm 8.9 \mu\text{M}$ and V_{max} of $3.8 \pm 0.3 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for $3\alpha\text{-NBD-DCA}$ and K_m of $45.7 \pm 3.5 \mu\text{M}$ and V_{max} of $6.1 \pm 0.2 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for $3\beta\text{-NBD-DCA}$, respectively. The calculated affinities suggest that the same transporter protein is involved in the translocation of the DCA analogues at the alveolar barrier. In the case of Caco-2 cells, the high diastereoselectivity of the processes as well as the Eadie-Hofstee transformation suggest that the active transport process was also mediated by a single transporter site. The kinetic parameters were estimated to be $K_m = 42.3 \pm 13.0 \mu\text{M}$ and $V_{\text{max}} = 2.8 \pm 0.4 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for $3\alpha\text{-NBD-UDCA}$ and $K_m = 28.2 \pm 7.5 \mu\text{M}$ and $V_{\text{max}} = 1.8 \pm 0.2 \text{ nmol}/(\text{mg protein} \cdot \text{min})$ for $3\alpha\text{-NBD-DCA}$, respectively. In comparison to A549 cells, binding affinities of BA are very similar indicating a related transporter protein involved. However, it cannot be ignored that certain chemical features are preferred in the two used cell culture models. Interestingly, 3α - compounds are actively taken up into Caco-2 cells, whereas this stereochemical binding is not necessarily needed when uptake into A549 cells was investigated where 3α - and β -compounds were taken up efficiently. Here, only the position of the OH group seems to be determining which might not rule out that the same transporter protein is involved.

The use of amino-NBD bile acids as fluorescent tools goes back almost 20 years to the synthesis, characterisation, distribution and metabolism of 3-, 7- and 12-aminoNBD analogues of CA (Schneider et al., 1991; Schramm et al., 1991; Schramm et al., 1993).

In an interesting re-exploration of some of these compounds, Rohacova et al. reported that 3 α , 3 β , 7 α and 7 β -NBD CA accumulate in freshly isolated rat hepatocytes in a manner that was transporter mediated, because uptake was markedly attenuated by troglitazone, an inhibitor of hepatic NCTP and OATP (Rohacova et al., 2009). While 3 α -NBD CA was efficiently taken up, all of the isomers underwent active transport. The same group recently reported on some transport characteristics of a series of 3- and 7 α / β CA dansyl analogues in hepatocytes (Rohacova et al., 2009b). In this series the 7 α - and β compounds appeared to be taken up to similar extents, and, although there was a marked difference in extent of uptake of the 3-isomers, there was still significant active uptake of the 3 β -analogue. Analogues of chenodeoxycholic acid (CDCA), CA, UDCA, DCA and lithocholic acid incorporating a 24-(Ne-NBD) lysine were reported to be transported via OATP1B1 and OATP1B3-expressing HepG2 cells. The CDCA analogue was most efficiently transported by both OATP subtypes with K_m values one order of magnitude lower than observed in the present study for the 3 α - NBD compounds (Yamaguchi et al., 2006). Fluorescent-lysyl analogues should properly be considered as reporters for the corresponding bile acid glycine conjugates since the terminal acid group is similarly placed with respect to the steroidal scaffold. In these compounds the pentyl-N(e-NBD reporter group replaces one of the hydrogen atoms on the glycyl methylene carbon. This arrangement, which substantially increases mass and steric bulk, produces high affinity substrates for OATPs (and ASBTs) but this kind of reporter is not suitable for investigating mechanistic questions related to unconjugated bile acids. It is important to note in this context that free DCA and UDCA possess chemical properties such as size, polarity and pKa that are distinct from their glycine and tauro conjugates and they cause different biological effects (Hofmann et al., 2008).

Bile acids are transported via several transport proteins such as NTCP (SLC10A1), ASBT (SLC10A2), BSEP (ABCB11), OSTa/OSTb and OATP (Klaassen et al., 2010; Hagenbuch et al., 2008). Of these, NTCP, ASBT and BSEP are reported to be inconsistently expressed in the Caco-2 literature (Hilgendorf et al., 2007; Sai et al., 2006). Moreover, the free bile acids were not substrates for hASBT-transfected MDCK cells, although they inhibited hASBT translocation of taurocholate with low μM potency (Balakrishnan et al., 2006b). The presence of the basolateral transporters OSTa and OSTb in Caco-2 cells was recently demonstrated by Ming et al. on mRNA level (Ming et al., 2011). Several groups have shown numerous OATP transporters to be expressed in Caco-2 cells particularly OATP2A1, OATP4A1 and OATP2B1 (Endter et al., 2009; Sai et al., 2006; Tamai et al., 2000). OATP2B1 is highly expressed in the apical cell membrane, making the transporter the most likely candidate for the uptake observed in Caco-2 cells in the present study. More evidence could be provided by the undertaken pH-experiments in Caco-2 cells showing a significant dependence of the uptake on the extracellular pH. OATPs have been reported to be pH-dependent transporter proteins and suggested to be anion exchangers (Leuthold et al., 2009; Roth et al., 2012; Kobayashi et al., 2003; Nozawa et al., 2004). HCO_3^- efflux and therefore OATP activity is stimulated when extracellular H^+ concentration is increased.

Previously, OATP2B1 was found to be expressed in the jejunal epithelium and has been described to mediate uptake of taurocholate with a K_m of 72 μM , which is in the same order of magnitude as observed here (Nozawa et al., 2003).

The presence of OATPs in the lung is still rather unexploited. To date, gene array analysis and mRNA expression studies were reported (Bleasby et al., 2006; Endter et al., 2009; Seki et al., 2009) confirming the presence of several members of the OATP

family, e.g. OATP2A1, OATP2B1, OATP3A1 and OATP4A1. It is noteworthy that Seki and colleagues showed functional expression of OATP2B1 in A549 cells. They confirmed the involvement of OATP2B1 in the uptake of amiodarone at the human alveolar epithelium by performing knockdown of the transporter protein. High mRNA expression of OATP1B3 was found; however, no significant inhibition effect of the inhibitor estradiol-17 β -glucuronide could be seen and therefore was ruled out as a major contributor (Seki et al., 2009).

8.5 CONCLUSIONS

3 α - and 3 β - NBD -amino derivatives of DCA and UDCA have been prepared as fluorescent probes for investigating the contrasting effects of the parent bile acid compounds for detection of organic anion-transporting peptide mediated processes. The compounds possess adequate aqueous stability and are non-toxic in several cell lines. They have similar fluorescent characteristics to previously reported NBD analogues. The uptake of the compounds into Caco-2 cells was strikingly diastereospecific; whereas the 3 α -compounds taken up by active transport, the 3 β -compounds were not. In A549 cells, uptake of the compounds was determined by the position of the OH group; the stereochemical binding of the NBD was not of particular importance. In both cell culture models, Eadie-Hofstee analysis indicated that the active transport was mediated by a single protein.

However, to identify the transporter site which is mainly mediating the uptake of fluorescent bile acids, further uptake studies in relevant heterologous expression systems and in the presence of specific inhibitors are needed. 3 α -NBD DCA warrants additional study as a tool for functional activity studies associated with organic anion transporter proteins. The specificity of the interactions suggests that the α -compounds are able to mimic the natural bile acid parents. The 3 α -UDCA/DCA pair will be employed to investigate mechanistic aspects of the functional antagonism exhibited by these compounds in intestinal biology.

CHAPTER 9

Nanoparticles attenuate P-glycoprotein (MDR1) function in A549 human alveolar epithelial cells

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ABSTRACT

P-glycoprotein (P-gp) is a well characterised membrane transporter relevant to drug disposition and multi-drug resistance. This study aimed to investigate the effect of latex nanoparticles (NPs) on the release of the P-gp substrate, rhodamine 123 (Rh123) using the human alveolar epithelial cell line, A549.

Expression and function of P-gp were confirmed in A549 cells. Rh123 release studies were carried out in the presence of known inhibitors of P-gp function (cyclosporine A and verapamil), ATP depletion (NaN_3/DOG) and nanoparticles (NP) with different surface modifications, ζ -potentials and sizes (plain, carboxylated and amine- and sulphate-modified NPs). The cytotoxic effect of NPs on A549 cells was evaluated by MTT assay. The effects on P-gp levels after acute exposure to NPs were investigated by Western blot analysis of A549 cell lysate and supernatant.

Cellular retention of Rh123 was significantly ($P < 0.05$) increased in the presence of carboxylated (100 nm) and amine-, and sulphate-modified NPs. A slight, but not significant decrease in Rh123 release could also be observed for plain latex and carboxylated (500 nm) NPs. The MTT assay demonstrated that most NPs caused only low levels of cytotoxicity (78-88% cell viability), the positively charged amine-NPs, however, were considerably cytotoxic. Western blot showed that the nanoparticles did not cause any cell membrane disruption. Our findings suggest that nanomaterials can attenuate membrane transporter function depending on size and surface properties and hence, might influence disposition of xenobiotics as well as endogenous substrates.

9.1 INTRODUCTION

P-glycoprotein/MDR1 (P-gp) is a 170 kDa membrane glycoprotein, encoded by the *ABCB1* gene. It is known to play an important role as an efflux pump in drug disposition, particularly in cancer chemotherapy (Baguley, 2010). P-gp is also expressed in many tissues under physiological conditions, indicating their major role in cellular transport of a variety of endogenous substrates and therapeutic agents (Baguley, 2010). Presently, over 100 compounds have been identified to be P-gp substrates, of which the majority were basic lipophilic amines. The lung is thought to accumulate lipophilic amines and the similarity in substrate specificity of P-gp strongly suggests that P-gp mediated efflux plays an important role in pulmonary accumulation (Bosquillon, 2009, Endter et al., 2007).

In this Chapter, we aim to investigate in how far nanoparticulates affect the functionality of transport systems in particular P-gp and OCTs and which particle properties govern these interactions. Commercially available, insoluble latex nanoparticles with various surface and size characteristics were studied in an *in vitro* model based on the human alveolar type 2 cell-like adenocarcinoma cell line, A549.

9.2 MATERIALS AND METHODS

9.2.1 Materials

A549 cells (ATCC CL-185) were obtained from LGC Promochem (Teddington, UK). All cell culture plastics were obtained from Greiner (Frickenhausen, Germany), with the exception of Lab-Tek chamber slides (Nunc, Roskilde, Denmark). Nanoparticles (L3405, L3280, LB1, L9902 and L0780; properties are summarised in table 1), mouse monoclonal anti-P-gp antibody (clone F4) and all other chemicals and reagents were purchased from Sigma- Aldrich (Dublin, Ireland).

Table 1. Physicochemical properties of nanoparticles used.

Latex bead nanoparticles (Sigma-Aldrich order no.)	Abbreviation	Fluorescence label (excitation/emission)
Carboxylate-modified, 2.5% solids, 100 nm (L3405)	carboxy (100)	(575 nm / 610 nm)
Carboxylate-modified, 2.5% solids, 500 nm (L3280)	carboxy (500)	(575 nm / 610 nm)
Unmodified, 10% solids, 100 nm (LB1)	latex	none
Amine-modified, 2.5% solids, 50 nm (L0780)	amine	(360 nm / 420 nm)
Sulphate-modified, 2.5% solids, 100 nm (L9902)	sulphate	(575 nm / 610 nm)

9.2.2 Cell culture

A549 (American Type Culture Collection CL-185; LGC Promochem, Teddington Middlesex, UK) cells are derived from a human pulmonary adenocarcinoma (Giard et al., 1973). Due to phenotype similarity to type II alveolar epithelial cells this cell line has been widely utilised in studies of alveolar epithelium function (Sporty et al., 2008). Cells of passage numbers 66 to 82 were used in this study and were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM:F-12) supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37°C in 5% CO₂ atmosphere and culture medium was exchanged every 48 h.

9.2.3 Rhodamine 123 release studies

Rhodamine 123 (Rh123) release studies were performed using A549 cell layers cultured for 5 days in 24-well plates at a density of 40,000 cells/ cm². Cell layers were washed twice with pre-equilibrated Krebs-Ringer bicarbonated buffer (KRB; composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, 0.81 mM MgSO₄ and 5.55 mM glucose; pH 7.4), before being loaded with a 10 µM solution of Rh123 in KRB for 30 min. Next, the Rh123 solution was replaced with fresh pre-warmed KRB containing the nanoparticles (100 µg/ml) or pharmacological inhibitors [cyclosporine A (CsA) 10 µM; verapamil 10 µM; NaN₃/2-deoxy-D-glucose (DOG) 10 mM]. The cell layers were then incubated for 30 min at 37°C in 5% CO₂ atmosphere, before being washed twice with ice-cold KRB and lysed with a 1% solution of Triton X-100 in KRB.

In the case of ASP⁺ uptake studies, cell monolayers were washed twice with pre-warmed KRB, then incubated with ASP⁺ solution (10 µM) in the presence or absence of different concentrations of nanoparticles (100 µg/ml) for 30 min at 37°C. To stop the reaction, cell monolayers were similarly washed twice with ice-cold KRB and afterwards lysed with Triton X-100.

Fluorescence of samples was analysed in 24-well plates using a fluorescence plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively. The samples were diluted with KRB, where appropriate. Rh123 release from untreated A549 cell layers was used as control. For standardisation, the total protein amount of cell layers was determined by bicinchoninic acid assay according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, USA).

Half-saturation constant (K_m) and maximum release rate (V_{max}) of Rh123 were calculated by fitting the release rate (v) to the following equation by means of non-linear least squares regression analysis according to the Michaelis-Menten equation (Eadie- Hofstee plot):

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

where (S) was [Rh123].

9.2.4 Western blotting

A549 cell layers were grown as described above and for immunoblotting cell supernatant (CS) was collected by aspiration after 5 days in culture. Cell lysate (CL) was obtained by washing the cell layers three times with ice-cold phosphate saline buffer (PBS), followed by cell homogenisation in 60 μ l per well freshly prepared lysis buffer, containing aprotinin (6%) and leupeptin (1%). Samples of CS and CL were sonicated twice for 20 s and centrifuged at 9279 g for 20 min. The total protein amount was determined by Bio-Rad protein assay (Hercules, USA) with bovine serum albumin (BSA) used as standard. Western blotting was performed after immediately after sample preparation. Samples were standardised to equal protein concentrations, 6 μ l of loading buffer were added and the mixture was heated up to 95°C for 5 min, before 20 μ l of each sample was loaded onto a 6% SDS gel. Electrophoresis was performed for 2 h at 110 V. The protein bands were transferred onto PVDF membranes (Bio-Rad) at 25 V for 30 min. Blots were blocked in PBS containing 5% (w/v) BSA for at least 1 h at room temperature, before overnight incubation with the anti-P-gp primary antibody (1:100) at 4°C. Membranes were then washed with PBS and the secondary goat anti-mouse IgG antibody (Promega, Medical Supply, Ireland) in a concentration of 1:13,500 was added for 1 h at room temperature. Peroxidase activity was detected with

Immobilon Western Chemiluminescent HRP substrate (Millipore). Signals were documented using a ChemiDoc system (Bio-Rad).

9.2.5 Analysis of particle size and ζ -potential

Physicochemical properties (i.e., size and ζ -potential) of the nanoparticles were measured before and after the release experiments using a Zetasizer Nano ZS (Malvern, Malvern, UK) at 25°C and a scattering angle of 173°. The samples containing nanoparticles were placed in a standard disposable capillary cell (DTS1060, Malvern UK) for size measurements and ζ -potential, respectively.

9.2.6 Cell viability assay

The viability of A549 cells was determined by MTT (3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cells were seeded at a density of 30,000 cells/cm² in 96-well plates and left for 24 h to attach. After 30 min of incubation with nanoparticles (100 µg/ml) suspended in KRB, the cell layers were washed twice with KRB, before 100 µl of fresh KRB and 10 µl of MTT solution (5 mg/ml in KRB) were added to each well. After further incubation at 37°C for 3 h, the supernatant was removed and cells were dispersed in 50 µl DMSO. Absorbance was measured at 570 nm by using a microplate reader (FLUOstar Optima).

9.2.7 Confocal laser scanning microscopy

A549 cell layers were grown on chamber slides at 35,000 cells/cm² for 5 days, before cells were being fixed for 10 min with 2% (w/v) paraformaldehyde and incubated for 10 min in 50 mM NH₄Cl, followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100 in PBS. After 60 min incubation with 200 µl of a 1:500 dilution of monoclonal

mouse anti-P-gp antibody, the cell layers were washed three times with PBS, before incubation with 100 μ l of a 1:200 dilution of Alexa Fluor 488-conjugated goat anti-mouse F(ab')₂ fragment (Invitrogen) in PBS containing 1% (w/v) BSA. Propidium iodide (1 μ g/ml in PBS) was used to counterstain cell nuclei. In the case of F-actin staining, cells were initially incubated for 30 min with nanoparticles (100 μ g/ml) suspended in KRB. TRITC-phalloidin was used at a concentration of 500 ng/ml in PBS. After 30 min of incubation, the specimens were again washed three times with PBS and embedded in FluorSave anti-fade medium (Merck, Nottingham, UK). Images were obtained using a confocal laser scanning microscope (CLSM, Zeiss LSM 510, Göttingen, Germany) with the instrument's settings adjusted so that no positive signal was observed in the channel corresponding to the fluorescence of the isotypic controls.

9.2.8 Statistical analysis

Each experiment was repeated at least in triplicate with cells from three different passages. Results were expressed as mean $\pm$ SD of at least three experiments. Two groups were compared using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test. $P < 0.05$ was considered as significant.

9.3 RESULTS

9.3.1 P-gp expression, localisation and activity in A549 cells

Immunocytochemical staining, performed after 5 days of culture of *A549* cell layers, showed moderate expression of P-gp in the plasma membrane (Figure 1A). In addition, a punctuate staining pattern typical for intracellular vesicles or similar sub-structures was observed (Figure 1A). Consistent with this observation was a moderate signal of immunoreactive P-gp detected by immunoblot (Figure 1B).

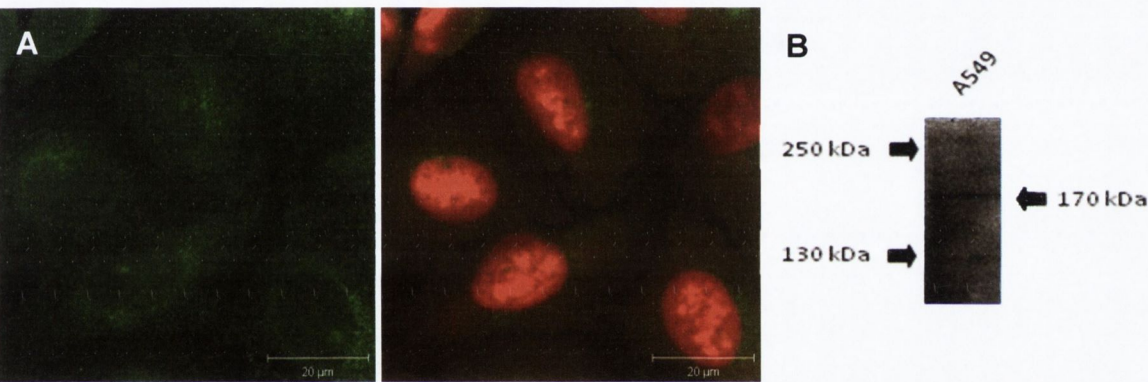


Figure 1. Expression of P-glycoprotein/MDR1 (P-gp) in plastic-grown A549 human alveolar epithelial cell layers after 5 days of culture Cells were plated at a density of 3.5×10^4 cells per cm^2 and cultured under liquid covered culture conditions. A) Immunolabelling of P-gp. Staining for P-gp (green) is shown using confocal laser scanning microscopy. Nuclei were counterstained with propidium iodide (red). Scale bars represent μm . B) Immunoblot analysis of P-gp. A single band of 170 kDa can be detected in A549 cell lysate.

To study the P-gp function, A549 cell layers were loaded with Rh123 and the release of the substrate was studied at 37°C and 4°C , as well as in the presence of P-gp inhibitors (i.e., CsA and verapamil) and attenuators of energy dependent translocation events by depletion of ATP (i.e., NaN_3 and DOG). The difference of fluorescence intensities between the measurements at 37°C and 4°C was calculated (depicted in Figure 2A) and the obtained activity was plotted as an Eadie-Hofstee plot (a linearisation of the

Michaelis-Menten equation). The linear plot depicted in Figure 2B indicates a single transporter being responsible for the efflux of Rh123. P-gp mediated efflux activity was saturable and kinetic parameters were determined as follows: K_m value of Rh123 = $32.91 \pm 4.49 \mu\text{M}$ and $V_{\text{max}} = 0.057 \pm 0.004 \text{ pmol/mg protein}$.

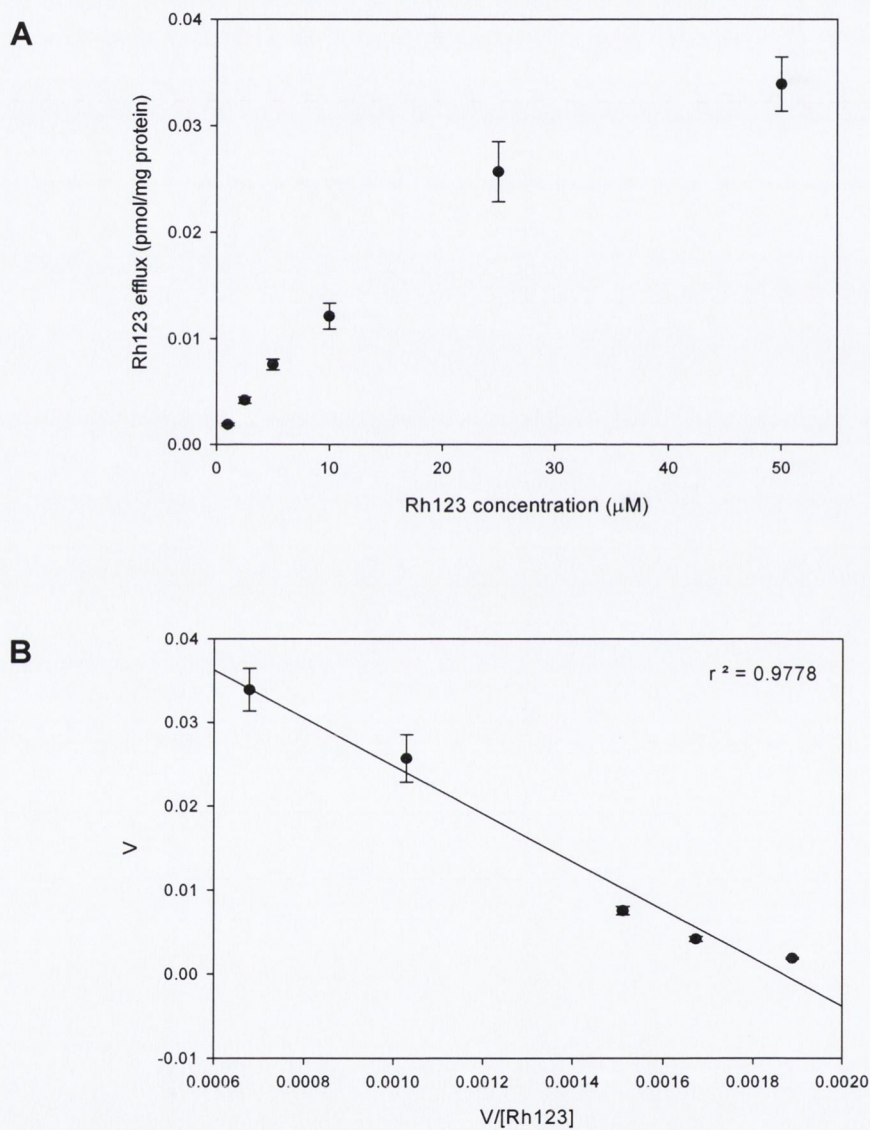


Figure 2. Kinetic analysis of rhodamine 123 (Rh123) release from A549 cell layers after 5 days of culture. A) Release of Rh123 was measured for 30 min at 37°C and 4°C. The efflux was calculated as the difference of fluorescence signals obtained at the two temperatures and standardised against the total protein amount. B) Rh123 release data were transformed to an Eadie-Hofstee plot. The single straight line indicates a single transport mechanism for Rh123 release from A549 cells. Results are expressed as means $\pm$ SD ($n = 3$).

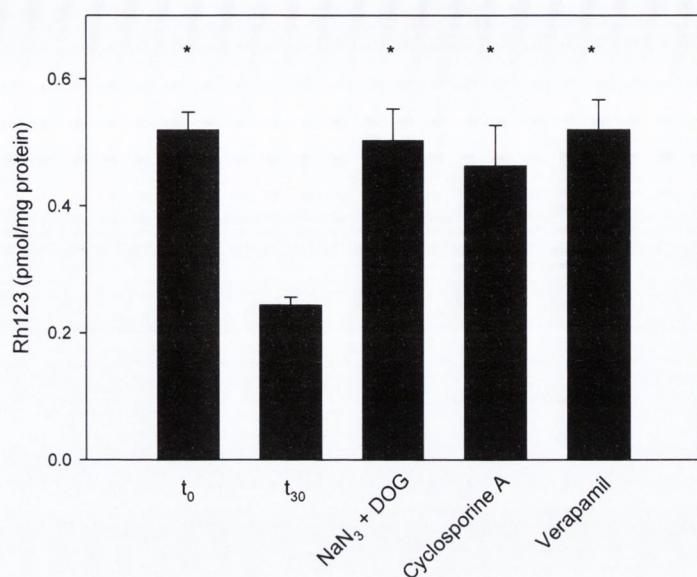


Figure 3. Effect of cyclosporine A (10 μM), verapamil (10 μM) and NaN_3 /2-deoxy-D-glucose (DOG; 10 mM) on rhodamine 123 (Rh123) release from A549 cell layers cultured for 5 days. Cell layers were loaded with Rh123 (10 μM) for 30 min (t_0) and fluorescence was measured after another 30 min of release (t_{30}). Results are expressed as means $\pm$ SD from 6 independent experiments. * $P < 0.01$ versus control buffer alone.

Cyclosporine A and verapamil almost completely suppressed Rh123 release from A549 cell layers (Figure 3). A similar effect was observed when A549 cells were incubated with NaN_3 plus DOG (Figure 3).

9.3.2 Rh123 efflux in the presence of nanoparticles

Rh123 release studies were then carried out with insoluble nanoparticles of different sizes and surface charges suspended in the incubation buffer at concentrations of 100 $\mu\text{g/ml}$. Figure 4 shows the effect of the nanoparticles on Rh123 release from A549 cell layers. Incubation with carboxy (100 nm), amine and sulphate nanoparticles resulted in significantly ($P < 0.01$) higher intracellular Rh123 levels after 30 min, indicating an attenuating effect on release by inhibition of the P-gp transport system. Carboxy (500 nm) and unmodified latex particles also decreased Rh123 efflux, albeit those effects were not significant ($P < 0.05$).

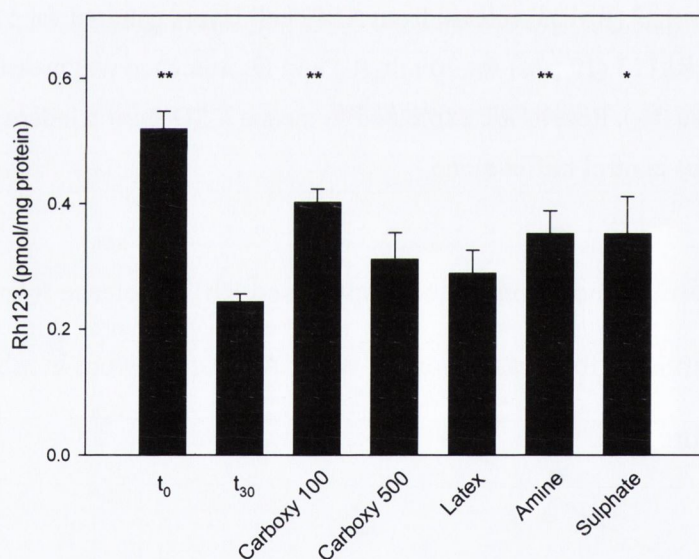


Figure 4. Effect of different nanoparticles (100 $\mu\text{g/ml}$) on rhodamine 123 (Rh123) release from A549 cell layers cultured for 5 days. Cell layers were loaded with Rh123 (10 μM) for 30 min (t_0) and fluorescence was measured after another 30 min of release (t_{30}). Results are expressed as means $\pm$ SD from 6 independent experiments. * $P < 0.05$, ** $P < 0.01$ versus control buffer alone.

9.3.3 Western blotting analysis

The level of P-gp was quantified in A549 cell layers and in cell supernatant by Western blotting (Figure 5). No obvious changes in cellular P-gp levels, compared to control, were observed after incubation with the different nanoparticles (Figure 5A). Extraction of P-gp from the membrane into the cell supernatant could also be ruled out, as no protein signals were obtained in the investigated samples (Figure 5B).

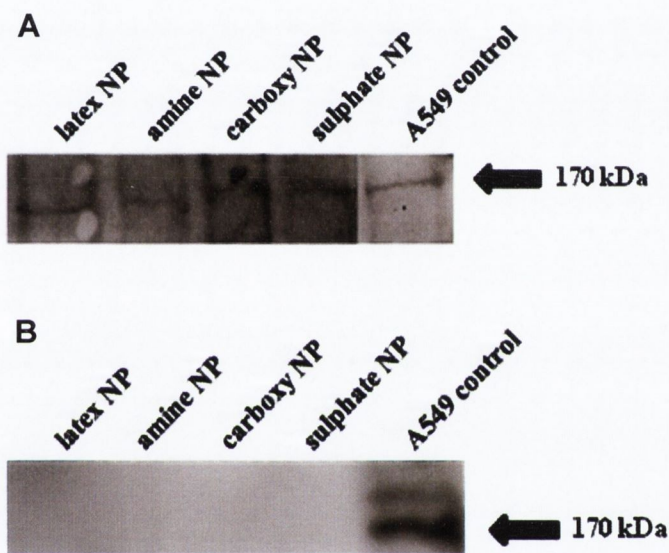


Figure 5. Analysis of P-glycoprotein/MDR1 protein levels in A549 cells and supernatant. A549 cell layers were cultured for 5 days, before being incubated with different nanoparticles (100 µg/ml) for 30 min. Protein was extracted from cells and cell supernatant and probed with a monoclonal anti-P-gp antibody. A) Representative immunoblot of cell lysate. A single band of 170 kDa can be detected in A549 cell lysate. B) Representative immunoblot of cell supernatants. No signal could be obtained.

9.3.4 Physicochemical characteristics of nanoparticles

Latex nanoparticles with various surface charges, surface modifications and sizes (see Table 1) were used in Rh123 release studies. Table 2 shows the changes in ζ -potential and particle size when analysing the NPs before and after release studies.

Prior to experiments, carboxy 100 and 500, latex and sulphate nanoparticles were found to be negatively charged between with their ζ -potential values ranging from -22 to -63 mV. Amine-modified particles showed a net positive charge of 61 mV.

The particle sizes measured in our lab were in good agreement with the supplier's given specifications.

Incubation with A549 cell layers resulted in dramatic changes in both particle size and surface charge (Table 2). Whilst the ζ -potential was diminished among all nanoparticles, particle size increased significantly as a result of agglomeration. The highest increase was observed for amine-modified particles which grew from approx 50 nm to almost 4 μ m in diameter.

Table 2. Physicochemical properties of latex nanoparticles before and after rhodamine 123 release experiments.

Nanoparticles	Initial values			After Rh123 release		
	Size (nm)	PdI	ζ -potential (mV)	Size (nm)	PdI	ζ -potential (mV)
carboxy 100	120.8 $\pm$ 0.4	0.02 $\pm$ 0	-45.0 $\pm$ 0.5	563.4 $\pm$ 23.3	0.56 $\pm$ 0.1	-20.6 $\pm$ 0.5
carboxy 500	476.8 $\pm$ 9.2	0.07 $\pm$ 0	-63.1 $\pm$ 0.4	2125.0 $\pm$ 117.9	0.32 $\pm$ 0.1	-15.1 $\pm$ 0.8
latex	117.1 $\pm$ 1.2	0.04 $\pm$ 0	-48.5 $\pm$ 0.6	1715.0 $\pm$ 56.2	0.62 $\pm$ 0.1	-42.2 $\pm$ 0.8
amine	56.7 $\pm$ 0.8	0.05 $\pm$ 0	61.3 $\pm$ 5.2	3943.0 $\pm$ 220.9	0.73 $\pm$ 0.3	11.0 $\pm$ 0.8
sulphate	131.1 $\pm$ 2.1	0.01 $\pm$ 0	-22.3 $\pm$ 1.1	1422.0 $\pm$ 58.7	0.52 $\pm$ 0.1	-10.0 $\pm$ 0.7

9.3.5 ASP⁺ uptake in the presence of nanoparticles

ASP⁺ uptake studies were performed using the same nanoparticles as for Rh123 release studies (except carboxy 100 nm). Nanoparticles were suspended in the incubation buffer

at concentrations of 100 $\mu\text{g/ml}$. Figure 6 shows the effect of the nanoparticles on ASP^+ uptake into A549 cell layers. Incubation with carboxy (500 nm), unmodified latex and amine nanoparticles resulted in significantly ($P < 0.01$) decreased intracellular ASP^+ levels after 30 min, indicating an inhibitory effect on the OCT transport system. Amine NP decreased ASP^+ uptake to 15% compared to control ASP^+ uptake, whereas carboxy NP and latex NP could achieve to diminish ASP^+ levels to 50% and 83%, respectively. Sulphate particles also decreased ASP^+ uptake, but this effect was not significant ($P < 0.05$).

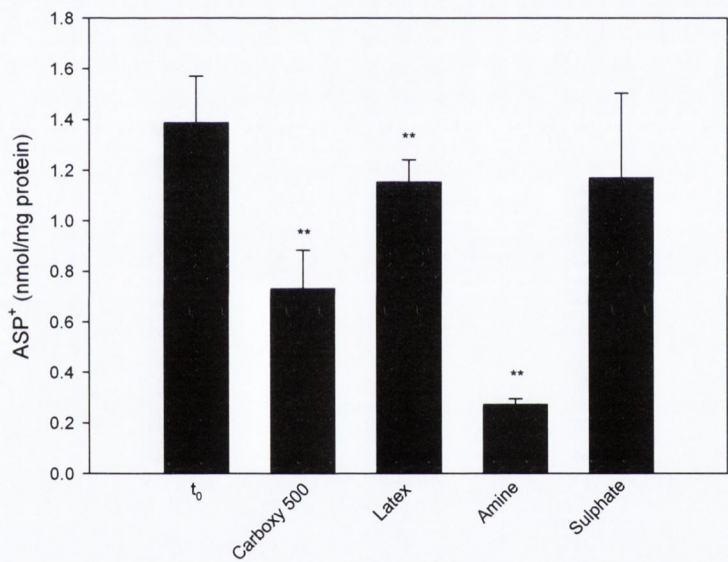


Figure 6. Effect of different nanoparticles (100 $\mu\text{g/ml}$) on ASP^+ uptake into A549 cell layers. Cell layers were loaded with ASP^+ (10 μM) and incubated with the nanoparticles for 30 min (t_0). The fluorescence was measured directly after the experiments. Results are expressed as means $\pm$ SD ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus control buffer alone.

9.3.6 Physicochemical characteristics of nanoparticles in ASP^+ studies

In line to Rh123 release studies, incubation of nanoparticles with A549 cell layers in the presence of ASP^+ resulted in tremendous changes in both particle size and surface charge.

Physiochemical properties are summarised in Table 3 showing the analysis of the NPs before and after uptake studies. Whilst the ζ -potential was diminished among all nanoparticles, particle size increased significantly as a result of agglomeration. The highest increase was observed here for unmodified latex particles which grew from 117 nm to almost 6 μ m in diameter.

Table 3. Physicochemical properties of latex nanoparticles before and after ASP⁺ uptake experiments.

Nanoparticles	Initial values			After ASP ⁺ uptake		
	Size (nm)	PdI	ζ -potential (mV)	Size (nm)	PdI	ζ -potential (mV)
carboxy 500	476.8±9.2	0.07±0	-63.1±0.4	1043±18.3	0.29±0.1	-15.3±0.9
latex	117.1±1.2	0.04±0	-48.5±0.6	6675±269	0.27±0.1	-19.2±0.7
amine	56.7±0.8	0.05±0	61.3±5.2	2030±93.5	0.34±0.1	9.0±0.6
sulphate	131.1±2.1	0.01±0	-22.3±1.1	4048±1099	0.71±0.1	-15.0±0.8

9.3.7 Cytotoxicity assay

In order to investigate, if the observed effects resulted from cytotoxic events caused by the nanoparticulates, the viability of A549 cells was tested by MTT assay. Cells were incubated with NPs at concentrations of 25, 50 and 100 µg/ml for 30 min (Figure 7). Carboxy (500 nm), latex and sulphate particles marginally reduced the cell viability to 79±7%, 78±14% and 84±13%. Amine nanoparticles, however, showed a pronounced and concentration-dependent cytotoxicity, with only 47±1% cell viability at the highest concentration after 30 min. It should be noted that carboxy (100 nm) nanoparticles have been discontinued by the supplier prior to the onset of this study.

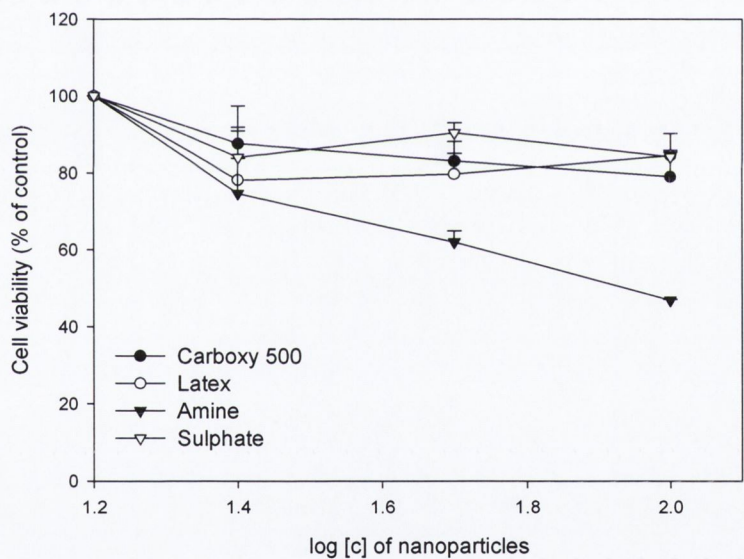


Figure 7. Cytotoxicity of nanoparticles. A549 cell layers were cultured for 5 days, before being incubated with different nanoparticles (100 µg/ml) for 30 min. Cell viability was assessed by MTT assay and normalised to the level of untreated cells. Results are expressed as means ± SD (n = 6).

9.3.8 Confocal laser scanning microscopy

The interactions of nanoparticles with A549 cell layers were measured using confocal laser scanning microscopy (Figure 8). TRITC-labelled phalloidin was used to visualise the cytoskeleton. Agglomerated carboxy and sulphate NPs appeared in the same channel due to their Texas-red label (Figure 8C and 8E). It is evident from the CLSM micrographs that none of the nanoparticles were taken up into the cells or caused significant disruption of the cytoskeleton.

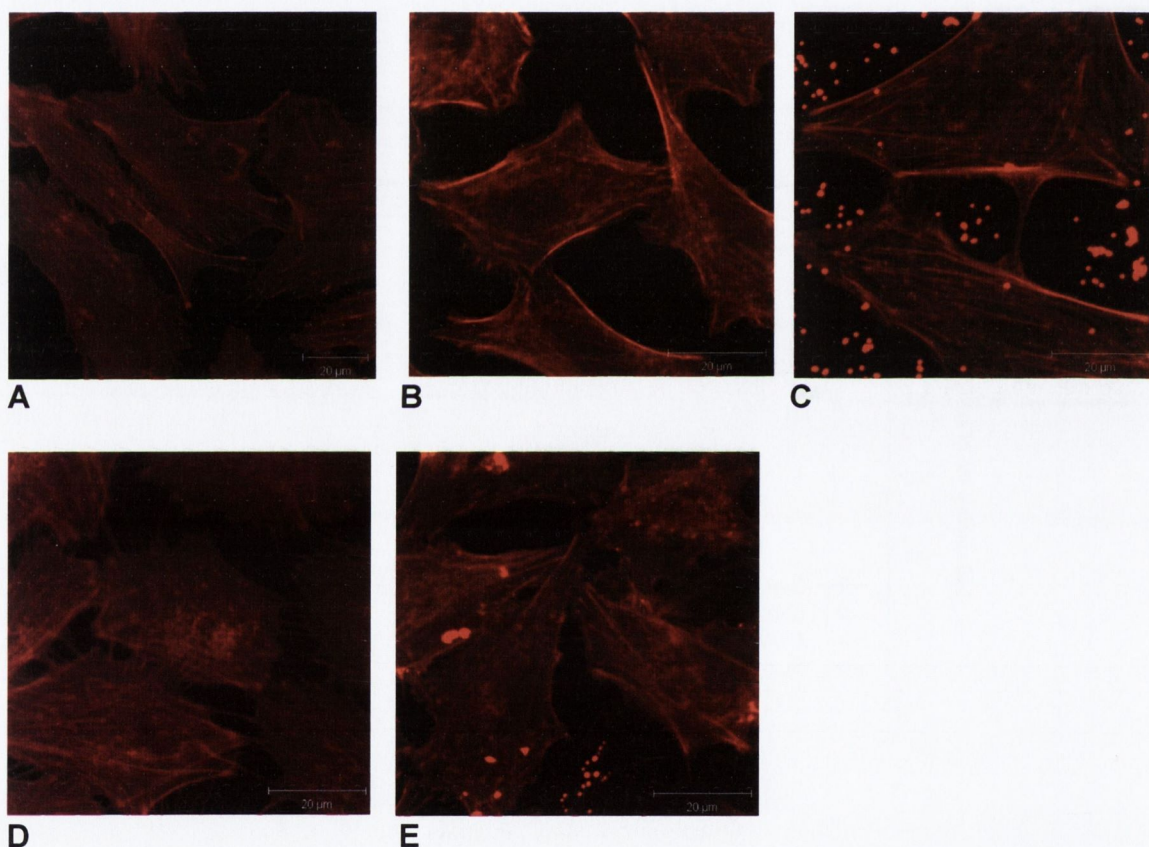


Figure 8. Visualisation of F-actin in plastic-grown A549 human alveolar epithelial cell layers after 5 days of culture. Cells were plated at a density of 3.5×10^4 cells per cm^2 and cultured under liquid covered culture conditions. Cells were fixed and labelled using TRITC-phalloidin (red), after being incubated with different nanoparticles (100 $\mu\text{g}/\text{ml}$) for 30 min. A) Control, B) unmodified latex nanoparticles, C) carboxylated nanoparticles 500 nm, D) amine-modified nanoparticles and E) sulphate-modified nanoparticles. In C and E nanoparticles are visible due to their Texas red label. Scale bars represent μm .

9.4 DISCUSSION

Consistent with earlier observations (Hamilton et al., 2000; Lehmann et al., 2001) our data confirmed that the A549 cell line functionally expressed P-glycoprotein. Rh123 efflux was found to be mediated by a saturable, one-carrier mechanism and increased accumulation of Rh123 was observed in the presence of verapamil, cyclosporine A and NaN₃/DOG, agents known to inhibit P-gp function and deplete ATP, respectively.

It has also been suggested that nanoparticles have the ability to accumulate in cells without being recognised by P-glycoprotein, one of the main mediators of multidrug resistance, resulting in the increased intracellular concentration of drugs (Lee et al., 2005). When studying the interaction of insoluble, latex-based nanoparticles of different surface modification, ζ -potential and particle size with P-gp, we found that Rh123 efflux from A549 cell layers was inhibited in a size and surface-chemistry dependant manner that, in some cases, was of similar scale as the attenuation observed with the pharmacological agents. It can be speculated that nanoparticles might directly attenuate P-gp function and inhibit OCT function. In particular small, carboxylated and sulphated NPs were able to reduce P-gp-mediated efflux of Rh123 from A549 cells. ASP⁺ uptake into A549 cells was markedly inhibited in the presence of carboxylated, latex and amine NPs. Whilst similar effects were also observed for amine-modified particles, it cannot be ruled out that this effect resulted from the particles' acute cytotoxicity rather than from direct P-gp or OCT inhibition.

Recently, Arvizo *et al.* (Arvizo et al., 2010) reported that gold nanoparticles, depending on their surface charge, caused changes in membrane potential. Nevertheless, P-gp function has repeatedly been observed to be independent of membrane potential (Luker

et al., 2001). McCarthy and co-workers (McCarthy et al., 2009) have shown that when Calu-3 cell monolayers were acutely exposed to carbon nanoparticles, there was an inhibition of forskolin induced I_{sc} activation through CFTR. They concluded that size and charge were essential factors in determining the extent of this effect and that cholesterol and specialised membrane domains at the cell surface play a pivotal role. Thus, a more direct interaction between the nanoparticles and the membrane or even the ABC-transporter can be suggested to be responsible for the observed effects.

We observed that positively charged nanoparticles had the highest cytotoxicity which is consistent with previous reports (Arvizo et al., 2010, Lundqvist et al., 2008), whereas negatively charged nanoparticles induced only marginal cell death. Thus, whilst nanoparticles potentially change the conformation of the cell membrane, resulting in a disruption of cellular processes and membrane integrity (Lynch et al., 2007), most interactions with the cell membrane do not cause any membrane disruption. This was confirmed by Western blot analysis of the cells and their supernatant.

Significant changes in the size of nanoparticles, once they were in contact with the cell supernatant were shown. In most cases, the surface of nanoparticles is covered immediately after contact with biological fluids by several biomolecules (Lynch et al., 2009). The particles' surface interacts with its dispersion medium in a complex fashion, involving conflictive forces, especially between particles, ions and biological substances in the medium such as proteins. This interplay affects both the hydrodynamic size and the ζ -potential of the nanoparticles and can have an impact on the agglomeration status of the particle itself (Lynch et al., 2009).

To further our understanding of nanomedicines, it is necessary to investigate the nanomaterial's physicochemical properties and how these properties influence their uptake and biodistribution. This is the first study comparing pharmacological P-gp inhibitors with nanoparticulates. The attenuation of Rh123 release, and hence, P-gp function by nanoparticles, is an interesting observation, which might further increase the interest in nanoparticulate drug delivery systems, particularly for the treatment of cancer and to overcome biological barriers imposed by efflux pumps, such as P-glycoprotein.

9.5 CONCLUSION

Our findings suggest that nanomaterial can attenuate membrane transporter function depending on size and surface properties and hence, might influence disposition of xenobiotics as well as endogenous substrates.

CHAPTER 10

CONCLUSIONS

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Although much remains to be explored regarding the functional characterisation of membrane drug transporters in the lung, the presented work provides novel insights into expression of key transporter proteins, their functional activity and potential drug-transporter interactions at the air-blood barrier.

I Membrane transporters, e.g., members of the SLC- (*SLCO*, *SLC22* and *SLC47*) and ABC-super family (P-gp/MDR-1) have been confirmed to be present in different *in vitro* models representing various sections of the lung epithelium in a very distinct and complex fashion. We reported pioneering data on the protein expression of drug transporters in freshly isolated human alveolar epithelial cells. In particular, we confirmed the presence of all five clones of *SLC22A1-5* transporters at the alveolar epithelium *in vitro* (continuously growing cell lines and primary culture) and in bronchiolar epithelial cells, H441, whereas the absence of OCT2 was confirmed in Calu-3 and 16HBE14o- bronchial epithelial cells. The presence of MATE1 (*SLC47A1*) was confirmed in A549, H441 and Calu-3 cells. The protein expression pattern of OATPs revealed high expression levels of OATP2A1, OATP3A1 and OATP4A1 in alveolar (primary cell culture and A549) cells, whereas lower levels of transport proteins were detected in 16HBE14o- and Calu-3 cells. OATP1B3 was only weakly expressed in all investigated cell types. Additionally, the expression of P-gp was found in A549 and H441 cells.

There is, however, considerable discord about the exact subcellular localisation of drug transporter proteins. One reason for this is the paucity of reliable, commercially available antibodies.

II Studying drug transport processes in an organotypic *in vitro* model is far more complex than in expression systems transfected with only one or two transporters. Due to the fact that multiple organic cation transporters were found to be expressed at the respiratory epithelium, many experiments were carried out to understand translocation processes. The major findings of the functional analyses were that alveolar A549 cell monolayers showed an uptake pattern for organic cations distinctly different from bronchiolar H441, bronchial 16HBE14o- and Calu-3 as well as intestinal Caco-2 monolayers. In A549 cells we found evidence that more than one transporter is involved, possibly explaining the higher uptake rate of organic cations. A conclusion can be drawn that the uptake of organic cations was mediated mainly by OCT1 and OCT2 at the alveolar epithelium. An OCT-driven uptake of organic cations has been shown at the bronchial and bronchiolar epithelium at lower levels with a hypothetical involvement of OCT1. OCTN2 mediates acetylcarnitine uptake in alveolar and bronchial epithelial cells. Although we studied the contribution of transporter subtypes at the alveolar epithelium, it still remains to be confirmed if OCT1 is the major transporter at the bronchial epithelium. Besides, we found high protein abundance of OCT3 in all cell culture models, whereas on a functional level, no evidence was found of any involvement of OCT3. Furthermore, the role of OCTN1 in the translocation of TEA remains unclear. In this work, only a limited set of RNAi studies was carried out in alveolar epithelial cells. Nevertheless, subsequent knockdown studies in bronchial and bronchiolar epithelial cells would be of pivotal importance to define the contribution of the individual transporter proteins to the uptake and efflux of e.g., organic cations. Unfortunately, knockdown studies in cell culture models forming tight junctions are rather demanding in terms of knockdown-efficiency. Due to the fact that

these polarised cell monolayers do not allow high uptake efficiency of the transfection reagent and concentration and experimental settings used need more optimisation steps.

In addition, in A549 and H441 cells, we have shown P-gp and MATE1 expression, both transporter proteins might be involved in secretion of organic cations from the epithelium. In Calu-3 cells, MATE1 is possibly a key transporter. To further investigate this matter, knockdown studies are necessary.

Currently, some characteristics of organic cation uptake at the lung epithelial barrier cannot be fully elucidated, due to the fact that even more membrane transporters might be involved, such as proteins encoded by *SLC5*, *SLC6*, *SLC7*, *SLC29*, *SLC44* and *SLC47* gene families, in particular *SLC47A1* (MATE1) (Salomon and Ehrhardt, 2012). It cannot be ruled out that active processes play an important role in the uptake of organic cations in addition to passive diffusion, in particular at the bronchiolar and bronchial epithelium.

III Whilst routinely used as a surrogate for human pneumocytes *in vitro*, A549 cells lack the ability to form electrically tight monolayers of polarised cells. Therefore, we characterised H441 cells as an *in vitro* model for transport studies of distal lung epithelial barrier. In this respect, this study provides a first overview about drug transporter expression and their functionality in H441 epithelial cells. H441 are the first cell line of human distal lung descent with the ability to form electrically tight monolayers with polarised cells. We confirmed the expression of a number of key drug transporters and P-gp function, as determined by rhodamine 123 transport was shown. It is noteworthy that there were obvious parallels between A549 and H441 cell culture models and human alveolar epithelial primary cells. Hence, H441 cells are a good

candidate as an *in vitro* model for transport studies of distal lung epithelial barrier. To study drug absorption *in vitro* it is essential to be able to choose between appropriate and established cell culture models. Unfortunately, human bronchial epithelial primary cells were not included in this Thesis, hence, it remains unclear which model (Calu-3 or 16HBE14o- cells) reflects most closely *in vivo* characteristics.

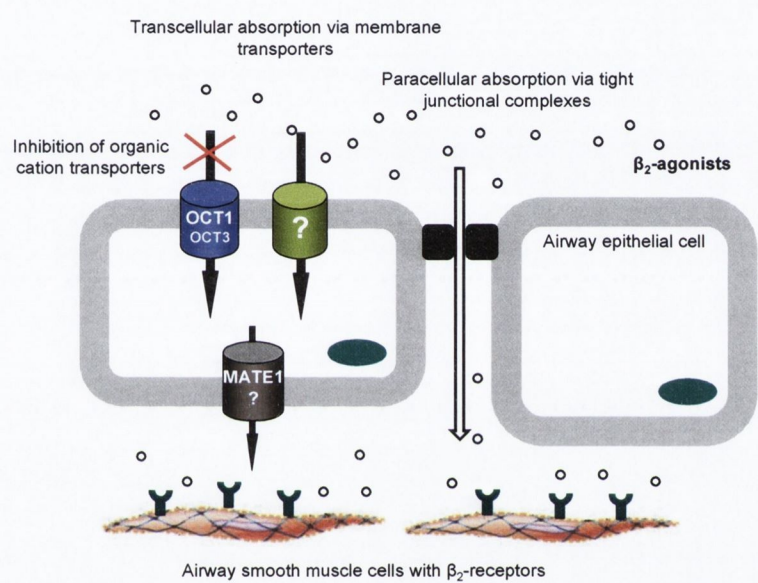


Figure 1. A scheme of possible absorption pathways of β_2 -agonists across the lung epithelial barrier is shown. Both, transcellular and paracellular absorption mechanisms, might be involved. Beta-2 agonists have been shown to remarkably inhibit OCT1, whereas OCT3 was less affected. An active uptake mechanism of salbutamol was found at the alveolar epithelium, but the responsible transport was not yet identified. A possible involvement of MATE1 to the efflux of salbutamol was speculated (it is of note that the localisation of transporters presented here do not reflect the actual cellular localisation found in respiratory cell culture models; the scheme was prepared in order to simplify and depict obtained data).

IV Drug transporters certainly play a role in physiology and drug distribution at the molecular/ cellular level in the lung and their involvement in pulmonary drug

disposition is highly likely. Beta-2 agonists such as salbutamol, formoterol and salmeterol as well as inhaled corticosteroids were able to attenuate the uptake of organic cations, highlighting possible drug-drug interactions at the respiratory epithelial barrier.

For the first time, we provided evidence that polyspecific organic cation transporters in particular, hOCT1, are targets for specific inhibition by β_2 -agonists with IC_{50} values of 22.3 μ M for formoterol and of 47.8 μ M for salmeterol (Figure 1). All three β_2 -receptor agonists competitively inhibit uptake of [14 C]-TEA, but not [3 H]-acetylcarnitine at the alveolar epithelium. The uptake of salbutamol, however, was not mediated by the five cloned organic cation transporters. The identification of this elusive transporter protein also remains to be answered.

V We also found a functional active verapamil transporter in A549 epithelial cells which cannot be attributed to the classical members of the organic cation transporter family. It will be interesting to investigate the responsible mechanism in detail in the future.

VI Besides organic cation translocation, we found evidence for active bile acid uptake using isomeric 3-desoxy 3-NBD-amino derivatives of UDCA and DCA at the intestinal and alveolar epithelium and it can be hypothesised that OATP2B1 is mainly involved in this process. An interesting finding was that the uptake of bile acid analogues was clearly dependent on chemical features such as diastereoselectivity. Also in the case of investigated bile acid analogues, further uptake studies in relevant heterologous expression systems are needed to identify the respective transporter site.

The aim was, nevertheless, to assess the suitability of bile acid analogues to study functional activity in human intestinal and alveolar epithelial cells.

VII P-glycoprotein (P-gp) is a well characterised membrane transporter relevant to drug disposition and multi-drug resistance. It has been suggested that nanoparticles have the ability to accumulate in cells without being recognised by P-glycoprotein. It was the first study comparing pharmacological P-gp inhibitors with nanoparticulate systems where we observed an attenuation of Rh123 release, and hence, P-gp function by nanoparticles, which might further increase the interest in nanoparticulate drug delivery systems, e.g., in the context of anticancer therapy.

Collectively, it is obvious that expression profiling of transporter proteins in the entire lung needs to be completed, before it will be possible to optimise existing *in vitro* and *ex vivo* models. 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 (Shitara et al., 2006; Inui et al., 2000)), which will allow correlation between *in vitro* results and *in vivo* data. Moreover, species differences between current experimental animal models and human patients need to be extensively studied.

To successfully relate, if a single transporter is involved in the absorption of a certain drug at a certain location in the lung, selective inhibitors are needed. However, a general lack of highly specific inhibitory compounds is obvious and urgently needs to be addressed. Particularly, in the case where the transporter sites in question show a

high degree of substrate overlap, and where no specific inhibitors are available, it is difficult to finally define the contribution of the individual transporter proteins. Some of the studies that investigated the role of organic cation transporters on the transport of inhaled drugs used very high concentrations of the compound of interest. This might partly explain contradictory evidence currently found in the literature. It appears therefore crucial to, whenever possible, choose concentrations that are similar to what is expected *in situ*. Another important tool to analyse transporter contribution to drug absorption is the use of RNAi and knock-out techniques. Unfortunately, RNAi studies are only slowly emerging in this field, with their focus mainly on OCT3 (Zhu et al., 2012; Horvath et al., 2011). Taken into account the re-kindled discussion about β_2 -receptor polymorphisms (Cancado et al., 2011), investigating the role of SNPs of transporter proteins might also be of interest for future research. Sakata et al. functionally characterised five SNPs in the OCT3 gene and found that three of these variants were linked to reduced transport activity. This report raises questions about how SNPs contribute to inter-individual variations in pulmonary drug absorption (Sakata et al., 2010).

In summary, it can be said that respiratory drug transporter research has really only started. There are still many unanswered questions regarding the characterisation of membrane drug transporters at the pulmonary epithelium, but with this work I aimed to answer some of them. However, the importance of organic cation (and other) transporters in the lung has finally been recognised and with the available techniques, it shall be possible to close the gap between the lung and other organs quickly.

APPENDICES

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II. LIST OF ABBREVIATIONS

16HBE14o-	Human bronchial epithelial cell line
A549	Human alveolar epithelial adenocarcinoma cell line
A-to-B	Apical-to-basolateral
ABC	ATP-binding cassette
AFC	Alveolar fluid clearance
AIC	Air-interface culture
ANOVA	Analysis of variance
ASP ⁺	4-(4-(dimethylamino)styryl)-N-methylpyridinium
iodide	
ATP	Adenosine triphosphate
ATI	Human alveolar epithelial type-I cell
ATII	Human alveolar epithelial type-II cell
BA	Bile acid
B-to-A	Basolateral-to-apical
BCA assay	Bicinchoninic acid assay
BEAS-2B	Human bronchial epithelial cell line
BPE	Bovine Pituitary Extract
BSA	Bovine serum albumin
BSA-FAF	Bovine serum albumin- Fatty Acid Free
Caco-2	Human colorectal epithelial adenocarcinoma cell
line	
Calu-3	Human bronchial epithelial adenocarcinoma cell line
cAMP	Cyclo-adenosine monophosphate
CA	Cholic acid
CDCA	Chenodesoxycholic acid
CLSM	Confocal Laser Scanning Microscopy
CMC	Critical micelle concentration
COPD	Chronic obstructive pulmonary disease
DLS	Dynamic light scattering
DMEM	Dulbecco`s Modified Eagle`s Medium
DMSO	Dimethyl sulfoxide

e.g.	<i>exempli gratia</i> , for example
ed.,	Editors
ECF	Extracellular Fluid
EDTA	ethylene diamine tetra-acetic acid
Eq.	Equation
et al.	<i>et alii</i> , and others
etc.	<i>et cetera</i> , and other things
FBS	Foetal bovine serum
FNa	Flourescein sodium
H441	human respiratory epithelial adenocarcinoma cell
line	
hAEpC	human alveolar epithelial primary cells
HEK-293	Human Embryonic Kidney 293 cells
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic
acid	
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish peroxidise
i.e.	<i>id est</i> , in other words
ICC	Immunocytochemistry
IFM	Immunofluorescence microscopy
KRB	Krebs-Ringer Buffer
LCC	Liquid covered culture
MDR	Multidrug resistance protein
MEM	Eagle`s Minimum Essential Medium
MPP ⁺	1-Methyl-4-phenylpyridinium iodide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NBD bile acid	a NBD-labeled fluorescent bile acid
NBD-F	4-fluoro-7-nitrobenzofurazan
NEAA	Non-essential amino acids
OATP	Organic anion transporting polypeptide

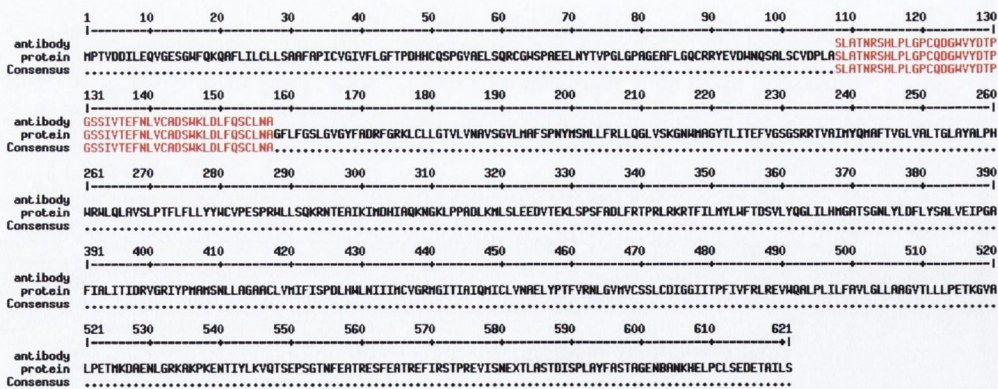
OCT	Organic cation transporter
OCTN	Organic carnitine transporter
OTT	over-the-top
p.	Page
P_{app}	apparent permeability coefficient
PBS	Phosphate-buffered saline
PdI	Polydispersity Index
PG	Prostaglandin
PGT	Prostaglandin-Transporter
P-gp	P-glycoprotein
q-PCR	quantitative polymerase chain reaction
Rh123	Rhodamine 123
RNA	ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SAGM	small airways growth medium
SD	Standard deviation
SEM	Standard error of the mean
SDS-Page	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
shRNA	Small-hairpin RNA or short-hairpin RNA
siRNA	Small-interfering RNA or short-interfering RNA
SLC	Solute carrier protein
SLCO	organic anion transporting polypeptide transporters
SNP	Single-nucleotide polymorphism
TCD	Trinity College Dublin
TEA	Tetraethylammonium
TEER	Transepithelial electrical resistance
TRIS	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethyl rhodamine iso-thiocyanate
vs.	<i>versus</i>
ZO-1	<i>zona occludens</i> -1, tight junction protein

III. PROTEIN SEQUENCING OF UTILISED ANTIBODIES

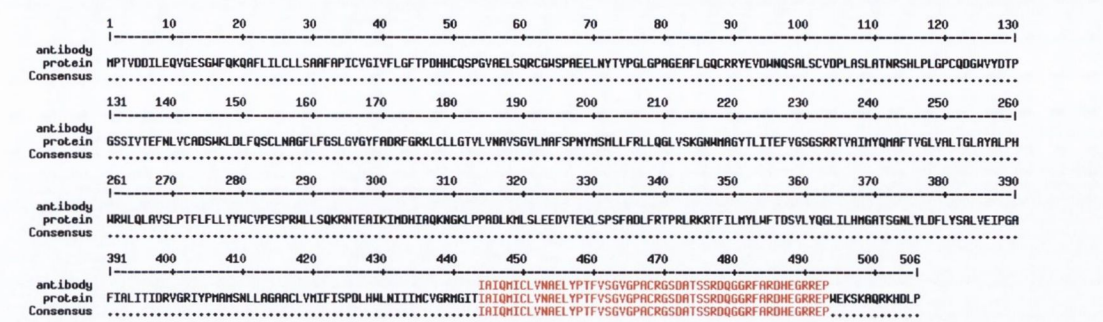
We analysed in how far SLC transporter proteins and the correspondent anti-SLC antibodies shared homology in common domains. In particular, the protein binding sites of antibodies were identified using BLAST® NCBI Data Base by detecting the positions of amino acids that define the length of the protein sequence (information provided by manufacturer). FASTA sequences of the total transporter protein sequence and the detected antibody binding site were aligned using MultAlin software to graphically illustrate the position of the binding site (online available- <http://multalin.toulouse.inra.fr/multalin/>). If needed, UniProtKB software was used first to identify Swissprot Id, then FASTA-sequence of the protein was copied into BLAST (Protein sequencing was only performed for antibodies where information was available from the company).

1. OCT1

1.1. Anti-SLC22A1: AV41516 SIGMA- extracellular loop

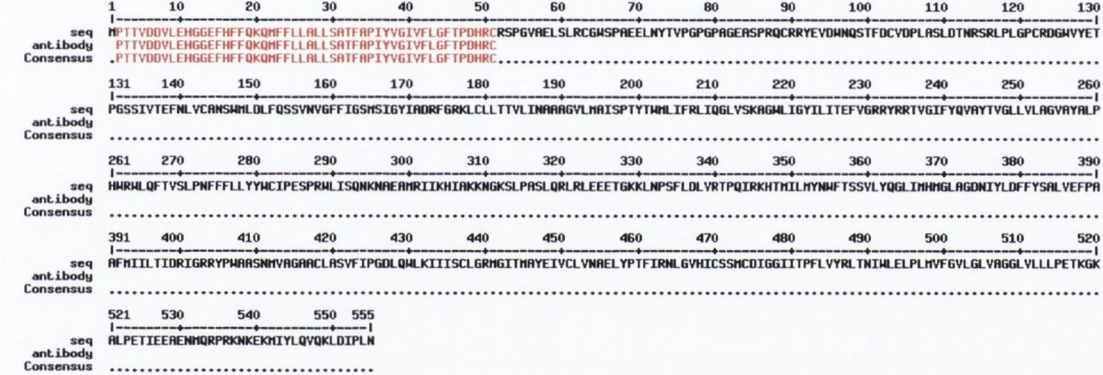


1.2. Anti-SLC22A1: ARP41640_T100 AVIVA-C-terminal region

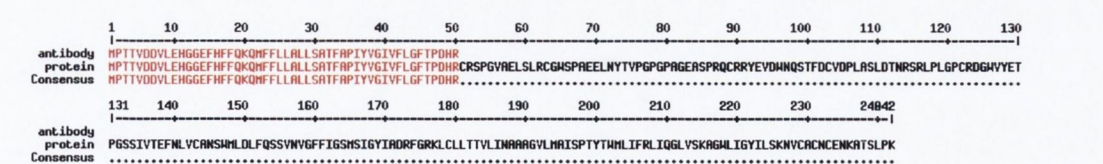


2. OCT2

2.1. Anti-SLC22A2: AV43847 SIGMA- N-terminal region



2.2. Anti-SLC22A2: ARP43847_T100 AVIVA- N-terminal region



3. OCT3

3.1. Anti-SLC22A3: AV44026 SIGMA- N-terminal region

seq	1	10	20	30	40	50	60	70	80	90	100	110	120	130
antibody	NPSFDEALQRYGEGRFQRRVFLLLCLTGVTFAFLFVGYYFLGTQPDHYMCRGPSAAALAEKCGMSPEEEN													
Consensus	NRTAPASRGPEPPERARRGRCQRYLLEANDSASATSALSCADPLAAPPNRSAPLYPCRGG													
seq	131	140	150	160	170	180	190	200	210	220	230	240	250	260
antibody	KRYAQAHSTIYSEFDLYCVNAHMLDLTQAILNLGFLTGAFTLGYADRYGRIVYLLSCLGVGVTGVYVAFAPNFPVYIFRFLQGVFGKGTNHTCYVIVTEIVGSKQRRIVGVIQHFFLGIIILPGI													
Consensus														
seq	261	270	280	290	300	310	320	330	340	350	360	370	380	390
antibody	RYFIPNAQGIQLAITLPSFLLYYVVPESPRMLITRKKGKQALQILAKKACMGKYLSSNYSEITVDEEYNSPSFLDLVRTPQMRKCTLLNFAMFTSAVVYQGLVHRLGIIGGLYIDFFISGVVE													
Consensus														
seq	391	400	410	420	430	440	450	460	470	480	490	500	510	520
antibody	LPGALLILLTIERLGRRLPFASNIYVAGVACLVTAFLEGGIAHLRTTATLGRLGITHAFIEIVYLVNSELYPITLRNFGVSLCSGLCDFGGIAPFLFLRLAARVHLELPLIFGILASICGGLVHLLPET													
Consensus														
seq	521	530	540	550	556									
antibody	KGIALPETVDDVEKLGSFHSCKGRNKKTPVSRSHL													
Consensus														

4. OCTN1

4.1. Anti-SLC22A4: OAAB03385 AVIVA- C-terminus

protein	1	10	20	30	40	50	60	70	80	90	100	110	120	130
antibody	NRDYDEVTAFLGEGPFQRLIFFLLSASIIPNGFNHGSVFLAGTPEHRCRVPDRAHMLSSAHNNHVPPLALRDGREVPHSCRYRLATIANFSALGLEPGRDVLDGLEQESCLDGEFSQDYLSTVTV													
Consensus														
protein	131	140	150	160	170	180	190	200	210	220	230	240	250	260
antibody	EAMLVCEDDAKVPLTISLFFVGVLLGSFVSQQLSDRFGRKNVLFATHAVQTGFSFLQIFSISEHFTVLFVIVGAGQISNYVHFILGTEILGKSVRIIFSTLGVCTFFAVGYHLLPLFAFYTRDWRHLL													
Consensus														
protein	261	270	280	290	300	310	320	330	340	350	360	370	380	390
antibody	LALVPGVLCVPLMHFIPESPRMLISQRFREDEDIIQKAKKNNHIAVPHYIFDSVEELNPLKQKAFILDLFRTNIAININSLHMLTSVGYFALSLDAPNHLGDAYLNCFLSALIEIPAYITAH													
Consensus														
protein	391	400	410	420	430	440	450	460	470	480	490	500	510	520
antibody	LLRLTPRYTIAHVLFGGGVLLFIQLVPVDYFLSIGLVHKGFLITSFASHLYVFTRELYPITLRNHHVGVTSASRVGSIAPYFVYLGAYNHLPIYVNGSLTVLIGILTLFFPESLGHLPETLE													
Consensus														
protein	521	530	540	551										
antibody	QKQKVKHFRSGKKTRDSHEEENPKYLITAF													
Consensus	QKQKVKHFRSGKKTRDSHEEENPKYLITR													

5. OCTN2

5.1. Anti-SLC22A5: OAAB05449 AVIVA- center region

protein	1	10	20	30	40	50	60	70	80	90	100	110	120	130
antibody	NRDYDEVTAFLGEGPFQRLIFFLLSASIIPNGFTGLSSVFLIATPEHRCRVPDRAHMLSSAHNNHVPPLALRDGREVPHSCRYRLATIANFSALGLEPGRDVLDGLEQESCLDGEFSQDYLSTVTV													
Consensus														
protein	131	140	150	160	170	180	190	200	210	220	230	240	250	260
antibody	EAMLVCEDDAKVPLTISLFFVGVLLGSFVSQQLSDRFGRKNVLFYTHAQGTGFSFLQIFSKEHNFVVLVFLVGHGQISNYVHAFVLGTEILGKSVRIIFSTLGVCTFFAVGYHLLPLFAFYTRDWRHLL													
Consensus														
protein	261	270	280	290	300	310	320	330	340	350	360	370	380	390
antibody	VALTHPGVLCVPLMHFIPESPRMLISQRFEEEREVIIRAKKANGIVVPSTIFDPSELQQLSSKKQDSHNILDLRTNIRMYTISHLNHTISVGYFLGLDTPNLHGDIYVNCFLSAHVEYPAYVLA													
Consensus	AKANGIVVPSTIFDPSELQQLSSKKQDSHN													
protein	391	400	410	420	430	440	450	460	470	480	490	500	510	520
antibody	MLLLQYLPRRYSHATRALFLGGSVLLFNQLVPPDLYLATVLVHVGKFGVTAASFHYVYVYTAELYPITVRNHHGVSSASRLGSLSPYFVYLGAYDRFLPYLLHGLTILTRILTLFLPESFGTLPDT													
Consensus														
protein	521	530	540	550	557									
antibody	IDQHLRVKGRKHKTPSHRAHLKDGQERPTILKSTAF													
Consensus														

6. MATE1

6.1. Anti-SLC47A1: OAAB02770 AVIVA- C-terminus

	1	10	20	30	40	50	60	70	80	90	100	110	120	130
protein	HEAPEEPAPYRGGPETLEVRGSRCLRLSNFREELRALLVLAGPAFLVQLMYFLISFISVFCGHLGKLEDAVTLAIRVIMVTGYSYGFGLSSACDTLISQTYGSQLKHVGVILQRSALVLLCCFPC													
antibody														
Consensus														
	131	140	150	160	170	180	190	200	210	220	230	240	250	260
protein	NALFLNTQHILLFRQDPDVSRLTQTYVTIFIPALPATFLYMLQVKYLLNQGIVLPQIVTGVARMLVNALANYLFLHQLHLGVIGSALANLISQYTLALLFLYILGKXKHQATWGGHSLECLQDARSFL													
antibody														
Consensus														
	261	270	280	290	300	310	320	330	340	350	360	370	380	390
protein	RLAIPSMILMCHENHAYEVGSFLSGILGMVELGAQSIYVELRIIYYMYPAGFSYRASVYRGNALGAGDNEQARKSSVSLITVLFAVAFSVLLLSCKDHVGYIFTTORDIINLVRAQVVIYRVVSHLFEA													
antibody														
Consensus														
	391	400	410	420	430	440	450	460	470	480	490	500	510	520
protein	LACTSGVLRGSGNQKYGRAIYNTIGYYVGLPIGIALMFATTLGVNGLHSGIITCTVFQAVCFLGFIIQLNKKKACQQAQVHANLKVNNVPRSGNSALPQDPLHPGCPENLEGILTNDVGKTGEPQSDQQ													
antibody														
Consensus														
	521	530	540	550	560	570								
protein	NRQEEPLPEH													
antibody	NRQEEPLPEH													
Consensus	NRQEEPLPEH.....													

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