Drug accumulation in alveolar macrophages: *In vitro* and *in silico* assessment of the contribution of lysosomal trapping

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Abstract

Alveolar macrophages (AMs) are involved in phagocytosis and clearance of inhaled particles including drugs from the lungs which may result in lack of efficacy of new drug candidates. In addition, drug accumulation in AMs is associated with safety concerns due to formation of foamy AMs which can occur as a consequence of “trapping” of cationic amphiphilic drugs in the lysosomes (e.g., in phospholipidosis). Currently minimal in vitro data exist on accumulation and lysosomal trapping of respiratory drugs in AMs. Ten drugs were selected for the present project, namely clarithromycin, rifampicin, ciprofloxacin, formoterol, fenoterol, terbutaline, budesonide, ipratropium and tiotropium bromide and imipramine (positive control for lysosomal trapping). Rationale for inclusion was that these were respiratory drugs with a wide range of physicochemical properties and therapeutic classes, have a potential for lysosomal accumulation and limited in vitro accumulation data reported.

The aims of this project were to characterise the accumulation of selected drugs in rat AM cell line NR8383 and primary human AMs and investigate the contribution of lysosomal trapping to their cellular accumulation. Total uptake clearance (CL_{uptake}) of drugs was assessed up to 10 minutes at 5 µM drug concentration at 37 and 4°C to delineate active uptake (CL_{active}) and passive diffusion (CL_{diff}) clearances. Cell accumulation was determined from cell-to-medium concentration ratios (K_p) at 37°C. Lysosomal trapping of drugs was evaluated by the reduction in CL_{uptake} and K_p of drugs in the presence of NH_4Cl, monensin and nigericin which abolish intracellular pH gradients. Wide range of CL_{uptake} in both NR8383 (0.04 - 16.7 µL/min/10^6 cells) and human AMs (0.02 - 66.2 µL/min/10^6 cells) was evident; the lowest and highest values were observed for terbutaline and imipramine, respectively. Uptake of most drugs was driven by an active process as the CL_{active}/CL_{diff} ratio ranged between 2 and >150-fold for imipramine and clarithromycin, respectively in NR8383. Similar trends were seen in human AMs where this ratio ranged between 2 and >500-fold for tiotropium bromide and clarithromycin, respectively. Comparison of CL_{uptake} and K_p data revealed a 3-fold bias between NR8383 and human AMs; in general values were higher in NR8383. Among all drugs, imipramine and clarithromycin (LogP >3, pK_a 9-9.5) were shown to significantly accumulate in the lysosomes of both cells; overall >60% reduction in CL_{uptake} and K_p was observed by the chemical agents. Overall, NR8383 was shown to be a useful in vitro model to study accumulation and lysosomal trapping of drugs.

Lysosomal trapping of investigated drugs was further assessed using a published in silico mechanistic cell model which was modified for AMs. Drug physicochemical data (logP, pK_a) and AM specific parameters where available (e.g., lysosomal volume, pH) were used to predict the extent of lysosomal trapping based on the pH differences between external medium, cytosol and lysosome. Cell and lysosomal drug concentrations were predicted, allowing the estimation of K_p for these compartments. The predictive performance of the model was evaluated by comparing the predicted K_{p,cell} with experimental K_{p,NR8383}. An overall 7-fold bias was observed; K_{p,NR8383} was under-predicted by 2.4 to approximately 14-fold for half of the drugs investigated. However, the model could correctly predict lysosomal trapping of basic clarithromycin and imipramine, and lysosomal non-targeting of the majority of basic, zwitterionic, neutral and positively charged drugs. The strengths and the limitations of the in silico model are highlighted, the areas for improvements identified and recommendations made for future applications of this approach to predict subcellular accumulation.


**Declaration**

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Activity</td>
<td>Activity</td>
</tr>
<tr>
<td>ATP-BC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>1-ABT</td>
<td>1-aminobenzotriazole</td>
</tr>
<tr>
<td>Activity coefficient</td>
<td>Activity coefficient</td>
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<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>ATI</td>
<td>Alveolar epithelial type-I cells</td>
</tr>
<tr>
<td>ATII</td>
<td>Alveolar epithelial type-II cells</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Concentration</td>
<td>Concentration</td>
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<td>Caco-2</td>
<td>Human epithelial colorectal adenocarcinoma cells</td>
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<tr>
<td>CAD</td>
<td>Cationic amphiphilic drug</td>
</tr>
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<td>Calu-3</td>
<td>Human bronchial epithelial cell line</td>
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<tr>
<td>CGM</td>
<td>Complete growth medium</td>
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<td>CL active</td>
<td>Active uptake clearance</td>
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<tr>
<td>CL uptake</td>
<td>Total uptake clearance</td>
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<tr>
<td>CL diff</td>
<td>Passive diffusion clearance</td>
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<tr>
<td>cLogP</td>
<td>Calculated log of the octanol-water partition coefficient</td>
</tr>
<tr>
<td>CNT</td>
<td>Concentrative nucleoside transporter</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
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<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DC</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug interaction</td>
</tr>
<tr>
<td>DMSO</td>
<td>Demethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>dx</td>
<td>Membrane thickness</td>
</tr>
<tr>
<td>Em</td>
<td>Membrane electrical potential</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELF</td>
<td>Epithelial lining fluid</td>
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<tr>
<td>F</td>
<td>Faraday constant</td>
</tr>
<tr>
<td>f</td>
<td>Fraction of the drug which can freely diffuse across membranes</td>
</tr>
<tr>
<td>Fa2N-4</td>
<td>Immortalised human hepatocytes</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>f_{u_{cell}}</td>
<td>Fraction unbound in the cell</td>
</tr>
<tr>
<td>f_{u_{med}}</td>
<td>Fraction unbound in the media</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>Gmfe</td>
<td>Geometric mean fold error</td>
</tr>
<tr>
<td>GST</td>
<td>Gluthathione S-transferase</td>
</tr>
<tr>
<td>16HBE14o-</td>
<td>Human bronchial epithelial cell line</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximum inhibitory concentration</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>J</td>
<td>Diffusive flux</td>
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<tr>
<td>J744</td>
<td>Mouse macrophage cell line</td>
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<tr>
<td>K</td>
<td>Partition coefficient</td>
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<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Lipid sorption parameter</td>
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<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;ow&lt;/sub&gt;</td>
<td>Octanol-water partition coefficient</td>
</tr>
<tr>
<td>K&lt;sub&gt;U1&lt;/sub&gt;</td>
<td>Apparent saturable uptake equilibrium constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;p,U1,max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;p&lt;/sub&gt; for saturable uptake</td>
</tr>
<tr>
<td>K&lt;sub&gt;p,max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;p&lt;/sub&gt; for total uptake</td>
</tr>
<tr>
<td>K&lt;sub&gt;p,min&lt;/sub&gt;</td>
<td>K&lt;sub&gt;p&lt;/sub&gt; for nonsaturable uptake</td>
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<td>Cell-to-medium concentration ratio under control conditions</td>
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<tr>
<td>K&lt;sub&gt;p+NH4Cl&lt;/sub&gt;</td>
<td>Cell-to-medium concentration ratio in the presence of NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
</tr>
<tr>
<td>L</td>
<td>Lipid fraction</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>LogD&lt;sub&gt;7.4&lt;/sub&gt;</td>
<td>Log of the octanol-water distribution coefficient at pH7.4</td>
</tr>
<tr>
<td>LogP</td>
<td>Log of the octanol-water partition coefficient</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
</tr>
<tr>
<td>LTR</td>
<td>LysoTracker Red</td>
</tr>
<tr>
<td>m</td>
<td>Mass</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance associated protein</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide reduced</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate reduced</td>
</tr>
<tr>
<td>N</td>
<td>Nernst-Planck equation</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>Ammonium chloride</td>
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<tr>
<td>NR8383</td>
<td>Rat alveolar macrophage cell line</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic anion transporter</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion transporting polypeptide</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>OCTN</td>
<td>Organic cation/carnitine transporter</td>
</tr>
<tr>
<td>P</td>
<td>Permeability</td>
</tr>
<tr>
<td>P&lt;sub&gt;app&lt;/sub&gt;</td>
<td>Apparent permeability</td>
</tr>
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<td>PEPT</td>
<td>Peptide transporter</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
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<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
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<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Log of the acid dissociation constant</td>
</tr>
<tr>
<td>PSA</td>
<td>Polar surface area</td>
</tr>
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<td>P450</td>
<td>Cytochrome P450</td>
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<tr>
<td>R</td>
<td>Universal gas constant</td>
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<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SA</td>
<td>Surface area</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>SLCO</td>
<td>Solute carrier organic anion transporter</td>
</tr>
<tr>
<td>$S_{\text{med}}$</td>
<td>Nominal substrate concentration in the media</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half-life</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine glucuronosyltransferase</td>
</tr>
<tr>
<td>$V_{d,ss}$</td>
<td>Volume of distribution at steady-state</td>
</tr>
<tr>
<td>$v$</td>
<td>Uptake rate</td>
</tr>
<tr>
<td>$V$</td>
<td>Volume</td>
</tr>
<tr>
<td>$V$-$H^+$-ATPase</td>
<td>Vacuolar-type proton adenosine 5'-triphosphatase</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximum uptake rate</td>
</tr>
<tr>
<td>$W$</td>
<td>Water fraction</td>
</tr>
<tr>
<td>$z$</td>
<td>Electric charge</td>
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</table>

The subscript “N” refers to neutral drug species
The subscript “D” refers to dissociated (ionised) drug species
The subscript “f” refers to free drug species
The subscript “s” refers to sorbed drug species
The subscript “lys” refers to lysosome
The subscript “mit” refers to mitochondria
The subscript “cys” refers to cytosol
The subscript “cell” refers to total cell
The subscript “medium” refers to total medium
The subscript “T” refers to total
The subscript “h,AM” refers to human alveolar macrophages
The subscript “NR8383” refers to NR8383 cells
Acknowledgements

Firstly, I would like to thank my supervisors Dr. Alex Galetin and Prof. Brian Houston for their invaluable support and guidance throughout the past four years. Thank you for giving me with the opportunity to complete my PhD within the Centre for Applied Pharmacokinetic Research (CAPkR).

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The Author

Ayşe Ufuk

In 2006, the author graduated from Ege University with a BSc degree in Biochemistry, majored in Biotechnology with first class honours. Following this, the author worked as a Biomedical Scientist for a year in a private clinical laboratory in Cyprus. Later on, the author continued with a postgraduate education and obtained a MSc degree in Clinical Biochemistry with Molecular Biology from the University of Surrey in 2008. Following graduation, the author did an internship at GlaxoSmithKline, Stevenage for 6 months where she was involved in pre-clinical formulation development activities within the Particle Sciences group. In September 2010, the author joined the Centre for Applied Pharmacokinetics Research at the University of Manchester to begin her PhD under the supervision of Prof. Brian Houston and Dr. Aleksandra Galetin. This PhD was awarded under the industrial CASE student award scheme in collaboration with the Biotechnology and Biological Sciences Research Council (BBSRC) and GlaxoSmithKline. The PhD research was completed in September 2014.
Chapter 1 Introduction

Inhalation is the major route of administration for drugs used to treat respiratory diseases while it also represents an excellent alternative to other therapeutic routes for systemically acting drugs (Taylor, 1990; Page et al., 2006; Leach, 2007; Alexander, 2009). This route of administration allows relatively small doses of drug being delivered directly to the airways to achieve high tissue concentration while it minimises undesirable systemic effects (Lipworth, 1996). The absorption of small lipophilic inhaled molecules from the lung into the systemic circulation is very rapid due to the large surface area of the lung, good permeability of pulmonary epithelia and the highly dispersed characteristic of aerosol particles. Moreover, relatively low levels of efflux transporters and metabolic enzymes present in the lung when compared to the gut and the liver also contribute to this rapid absorption (Patton et al., 2004; Patton and Byron, 2007). However, despite the recent advances in formulation and design of inhalation devices, there are still significant challenges facing the development of new inhaled medicines. These challenges include further understanding of pulmonary drug disposition, lack of efficacy and efficient delivery of the drug to its target (Forbes et al., 2011).

Lungs are complex organs with many different cell types contributing to their function. One of them are alveolar macrophages (AMs) which are responsible for removing undissolved inhaled particles and microorganisms from the lungs as part of their roles in lung protection. Therefore, they are considered as barriers to both drug-target interactions in the airways and drug absorption into systemic circulation (Lombray et al., 2004). Since alveolar macrophages are highly active in responding to foreign particulates introduced into the lungs, the environmental factors (e.g., smoking) and inflammatory diseases (e.g., asthma, chronic obstructive pulmonary disease (COPD)) may modify activity of AMs in drug clearance from the lungs, potentially impacting efficacy of inhaled drugs. Furthermore, efforts to develop inhaled drugs with low aqueous solubility to avoid systemic absorption and prolong residence in the airways can lead to increased accumulation of poorly dissolved drugs by AMs. As a consequence of this process, formation of foamy AMs has been observed in rat toxicity studies, the occurrence of which may significantly contribute to the attrition of inhaled drugs in the development process (Jones and Neef, 2012; Lewis et al., 2014).
Consequently, these issues highlight the need for readily available \textit{in vitro} systems for 
1) better understanding of respiratory drug accumulation (mechanisms and kinetics of 
accumulation and intracellular distribution) in AMs and 2) early screening of 
ocurrence of foamy macrophages to determine which compounds may have adverse 
effects (e.g., phospholipidosis) and avoid further progression of molecules with a 
limited chance in development (Forbes et al., 2011).

1.1 Lung structure and function

The primary function of the lungs is gas exchange, which consists of oxygenation of the 
blood and removal of CO$_2$ from the body. The lungs also play an important role as a 
primary barrier site against several pathogens, toxic gaseous agents and small 
 xenobiotic compounds (Cloutier and Thrall, 2010). The air is distributed into and out of 
the lungs by upper airways, which comprises nasal cavity and/or oral cavity, pharynx 
and larynx, and lower airways which includes all air passageways starting with trachea 
and ending with the smallest respiratory unit called alveoli (Germann and Stanfield, 
2002; Cloutier and Thrall, 2010).

1.1.1 Physiology of airway epithelium and endothelium

The respiratory tract can be functionally divided into two regions: conducting and 
respiratory airways (Germann and Stanfield 2002; Sporty et al., 2008). The conducting 
airways which provide a passageway through which air can enter and exit the 
respiratory airways, form the upper part of the respiratory tract and consist of nose, 
pharynx, larynx, trachea, bronchia and non-respiratory bronchioles (Germann and 
Stanfield, 2002; Sporty et al., 2008; Cloutier and Thrall, 2010). Conducting airways also 
function as a region to filter, warm and humidify the inhaled air (Sporty et al., 2008). 
The epithelium lining the conducting airways is columnar or cuboidal and is composed 
of several cell types including basal, goblet, ciliated, brush, serous, Clara and 
neuroendocrine cells (Bur and Lehr, 2008; Sporty et al., 2008). 
The respiratory airways, where the actual gas exchange occurs, consist of respiratory 
bronchioles, alveolar ducts and alveolar sacs (Germann and Stanfield, 2002; Cloutier 
and Thrall, 2010). The surface area of these airways is large and together with a very 
thin membrane, allows fast diffusion of gases and substances (Germann and Stanfield, 
2002). In the alveolar region, three cell types are present, namely, epithelial type-I
(ATI) and type-II (ATII) cells and non-epithelial AMs (Patton and Byron, 2007). The squamous ATI cells, primary site for gas exchange, occupy nearly 96 to 98% of the alveolar surface area and are about 0.26 μm in thickness (Bur and Lehr, 2008; Cloutier and Thrall, 2010). The much smaller cuboidal ATII cells, which synthesize pulmonary surfactant, occupy approximately 2 to 4% of the surface area (Cloutier and Thrall, 2010). These cells are the progenitor of ATI cells and differentiate into ATI cells after epithelial barrier injury (Groneberg et al., 2003; Patton and Byron, 2007). With 140 m² surface area, the alveolar epithelium lies on one side of the air-blood gas exchange barrier, while the other side of the barrier is occupied by the capillary endothelium with comparable surface area (Groneberg et al., 2003; Bur and Lehr, 2008). Capillary endothelium occupies about 40% of the total lung cellular composition. Such an extensive surface area and air-blood barrier thickness of only 0.1-0.5 μm allow rapid gas exchange by passive diffusion (Bur and Lehr, 2008). The third cell type in alveoli is AMs and these cells will be discussed in Section 1.2.2.3.

### 1.1.2 Pulmonary circulation

The lung has two separate blood supplies which are pulmonary and bronchial circulation. The pulmonary circulation comprises a capillary bed, which is the largest vascular bed in the body with a surface area of 70-80 m², receives 98-99% of the cardiac output. Pulmonary arteries, shown in Figure 1-1, bring deoxygenated blood from the right ventricle to the respiratory unit for gas exchange and pulmonary veins return the oxygenated blood to the left atrium for distribution to the rest of the body (Cloutier and Thrall, 2010).

The bronchial circulation arises from the aorta and nourishes the lung parenchyma. The bronchial arteries, which receive about 1-2% of the cardiac output, provide oxygenated blood to the lungs (from the trachea to the terminal bronchioles), nourish the walls of the bronchi, bronchioles, blood vessels and nerves, and perfuse the lymph nodes and most of the visceral pleura. Bronchial veins return a third of the blood to the right atrium and the rest to the left atrium via pulmonary veins (Cloutier and Thrall, 2010).
1.2 Drug absorption and distribution in the lung

1.2.1 Factors affecting drug absorption

Factors that affect particle deposition are considered to affect pulmonary drug absorption in general. The deposition of inhaled particles in the airways depends upon the particle characteristics (e.g., size, shape, density, electrical charge, hygroscopy), mode of inhalation (e.g., breathing pattern, flow rate, inspired volume, breath-holding time), particle source (e.g., solution, powder or suspension), morphology of the respiratory tract and the distribution of inspired aerosols in the lungs (Heyder et al., 1986; Martonen and Katz, 1993; Ariyananda et al., 1996).

Factors which affect inhaled drug absorption from the lungs include i) physiological factors such as air-blood barrier, morphology or geometry of airways, airway humidity, breath pattern and the presence of metabolic enzymes in the lung; ii) pathophysiological conditions of the respiratory system; iii) lung clearance mechanisms such as mucociliary clearance and pulmonary endocytosis; iv) the mechanism of absorption, i.e., whether it occurs by passive diffusion or via carrier-mediated transport; v) the drug physicochemical properties including molecular weight, partition coefficient, aqueous solubility, pH and osmolarity; and vi) physical characteristics of inhaled particles (e.g., size, shape) and their site of deposition (Schanker, 1978; Taylor, 1990; Gupta et al., 2010).
1.2.2 Absorption barriers within the lung

The lung consists of a set of barriers which play a role in host defence, but at the same time pose a hurdle for the delivery of drugs to their target sites within the lung. Therefore, inhaled drugs must first overcome these barriers, in order to be deposited and retained efficiently, as well absorbed from the respiratory tract. Among these pulmonary barriers, mucociliary clearance, alveolar macrophages and metabolism by drug metabolising enzymes act as non-absorptive clearance mechanisms (Tronde et al., 2008a).

1.2.2.1 Metabolism

Cytochrome P450 enzymes perform the oxidative metabolism of the majority of drugs and the endogenous compounds. The identification of the human lung CYP isoforms has been difficult due to a number of reasons, such as limited availability of suitable lung tissue and factors affecting enzyme stability (e.g., post-mortem handling of tissues and delay in freezing the tissues) (Kim et al., 2004). Another reason is that the level of P450 expression in human lung is relatively very low, compared to the liver and rodent lungs (Bend et al., 1985; Kim et al., 2004; Patton et al., 2004; Ehrhardt et al., 2008a). For instance, the most abundant liver enzyme CYP3A4 is expressed to a lower degree in the lung tissue and not in all tested individuals, whereas CYP3A5 is considered more important in the lungs (Olsson et al., 2011). As a consequence of this low metabolic activity and relatively rapid absorption, many small molecules have near complete pulmonary bioavailability (Olsson et al., 2011).

Several P450 enzymes and a number of conjugative enzymes have been detected at mRNA level in human lung tissue (Zeldin et al., 1996; Mace et al., 1998; Rylander et al., 2001; Wei et al., 2001; Nishimura et al., 2003; Kim et al., 2004; Castell et al., 2005; He et al., 2005; Berg et al., 2014). The location of most human lung P450s and their role in the disposition or efficacy of therapeutic drugs is still not well known (Ding and Kaminsky, 2003; Somers et al., 2007). However, it has been stated that they are expressed in bronchial and bronchiolar epithelium, Clara cells, ATII and AMs (Castell et al., 2005). For instance, immunohistochemical analysis of human lung tissue revealed localisation of CYP1A1 and CYP1B1 in AMs, ATI and ATII cells, Clara cells and ciliated columnar epithelial cells (Kim et al., 2004). Furthermore, CYP2J2 has been localised in AMs, airway epithelial cells (from trachea to bronchioles), bronchial and vascular smooth muscle cells and vascular endothelial cells (Zeldin et al., 1996). In the
case of phase II enzymes, sulfotransferase 2B1 (SULT2B1) has been localised (mRNA and protein) in human bronchial epithelial cells (He et al., 2005). Furthermore, the mRNA level and the activity of a number of UGTs have been detected in human lung microsomes and the tissue, respectively and they were much lower than the levels in the liver (Ren et al., 2000). In addition, the presence of several other phase II enzymes (mRNA level) has been identified in human lung parenchymal cells (Somers et al., 2007). The summary of phase I and II metabolic enzymes expressed in human lung parenchyma cells and AMs is given in (Table 1-1).

In agreement with previous studies, the expression and the activity of most phase I and phase II enzymes was significantly lower in lung parenchyma cells than in hepatocytes (Somers et al., 2007). The activity of CYP enzymes in parenchyma cells were between 1 and 10% of that in the hepatocytes, epoxide hydrolase and esterases were ~20%, and for some SULT enzymes, the activities were either similar or substantially higher in the parenchyma cells (Somers et al., 2007). In alveolar macrophages, a number of CYP2Cs, CYP2J2 and CYP3A5 have been found to be expressed while CYP3A4 was not detected. Contradictory data on the expression of CYP1A1, CYP1B1 and CYP2E1 have also been reported (Willey et al., 1996; Hukkanen et al., 1997; Piipari et al., 2000; Hukkanen et al., 2001). In addition, a number of SULT and GST enzymes were shown to be expressed in human AMs whereas UGTs were not detected.

The studies of both phase I and II metabolism in human lung and particularly in specific cell types within the lung such as AMs is important, as these enzymes are involved in the metabolism of both carcinogens (Shimada, 2006) and inhaled therapeutics (Somers et al., 2007) such as salbutamol, beclamethasone dipropionate, budesonide and formoterol (Jönsson et al., 1995; Pacifici et al., 1996; Pacifici et al., 1997; Tunek et al., 1997; Daley-Yates et al., 2001; Meloche et al., 2002; Zhang et al., 2002). The metabolism of these inhaled drugs has been demonstrated mainly in vivo or by in vitro studies using human lung and/or liver microsomes and cytosol. However, metabolism of inhaled drugs in AMs has not been demonstrated so far.

### 1.2.2.2 Surfactant and Epithelial lining fluid

The epithelial lining fluid (ELF) at the bronhioalveolar region is covered with a monolayer of lung surfactant which reduces the surface tension in the lung, thereby preventing the bronchioles and alveoli from collapsing, and helping them expand with inspiration of air (Dobbs, 1989; Ehrhardt et al., 2008b).
Table 1-1: Phase I and II metabolic enzyme expression in the human lung parenchyma cells and alveolar macrophages.

<table>
<thead>
<tr>
<th>Lung parenchyma cells</th>
<th>Alveolar macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,4,5,6-mRNA; 1,3,7-protein</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Low</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Low</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>ND</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>High</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Low</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>ND</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Low</td>
</tr>
<tr>
<td>CYP2C18</td>
<td>ND</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>High</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>High</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>ND</td>
</tr>
<tr>
<td>CYP2J2</td>
<td>High</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Low</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>High</td>
</tr>
<tr>
<td>CYP3A7</td>
<td>ND</td>
</tr>
<tr>
<td>NADPH oxidoreductase</td>
<td>ND</td>
</tr>
<tr>
<td>CES1</td>
<td>High</td>
</tr>
<tr>
<td>EPXH1</td>
<td>High</td>
</tr>
<tr>
<td>EPXH2</td>
<td>Low</td>
</tr>
<tr>
<td>SULT1A1</td>
<td>High</td>
</tr>
<tr>
<td>SULT1A2</td>
<td>High</td>
</tr>
<tr>
<td>SULT1A3/4</td>
<td>High</td>
</tr>
<tr>
<td>SULT1C2</td>
<td>High</td>
</tr>
<tr>
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<td>Low</td>
</tr>
<tr>
<td>SULT2B1/2</td>
<td>High</td>
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<tr>
<td>UGT1A1</td>
<td>Low</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Low</td>
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<tr>
<td>UGT1A6-1</td>
<td>High</td>
</tr>
<tr>
<td>UGT1A6-2</td>
<td>High</td>
</tr>
<tr>
<td>UGT2A1</td>
<td>Low</td>
</tr>
<tr>
<td>UGT2B4</td>
<td>High</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Low</td>
</tr>
<tr>
<td>UGT2B11</td>
<td>Low</td>
</tr>
<tr>
<td>GST 12</td>
<td>ND</td>
</tr>
<tr>
<td>GST mu(µ)</td>
<td>ND</td>
</tr>
</tbody>
</table>

+/- indicates the presence and absence of expression; ND: not determined

References: 1 Botto et al., 1994; 2 Willey et al., 1996 (NADPH, nicotinamide adenine dinucleotide phosphate; GST, gluthathione S-transferase; m, microsomal); 3 Zeldin et al., 1996; 4 Hukkanen et al., 1997; 5 Koskela et al., 1999; 6 Piipari et al., 2000; 7 Hukkanen et al., 2001; 8 Kim et al., 2004; 9 Somers et al., 2007 (expression was indicated as either Low: <100 gene copy per 100 ng of total RNA or High: >100 gene copy per 100 ng of total RNA; CES-Carboxylesterase, EPXH-epoxide hydrolase; SULT-sulfotransferase; UGT-uridine glucuronosyl transferase).
The lung surfactant consists of 80-90% lipids and approximately 10% surfactant-associated proteins (Ehrhardt et al., 2008b). Phospholipids represent predominant lipid component, of which 60-70% is phosphatidylcholine, 5-10% is phosphatidylglycerol and phosphatidylethanolamine and 3-6% is phosphatidylinositol and phosphatidylserine mixture. Daniel et al. (1995) suggested that lung surfactant may bind to cationic amphiphilic drugs (CADs), and hence contribute to their uptake by the lung tissue. In addition, the lung surfactant may cause large molecules to aggregate which could enhance their digestion by AMs, thereby compromising their dissolution into the surface liquid and subsequent absorption (Patton, 1996).

Epithelial lining fluid is a complex mixture of solutes and anti-inflammatory cells which lines the surface of terminal bronchioles and alveoli (Honeybourne and Baldwin, 1992). The schematic illustration of lung epithelia with ELF and their relative thicknesses is shown in Figure 1-2. The thickness of the ELF and mucus together in the airways will determine the rate of drug entry into the tissue. In addition, drug physicochemical properties will also affect its ability to penetrate through the mucus barrier (Bur and Lehr, 2008). Moreover, pH, osmolality, ions, proteins, lipids and other constituents of the ELF may play a role in drug absorption (Patton, 1996).

**Figure 1-2:** Schematic view of the epithelial cell size and surface fluid thickness in bronchial and alveolar region. In the conducting airways, the layer involving ciliated epithelium, ELF and mucus are collectively known as mucociliary clearance mechanism. The lining fluid in alveolar region contains no mucus and is not moved by cilia (Patton, 1996).
1.2.2.3 Alveolar macrophages

Alveolar macrophages are derived from circulating blood monocytes and represent the first line of defence against inhaled particles and pathogens (Dobbs, 1989; Bur and Lehr, 2008; Cloutier and Thrall, 2010). They mainly reside within the layer of alveolar surfactant and in the epithelial lining fluid of the alveoli but are also found, although less frequently, in the terminal airways and interstitial space (Sköld et al., 1993; Lohmann-Matthes et al., 1994; Cloutier and Thrall, 2010). The main function of these cells is to phagocytise inhaled foreign particles and pathogens deposited in the alveolar region. The AMs, together with phagocytosed particles, are either cleared by the lymphatic system or transported to ciliated regions of the airways where they are then cleared via the mucociliary clearance mechanism (Labiris and Dolovich, 2003; Patton and Byron, 2007; Cloutier and Thrall, 2010). Drugs which are deposited in the alveolar region and not cleared by AMs are absorbed into the pulmonary circulation (Labiris and Dolovich, 2003). Apart from their phagocytic function, AMs are also involved in the secretion of cytokines (e.g., interleukin-1α, tumour necrosis factor-α) to initiate inflammation, interactions with other cells of the immune system, secretion of enzymes which can degrade connective tissue and metabolism of surfactant (Reasor, 1981; Nicod, 2005). In addition, AMs can alter the alveolar absorption of deposited drug particles greatly (Bur and Lehr, 2008). They are considered as barriers not only for the interaction of inhaled small molecules with targets residing in the airways, but also for the absorption of larger macromolecules such as proteins. Lombry et al. (2004) demonstrated that the transport of human chorionic gonadotropin (hCG) (40 kDa) and immunoglobulin G (150 kDa) from the lung to the bloodstream was significantly hindered by AMs and the bioavailability of both proteins was increased by several fold in rats by depletion of AMs by liposome encapsulated dichloromethylene diphosphate. A substantial increase in both serum hCG and IgG was observed following AM depletion from the lungs. Furthermore, the authors suggested that the uptake of these soluble proteins by AMs occurred by adsorptive and/or fluid-phase endocytosis (i.e., pinocytosis) rather than receptor-mediated endocytosis (AMs have no receptors for hCG).
1.2.2.4 Epithelium
The epithelium, which is usually a single cell monolayer, is an important barrier to drug absorption. The cells of the epithelium vary greatly between the airway and the alveolar epithelium (Patton, 1996) and are covered in detail in Section 1.1.1. At the apicolateral sites of epithelial cells, tight junctions are located. These tight junctions regulate the transfer of water, ionic and neutral molecules and inflammatory cells paracellularly and contribute to the maintenance of cell homeostasis and polarity (Godfrey, 1997). Tight junctions can be considered as a barrier to drug absorption, as they restrict the passage of macromolecules (>40 kDa) through the paracellular pathway (Patton, 1996; Godfrey, 1997).

1.2.2.5 Interstitium and basement membrane
The basement membrane attaches the epithelial and endothelial cell layers in the lung, modulating the movement of fluid, molecules, particles and cells from the alveolar space and blood into the interstitium (Patton, 1996). Alveolar spaces are divided by interstitium which plays an important role in separating and binding together the specific cell layer in the tissue (Bur and Lehr, 2008; Cloutier and Thrall, 2010). They are both barriers against the absorption of drugs into systemic circulation.

1.2.2.6 Endothelium and lymphatics
Following the passage of molecules through epithelium and interstitium, the final barrier to systemic absorption is pulmonary vascular endothelium (Patton, 1996). It regulates the entry of vasoactive substances into the systemic circulation and is the major site for bradykinin and angiotensin I metabolism (Johnson, 1980; Ryan, 1984). The pulmonary lymphatic system removes fluid and protein from the lung interstitium, and prevents fluid being accumulated in the lung. One distinct feature of lymphatic capillaries is that they do not have tight junctions between them, in contrast to capillary of endothelial cells and lymphatic endothelial cells (Cloutier and Thrall, 2010).

1.2.3 Drug transport mechanisms across the lung barrier
1.2.3.1 Passive diffusion
The most important mechanism for the transmembrane movement of most drugs is passive diffusion in which the drugs follow the concentration gradient from high to low concentration either through the trancellular or paracellular pathway (Cohn, 1972).
Lipophilic compounds are generally transported through membranes by passive diffusion (Effros and Mason, 1983), while the transport of hydrophilic solutes appears to occur predominantly by diffusion through intercellular junction pores (Patton, 1996). Uptake of CAD imipramine in isolated perfused rabbit lung has previously been suggested to occur by both passive diffusion and active transport (Orton et al., 1973). Further studies have shown that at steady-state, accumulation of imipramine and a number of other basic lipophilic amines in the rabbit lung tissue consisted of both saturable and non-saturable components (Anderson et al., 1974). Passive diffusion has also been suggested to be involved in uptake of other CADs chlorphentermine and amiodarone in rat AMs in vitro (Heyneman and Reasor, 1986; Antonini and Reasor, 1991) and rifampicin in rabbit AMs (Johnson et al., 1980).

1.2.3.2 Carrier-mediated transport in the lungs

Carrier-mediated transport involves binding of a drug to a membrane carrier in a non-covalent manner and this process facilitates movement of the drug from one site of the membrane to another. One and most important characteristics of membrane transport is that it is an active process, as the energy is required for the movement of drug molecules against a concentration gradient (Cohn, 1972). Facilitated diffusion, another type of carrier-mediated transport, has not been reported to play a role in the transmembrane movement of drugs in the lung. The exception is the work by Heyneman and Reasor (1986), who suggested the involvement of facilitated diffusion in the uptake of chlorphentermine by rat AMs. The transport of hydrophilic molecules through the airway epithelium occurs either paracellularly (i.e., via tight junctions) or via active transport (Summers, 1991). In active transport, the uptake rate is saturable, i.e., the fraction of the dose absorbed decreases with increasing drug concentration. Additionally, the uptake of one drug molecule can be inhibited by another drug molecule which share common structural features and bind to the same specific carrier on the membrane (Patton et al., 2004).

Drug transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRPs), the organic cation transporter (OCT) and organic carnitine/cation transporter (OCTN), peptide transporter (PEPT), organic anion transporter (OAT) and organic anion transporting polypeptide (OATP) have all been identified in the lung. The list of drug uptake and efflux
transporter proteins expressed in human lung, their expression level, cellular distribution and localisation are summarised in Table 1-2 and Table 1-3, respectively.

1.2.3.2.1 Uptake transporters
OCTs (OCT1, OCT2, OCT3) and OCTNs (OCTN1, OCTN2) are both members of the solute carrier family 22. They all translocate a range of endogenous and exogenous molecules in a bidirectional manner. The OCTs translocate a range of organic cations and some neutral molecules in a Na\(^+\) independent manner (Gumbleton et al., 2011). In addition to the expression of OCT proteins (Table 1-2), the presence of mRNA transcripts of OCTs have been shown in human lung tissue by reverse transcription-polymerase chain reaction (RT-PCR) and gene microarray analyses (Lips et al., 2005; Bleasby et al., 2006). It has been indicated that large number of compounds can inhibit OCTs, yet many are not considered as substrates for these transporters (Gumbleton et al., 2011). For instance, the uptake of organic cation tetaethylammonium (TEA\(^+\)) by human OCT1 and OCT2 expressed in oocytes has been shown to be inhibited by budesonide (Lips et al., 2005). Budesonide has also been shown to inhibit cationic formoterol uptake in human bronchial smooth muscle cells expressing OCT3 (Horvath et al., 2007a). Consequently, formoterol has been suggested to be a substrate for OCT3 (Horvath et al., 2007a). Furthermore, both ipratropium and tiotropium bromide have been indicated to be substrates for both human and rat OCT1 and OCT2 based on the studies in human embryonic kidney 293 (HEK293) cells expressing these transporters (Nakanishi et al., 2011).

OCTN1 and OCTN2 can translocate zwitterionic carnitine (in Na\(^+\) and pH dependent manner) and other organic cations. The substrates for OCTNs show an overlapping specificity with those for OCTs, although relatively fewer substrates have been determined for OCTNs (Gumbleton et al., 2011). Both OCTN1 and OCTN2 have been reported to be involved in the transport of inhaled cationic formoterol, ipratropium and tiotropium bromide (Horvath et al., 2007b; Nakamura et al., 2010). More recently, low mRNA expression of OCTNs and OCT1 have been reported in granulo-macrophagic colony-stimulating factor-derived human macrophages (in vitro from human blood monocytes) (Moreau et al., 2011).

Peptide transporters PEPT1 and PEPT2 are members of the SLC15 family and transport di- and tripeptides, as well as various peptide-like drugs (Bosquillon, 2010; Gumbleton et al., 2011). They have broad substrate specificity and have the capacity to translocate
several non-peptidic drugs such as β-lactam antibiotics, angiotensin-converting enzymes inhibitors, anticancer and antiviral drugs (Yang et al., 1999; Sondergaard et al., 2008). While PEP1 is expressed mainly in epithelial cells of small intestine, renal tubules and bile duct, PEPT2 is expressed in a wider range of tissues (Bleasby et al., 2006). Their expression in tracheal and bronchial epithelial cells (Table 1-2) has been suggested to play a role in the transport of pulmonary peptides (Groneberg et al., 2002).

OATs belong to SLC22A family and are mainly responsible for the excretion and reabsorption of organic anions in the kidneys (Anzai et al., 2006). Among OATs, only OAT2 has been reported to be highly expressed in human lung by gene microarray analysis (Bleasby et al., 2006). However, none of the OAT transcripts have been detected in human lung (Bosquillon, 2010; Gumbleton et al., 2011).

Finally, OATPs are classified within the family of SLC21/SLCO as members of the solute carrier organic anion transporters (Hagenbuch and Gui 2008). There are eleven human OATPs among which some are tissue specific while others are expressed ubiquitously (Bleasby et al., 2006; Bosquillon 2010). RT-PCR and gene microarray analyses of human lung tissue indicated strong expression of OATP2A1, OATP3A1, OATP4A1 and OATP2B1 while no expression of liver specific OATP1B1 and OATP1B3 and other OATPs have been reported (Tamai et al., 2000; Bleasby et al., 2006). The cellular distribution and location of OATPs in the lung have not yet been investigated (Bosquillon 2010). More recently, Moreau et al. (2011) have reported high mRNA expression of OATP2B1 and intermediate expression of OATP3A1 and OATP4A1 in granulo-macrophagic colony-stimulating factor-derived human macrophages.

In addition to OCT1, OCTNs and OATPs mRNA data in human macrophages, high expression of a number of other transporters have been reported. These include concentrative nucleoside transporter 3 (CNT3) (substrates: antiviral zidovudine, didanosine), equilibrative nucleoside transporter 3 (same substrates for CNT3), monocarboxylate transporter 1 and 4 (substrates: β-lactam antibiotics, statins) and peptide histidine transporter 1 and 2 (substrates: histidine, di-and tri-peptides) (Moreau et al., 2011).
1.2.3.2  **Efflux transporters**

P-gp is a member of ATP-binding cassette (ABC) membrane transporters which act as ATP-driven drug-efflux pumps. P-gp is mainly expressed in the apical membrane of the enterocytes, hepatocytes, proximal renal tubules and at the blood-brain barrier (Giacomini et al., 2010). The level of P-gp expression in human lung is lower than in these respective organs (Bosquillon, 2010). Although its role in limiting oral drug absorption, preventing their entry into central nervous system and in drug-drug interactions is well established, the precise function of P-gp in the lung is not well known (van der Deen et al., 2005; Bosquillon, 2010). It has been suggested that P-gp may play a role in cellular defence in airways and in controlling mucus layer composition (Lechapt-Zalcman et al., 1997). In addition, it has been suggested that P-gp expressed in ATI epithelium can pump inhaled drugs and environmental toxicants from the cell back into the alveolar space, preventing its entry into the systemic circulation (Campbell et al., 2003). Gumbleton et al. (2011) have emphasized the role of P-gp in limiting systemic absorption of P-gp substrates which show low passive permeability through lung epithelium. The authors have also indicated that, although the rate of absorption of molecules will be influenced by P-gp efflux, the overall extent of absorption is not likely to be influenced due to low P-gp expression in the lungs combined with large surface area and good permeability characteristics of lung epithelium (Gumbleton et al., 2011). Budesonide and prednisone are known substrates for P-gp and interaction of P-gp with a number of other corticosteroids such as beclomethasone dipropionate and ciclesonide has also been demonstrated (Dilger et al., 2004; Cooray et al., 2006).

MRPs are another group of proteins belonging to the ABC superfamily of membrane transporters and function as organic anion efflux pumps (Bosquillon, 2010). Among the MRP1-9 proteins, MRP1 has been shown to be highly expressed in normal human lung tissue. Cellular localisation of MRP1 and MRP2 within lung tissue has been most well characterised relative to other MRPs (Table 1-3). Furthermore, these two MRP transporters have been studied the most in terms of their role in drug transport in general (Gumbleton et al., 2011). MRP1 is involved in the transport of glutathione, glucuronic acid and sulphate-conjugates, whereas MRP2 is responsible for the hepatobiliary excretion of drug conjugates, especially those with glutathione (Pfeifer et al., 2014). Although not representative of the intact lung tissue, positive expression and intracellular localisation of MRP proteins (from 1 to 5) have been reported in epithelial
cells isolated and cultured from the human lung tissue (Torky et al., 2005). The expression of MRPs has also been reported at the mRNA level (high for MRP1, moderate for MRP5 and low or not detectable for MRPs 2 to 4) in normal human lung tissue (Kool et al., 1997). While subsequent gene microarray analysis confirmed high expression of MRP1 and MRP5 and absence of MRP2 in the lungs, high and moderate expression of MRP3 and MRP4 expression have been reported, respectively (Bleasby et al., 2006). In terms of cellular expression, high level of MRP1 (mRNA) has been reported to present in human macrophages (Moreau et al., 2011).

Another ABC transporter protein which has a role in multidrug-resistance against therapeutic agents is BCRP (Bosquillon et al., 2010). There is little and contradictory data regarding BCRP expression level in the lung (Doyle et al., 1998; Bleasby et al., 2006; Bosquillon, 2010). While it has been suggested to have protective function against xenobiotics in tissues such as placenta and blood-brain barrier, its role the lungs has not been elucidated (Polgar et al., 2008).

Currently, there are limited functional data available for most of the transporters in the lung and their relevance for pulmonary pharmacokinetics (PK) is still unclear (Forbes et al., 2011; Gumbleton et al., 2011). Nonetheless, it is recognised that transporters in the lung may potentially influence drug absorption from airways to systemic circulation, lung residence times of inhaled drugs, their intracellular concentrations in pulmonary epithelial cells and consequently their efficacy and toxicity (Forbes et al., 2011).
Table 1-2: Summary of drug uptake transporter protein expression in human lung tissue.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Expression level</th>
<th>Cellular distribution</th>
<th>Cellular location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SLC Transporters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCT1</td>
<td>Weak</td>
<td>Ciliated bronchial epithelial cells</td>
<td>Apical</td>
<td>Lips et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td></td>
<td>Cytoplasmic</td>
<td></td>
</tr>
<tr>
<td>OCT2</td>
<td>Strong</td>
<td>Ciliated bronchial epithelial cells</td>
<td>Apical</td>
<td>Lips et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>Basal cells</td>
<td>Entire membrane</td>
<td></td>
</tr>
<tr>
<td>OCT3</td>
<td>Weak</td>
<td>Ciliated bronchial epithelial cells</td>
<td>Apical</td>
<td>Lips et al., 2005;Horvath et al., 2007a</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>Basal cells</td>
<td>Entire membrane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>Intermediate cells</td>
<td>Basolateral membrane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>Bronchial &amp; vascular smooth muscle cells</td>
<td>Apical</td>
<td></td>
</tr>
<tr>
<td>OCTN1</td>
<td>Strong</td>
<td>Tracheal epithelium</td>
<td>Apical</td>
<td>Horvath et al., 2007b</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>Alveolar epithelium</td>
<td>Apical</td>
<td></td>
</tr>
<tr>
<td>OCTN2</td>
<td>Weak</td>
<td>Airway epithelium</td>
<td>Apical</td>
<td>Horvath et al., 2007b</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>Alveolar epithelium</td>
<td>Apical</td>
<td></td>
</tr>
<tr>
<td>PEPT2</td>
<td>Strong</td>
<td>Tracheal/bronchial/bronchiolar epithelium</td>
<td>Apical</td>
<td>Groneberg et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>Type II pneumocytes</td>
<td>Cytoplasmic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>Submucosal endothelium</td>
<td>Cytoplasmic</td>
<td></td>
</tr>
</tbody>
</table>
Table 1-3: Summary of drug efflux transporter protein expression in human lung tissue.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Expression level</th>
<th>Cellular distribution</th>
<th>Cellular location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC Transporters</td>
<td>Weak</td>
<td>Bronchial/bronchiolar epithelium</td>
<td>Apical</td>
<td>Cordon-Cardo et al., 1990; van der Valk et al., 1990; Lechapt-Zalcman et al., 1997; Scheffer et al., 2002; Campbell et al., 2003; Endter et al., 2007</td>
</tr>
<tr>
<td>P-gp</td>
<td>Moderate but variable</td>
<td>Alveolar type-I cells*</td>
<td>Apical</td>
<td>Flens et al., 1996; Brechot et al., 1998; Scheffer et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Weak but variable</td>
<td>Bronchial epithelium</td>
<td>Apical</td>
<td>Sandusky et al., 2002; Scheffer et al., 2002</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>Goblet cells</td>
<td>Basal</td>
<td>Scheffer et al., 2002; Fetsch et al., 2006</td>
</tr>
<tr>
<td>MRP1</td>
<td>n/a</td>
<td>Bronchial epithelium</td>
<td>Basal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serous cells of bronchial mucosa</td>
<td>Basolateral</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alveolar macrophages</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>MRP2</td>
<td>Strong but variable</td>
<td>Bronchial and bronchiolar epithelium</td>
<td>Apical</td>
<td></td>
</tr>
<tr>
<td>BCRP</td>
<td>Weak</td>
<td>Bronchial epithelium</td>
<td>Apical</td>
<td>Scheffer et al., 2002; Fetsch et al., 2006</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>Endothelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>Seromucinous glands</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Strong</td>
<td>Alveolar pneumocytes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Contradictory data exist in the literature; # Positive and variable staining in AMs reported by van der Valk et al., 1990 and Scheffer et al. 2002, respectively; n/a: information regarding the expression level has not been reported.
1.2.3.3 Vesicle-mediated endocytosis and transcytosis
The presence of high numbers of small membrane invaginations or vesicles called caveolae in pulmonary capillary endothelial cells and ATI cells have often been highlighted in the literature (Patton, 1996; Gumbleton et al., 2000; Crandall and Matthay, 2001; Gumbleton, 2001; Schnitzer, 2001; Gumbleton et al., 2003; Ehrhardt et al., 2008b). While caveolae have been reported to be involved in the vesicular transport of both low molecular weight solutes and macromolecules in certain cell types, evidence for their significant endocytotic or transcytotic role in capillary endothelial and ATI cells has been reported to be limited (Gumbleton et al., 2000; Gumbleton et al., 2003).

1.3 Alveolar macrophages
1.3.1 Mechanisms of particle uptake in alveolar macrophages
All eukaryotic cells have developed a variety of mechanisms to internalise particles including small molecules and macromolecules and extracellular fluid and deliver them to specific organelles within the cytoplasm (Mukherjee et al., 1997). These processes are often called “endocytosis” in broader definition and include phagocytosis (which is receptor- and actin-mediated), pinocytosis (clathrin-mediated or non-clathrin mediated), clathrin-dependent receptor mediated endocytosis (or receptor-mediated endocytosis via clathrin coated pits) and clathrin-independent endocytosis (Silverstein et al., 1977; Mukherjee et al., 1997).

The processes which are thought to be involved in particle uptake in AMs are illustrated in Figure 1-3. Pinocytosis refers to the formation of smaller vesicles (<0.2 µm in diameter) for the uptake of fluid and dissolved solutes (e.g., lipoproteins) and macromolecules (e.g., enzymes, hormones) (Silverstein et al., 1977; Mellman, 1996; Aderem and Underhill, 1999). Clathrin-dependent receptor-mediated endocytosis involves internalisation of a variety of ligand-receptor complexes and takes place in all nucleated cells. Phagocytosis on the other hand occurs in specialised cells, often phagocytes, such as macrophages, monocytes and neutrophils. This process refers to internalisation of large particles and microorganisms into a cell (Mukherjee et al., 1997). This internalisation involves pseudopod (plasma membrane extensions) formation to engulf particles and cell surface receptor recruitment to recognise particles either directly or by opsonisation (opsonisation is a process that makes bacteria or
foreign particles easier to phagocytose and opsonins recognised by receptors include immunoglobulins and complement) (Mukherjee et al., 1997; Aderem and Underhill, 1999; Lydyard et al., 2011). Particle size of inhaled drug particles is an important factor in determining the effectiveness of phagocytosis: particle size of 1-3 µm has been found to be optimal for phagocytosis by AMs (Oberdörster, 1988; Chono et al., 2006). While the major clearance mechanism of soluble substances from the alveoli has been indicated to be transepithelial transport, endocytosis by AMs has also been indicated to contribute, in particular for large molecular weight hydrophilic compounds (Oberdörster, 1988). It has been demonstrated that phagocytosis is an energy-consuming process that requires ATP generated from either aerobic oxidative metabolism or anaerobic glycolysis (Silverstein et al., 1977). Indeed, several studies in primary AMs have investigated the contribution of these processes (by using inhibitors) in drug accumulation in AMs (Hand and King-Thompson, 1982; Hand et al., 1983; Heyneman and Reasor, 1986; Antonini and Reasor, 1991).

![Figure 1-3](image.png)

**Figure 1-3:** Schematic representation of the possible routes/mechanisms of particle uptake in alveolar macrophages.

Another active process known to be involved in transport of various endogenous and exogenous molecules including drugs into and outside of cells is transporter-mediated uptake (Bosquillon, 2010). Drug uptake and efflux transporters expressed in the lung were listed in Table 1-2 and Table 1-3, respectively. Finally, an uptake mechanism that is applicable to non-charged and hydrophobic molecules is passive diffusion. This
process has long been known to be involved in entry of many weak acids and bases in their unionised form into cells (de Duve et al., 1974).

1.3.2 Cell systems available to assess drug uptake in alveolar macrophages
Investigation of drug accumulation in alveolar macrophages has been performed both in vivo (in animals and human), and in vitro using either isolated primary AMs or cell lines, as detailed below.

1.3.2.1 In vivo assessment of drug accumulation in AMs
Numerous clinical studies in human and preclinical species have reported accumulation of different respiratory drugs in alveolar macrophages. In these studies, concentrations of antibiotics in AMs were assessed following isolation of the cells from the bronchoalveolar lavage fluid collected either i) during bronchoscopy (in clinical studies) or ii) after cannulation of animal lungs at desired times (often at steady-state) after oral drug administration (Conte et al., 1995; Patel et al., 1996; Rodvold et al., 1997; Togami et al., 2009; Togami et al., 2011). As AMs are covered with epithelial lining fluid and both have been indicated to be potential sites of bacterial infection, drug concentrations have often been measured in both AMs and ELF and compared relative to serum or plasma concentrations (not corrected for protein binding). Variable AM drug concentrations were evident both between subjects within the same study or between different studies which were comparable in terms of dosing regimen and AM collection times. For instance, 3-fold difference in AM-to-plasma concentration ratio of clarithromycin at steady-state has been reported in healthy subjects between two studies despite the same dosing regimen (Patel et al., 1996; Rodvold et al., 1997). In both studies, azithromycin concentration was also measured and 17-fold difference was observed in AM-to-plasma concentration ratio at steady-state between the studies (Patel et al., 1996; Rodvold et al., 1997). Much lower AM-to-plasma concentration ratio (12.4) was reported for ciprofloxacin in healthy subjects although different dosing regimen was used compared to that used for clarithromycin and azithromycin (Schuler et al., 1997). An AM-to-plasma concentration ratio of 16.3 was reported for rifampicin, although the analysis was performed in patients and a different dosing regimen was used relative to previously mentioned studies (Ziglam et al., 2002).
1.3.2.2 *In vitro* assessment of drug accumulation using alveolar macrophages

In *in vitro* assessment of drug accumulation using primary AMs, the cells are first harvested by pulmonary lavage before incubation with drugs *in vitro*. In most studies, the animals are sacrificed by exsanguination, trachea is isolated and cannulated with a tubing connected to a needle (Heyneman and Reasor, 1986; Antonini and Reasor, 1991). Lavaging can be performed while the lungs remain *in situ*, or after they are removed from the thorax (Reasor, 1981). The tissue is then lavaged with an appropriate buffer and the fluid is then collected followed by its centrifugation to pellet AMs. This process results in clean AM isolation, with 85-90% of the cells from lungs in preclinical species and >90% of the cells from human lungs recovered as AMs (Reasor, 1981). If AMs are obtained from humans, subjects undergo either fiberoptic bronchoscopy involving bronchioalveolar lavage as in *in vivo* studies (Hand et al., 1984) or surgery involving removal of a small lung tissue from which AMs are isolated following perfusion. However, obtaining AMs by bronchioalveolar lavage has some limitations including the risk of contamination of AMs by red blood cells, lymphocytes, polymorphonuclear cells and epithelial cells (Vestal et al., 1980), the potential loss of drug from AMs into extracellular fluid during collection (Honeybourne and Baldwin, 1992) and limited availability of patient population (particularly healthy volunteers) (Nave et al., 2007). In addition, if experiments are performed in AMs from animals, several animals may need to be sacrificed, especially when measurements at different time points are required.

In addition to primary AMs, NR8383 cell line can be used to study drug accumulation in AMs. NR8383 is a rat alveolar macrophage cell line originally derived from pulmonary lavage of a normal adult, male (500 g) Sprague-Dawley rat (Helmke et al., 1987; Helmke et al., 1989). The culture of the cell line was initiated in the presence of a gerbil lung cell conditioned medium and was propagated continuously for over 36 months. Between 221 and 228 days after initiation in culture, the rat AMs were observed to proliferate in a manner typical of transformed cell lines (Helmke et al., 1987). During the whole cell culture period, the NR8383 showed increased proliferation (shorter doubling times), performed phagocytosis, showed nonspecific esterase activity at all times, became less attached to glass or plastic surface and yielded greater number of floating cells. In addition to these properties, they have been shown to possess Fc receptors (a surface receptor protein involved in antibody binding to induce
phagocytosis), secrete interleukin-I and perform oxidative burst similar to their primary AM counterparts (Helmke et al., 1987; Helmke et al., 1989).

The NR8383 cell line has been used since then in research in investigating many different areas including; cytotoxic effects on lung tumour cells (Sawachi et al., 2010), internalisation, cytotoxicity, apoptosis and tumour necrosis factor-α expression in response to exposures to various industrial dusts (Attik et al. 2008), phagocytosis of particles used in drug delivery systems such as liposomes and microspheres (Jones et al., 2002; Chono et al., 2006; Hirota et al., 2007; Onoshita et al., 2010) and investigation of drug accumulation and subcellular distribution (Togami et al., 2009; Togami et al., 2010; Togami et al., 2011; Togami et al., 2013).

A number of different techniques were used in the past to study the uptake of drugs into primary animal alveolar macrophages. One of them involved using the oil-spin method (Johnson et al., 1980; Hand and King-Thompson, 1982; Hand et al., 1984). The method involved incubation of radiolabelled compounds with cells in suspension for certain incubation periods after which a volume of the incubation mixture was removed and placed on a silicone oil-formic acid gradient in a microcentrifuge tube. Cells in the top layer were then centrifuged through this silicone oil-formic acid and separated from the radioactive antibiotic solution. Both the lower layer containing cells dissolved in formic acid and the upper layer containing the culture medium and extracellular antibiotic were then solubilised, placed in vials and radioactivity was quantified by a liquid scintillation counter. Alternatively, following the separation, the centrifuge tubes were frozen at -50°C, the frozen layers were separated by cutting the tubes with a razor and the radioactivity in both layers were then measured. In another study by Heyneman and Reasor (1986), microscope coverslips, which had an incubation of an AM suspension and radio-labelled drug on them, were counted for radioactivity. Another uptake method was employed by Antonini and Reasor (1991) who plated primary rat AMs in tissue culture wells and incubated non-radiolabelled drug with the cells in wells for varying periods of time and then measured the drug concentration in cells.

In drug uptake studies with NR8383 cells, a number of different approaches have been used. In one of the studies, once the AMs and the drug formulation were incubated in a 24-well cell culture plate, the suspensions from each well were transferred in a tube containing Percoll for density-gradient centrifugation. Following centrifugation, the lower layer containing the cells was further processed and analysed for intracellular drug concentration (Onoshita et al., 2010). In a number of other studies, NR8383 cells
were allowed to adhere to cell culture plates followed by incubation with test compound (at single concentration) over 4 or 25 hours at 37 and 4°C. The cells were then collected and extracted, and the intracellular drug concentration was measured (Togami et al., 2009; Togami et al., 2010; Togami et al., 2011; Togami et al., 2013).

1.3.3 Determination of uptake kinetics in vitro

A recent review by International Transporters Consortium has summarised current methods for estimating uptake transporter kinetics in hepatocytes and highlighted both advantages and limitations of these methods (Zamek-Gliszczynski et al., 2013). One of these methods is the conventional two-step method (Poirier et al., 2008) in which the kinetic analysis of experimental data consists of two consecutive steps. In the first step, passive diffusion clearance (CL_{diff}) is estimated from the data obtained at 4°C experiments (performed parallel to 37°C) (Zamek-Gliszczynski et al., 2013). In the second step, CL_{diff} is used as a constant in Equation 1-1 that relates initial uptake rates to substrate concentration in order to determine the uptake kinetic parameters.

Equation 1-1
\[ v = \frac{V_{\max} \times S_{\text{med}}}{K_m \times S_{\text{med}}} + C L_{\text{diff}} \times S_{\text{med}} \]

where \( v \) is the initial uptake rate, \( V_{\max} \) is the maximum uptake rate and \( K_m \) is the Michaelis-Menten constant. \( S_{\text{med}} \) is the nominal substrate concentration in the media. The contribution of saturable active uptake clearance (CL_{active}) is consequently determined from the ratio of \( V_{\max} \) and \( K_m \). One of the limitations of this method is the altered membrane fluidity at 4°C (Frezard and Garnier-Suillerot, 1998) and the reliance of the kinetic parameter estimation on CL_{diff} measured at this low temperature (Ménochet, 2012). Furthermore, the method relies on data transformation for parameter estimation and does not consider the bidirectional nature of passive diffusion, intracellular binding and active efflux (Ménochet et al., 2012; Zamek-Gliszczynski et al., 2013).

In contrast, these issues have been addressed in a mechanistic compartmental model developed to dynamically assess changes in drug concentrations due to active transport, passive diffusion and intracellular binding processes occurring during uptake into plated hepatocytes (Ménochet et al., 2012). Unlike the conventional two-step model, CL_{diff} is estimated from the 37°C data and its bidirectional nature is considered. Furthermore, all kinetic parameters (\( K_m \), \( V_{\max} \), CL_{diff} and unbound fraction in the cell (f_{u,cell})) are
estimated simultaneously from the fitted concentration and time points without prior data transformation (Ménochet et al., 2012).

While assessment of drug accumulation in AMs has been commonly performed at single substrate concentration, investigation of drug uptake kinetics has remained limited to a number of studies. Previously, the analysis of the kinetic data was rather simplistic, involved linear transformation of the 37°C data and use of Lineweaver-Burk plots in an attempt to determine $K_m$ and $V_{max}$. However, the contribution of active uptake and passive diffusion clearances has not been determined (Hand and King-Thompson, 1982; Heyneman and Reasor, 1986). The interpretation of the results was often related to the saturation characteristics of drug uptake without attribution to any particular transporter as uptake transporters in AMs are largely unknown at present.

1.3.4 Lysosomal trapping of drugs in alveolar macrophages

Lysosomes are acidic organelles found ubiquitously within cells and are the major digestive compartments. The acidic environment of the lysosomal lumen (pH 4-5) enables optimal activity of lysosomal enzymes and facilitates the degradation of cellular and exogenous materials (Appelqvist et al., 2013). The acidic pH of the lysosomes is maintained by a lysosomal vacuolar-type proton pump or $V-H^+-ATPase$ which pumps protons from cytosol against their electrochemical gradient into the lysosomal lumen (Mego et al., 1972; Geisow, 1982; Feng and Forgac, 1992). Although almost all animal and human cells have lysosomes, they are particularly abundant in certain tissues such as liver, kidney, spleen and lung and cell types such as leucocytes and macrophages (MacIntyre and Cutler, 1988b).

It has been shown in a number studies performed in animal AMs that these cells accumulate CADs including imipramine, propranolol, chlorphentermine, amiodarone and chloroquine (Vestal et al., 1980; Wilson et al., 1982; Heyneman and Reasor, 1986; Antonini and Reasor, 1991; Daniel et al., 1995). This accumulation has been suggested to occur mainly due to sequestration of these drugs into lysosomes which are present abundantly in AMs (Vestal et al., 1980; MacIntyre and Cutler, 1988b; Antonini and Reasor, 1991; Daniel et al., 1995).

A simplified scheme of the generic cell and the lysosomal trapping of a basic drug is illustrated in Figure 1-4. CADs with $pK_a$ value close to neutrality are present to a large extent (~ 50%) in their neutral form at physiological pH (7.2-7.4) and can readily cross
plasma and lysosomal membranes by passive diffusion (Kazmi et al., 2013). Once they cross the lysosomal lipid bilayers and reach the acidic lumen, they become predominantly ionised. As a consequence of lysosomal membrane being relatively impermeable to the protonated species, they are subsequently trapped inside the lysosomes. As long as the low pH inside lysosomes is maintained, the basic compound will accumulate and achieve a very high concentration at steady-state relative to its concentration in the cytosol (Kaufmann and Krise, 2007). Drugs that accumulate in the lysosomes are often referred as “lysosomotropic” (Nadanaciva et al., 2011).

Lysosomal trapping is a reversible process and is linear at low concentrations of basic lipophilic drug. At high concentrations, lysosomal pH increases (to a value which depends on base concentration and time of exposure) due to the rate of base accumulation exceeding the rate of proton translocation mediated by V-H\(^+\)-ATPase from cytosol to lysosomal lumen. Subsequently, this situation leads to a reduction in the capacity of lysosomes to accumulate base further (MacIntyre and Cutler, 1988b; Logan et al., 2012). Another feature of lysosomal trapping is its dependence on the structural integrity of the lysosomal membrane due to the presence of V-H\(^+\)-ATPase pump. In addition, competition of two basic lipophilic drugs for trapping and impact on each other’s accumulation depending on the free concentrations and potencies of these drugs to increase lysosomal pH needs to be considered (MacIntyre and Cutler, 1988b).

**Figure 1-4:** Lysosomal trapping of a basic lipophilic compound illustrating its transport across cytosolic and lysosomal membrane and its ionisation as a result of pH differences between cellular compartments. Acidic pH of the lysosomes is maintained by V-H\(^+\)-ATPase pump on lysosomal membrane. Other cellular compartments are not shown for simplification of the scheme.
1.3.4.1 Mechanisms of lysosomal accumulation

Molecules may enter lysosomes by at least four possible mechanisms. The main route for entry is endocytosis which involves the cells internalising the plasma membrane along with cell surface receptors and soluble molecules (de Duve et al., 1974). Materials that are taken up in endocytic vesicles formed from the plasma membrane fuse with early endosomes (endosomes which are not matured). While majority of the endocytosed material including receptors is recycled back to plasma membrane, early endosomes retain the materials which need to be degraded while gradually maturing into late endosomes (contain lysosomal membrane proteins and acid hydrolyses). Through continuous trafficking between late endosomes (matured endosomes or pre-lysosomal compartments) and trans-Golgi network, endosomal material is delivered to lysosomes along with newly synthesized lysosomal enzymes and membrane proteins (Appelqvist et al., 2013). It is worth to note that the endosomes become more acidic as they go through maturation; early endosomes have a pH of ~6 which reduces to a range of 5-6 in late endosomes and further maturation leads to pH of 4-5 (Appelqvist et al., 2013). Therefore, the most acidic nature of lysosomes rationalises the accumulation of CADs in these organelles. In the case of AMs and other phagocytic cells, phagocytosis also plays an important role as another route of entry of larger molecules. The phagocytosed materials are taken up in phagosomes which then fuse with lysosomes for digestion (Cooper, 2000).

The second mode of entry of molecules into lysosomes is autophagy which is a catabolic process involving the degradation and turn-over of intracellular components (plasma membranes, organelles, proteins) (Cuervo et al., 2005). The third possible route of drug entry into AM is via passive diffusion (de Duve et al., 1974; Kaufmann and Krise, 2007). Although endocytosis is a very dynamic process, it has a relatively limited capacity to accumulate amines into lysosomes and passive permeation has been proposed as the major contributor to lysosomal accumulation of amine compounds (Kaufmann and Krise, 2007; Goldman et al., 2009). Finally, a number of drug transporter proteins associated with lysosomal membranes have been indicated as a possible mechanism for uptake of amines; however, the contribution of this mechanism to accumulation in lysosomes is currently thought to be minimal, analogous to endocytosis (Kaufmann and Krise, 2007).
1.3.4.2 *In vitro* methods to investigate lysosomal trapping

There are a number of methods and approaches that have been developed over the years in order to assess lysosomal sequestration of basic amines *in vitro*. All methods have certain advantages and limitations and the choice will depend on the purpose of the research conducted (Kaufmann and Krise, 2007).

One commonly applied technique is the use of fluorescence microscopy using fluorescent analytes that accumulate in the lysosomes. While this method is relatively easy to perform and can be applied to live cells to allow kinetic assessment of analyte accumulation, it has been reported that quantitation can be compromised by fluorescent quenching caused by binding events (Gigli et al., 1989).

The second commonly used approach involves homogenisation of cells and subcellular fractionation of the cell homogenates following drug uptake studies (Renard et al., 1987; Yoshida et al., 1987; Ishizaki et al., 1996; Togami et al., 2009; Togami et al., 2013). While separation of cytosolic and lysosomal fractions containing basic compounds is possible, the organelle/granule fraction obtained with centrifugation is often not purely lysosomal and may also contain other organelles or cellular components (Yoshida et al., 1987; Ishizaki et al., 1998b; Ishizaki et al., 1998a; Togami et al., 2009), although isolation of lysosomes from rat liver has been reported (Ishizaki et al., 1996; Ishizaki et al., 2000). Further disadvantages of this approach are insufficient or excess homogenisation of cells leading to inadequate separation of cytosolic and lysosomal fractions or damage to lysosomes causing leakage of lysosomal content (Renard et al., 1987). In addition, drugs can diffuse from lysosomes during the long homogenisation and fractionation procedure. The latter is not an issue if incubations with compounds are performed in isolated lysosomes following homogenisation and centrifugation steps. However, the results obtained using isolated lysosomes may not correlate well with data from intact cells in cases when multiple binding processes that occur outside of the lysosomes could alter lysosomal accumulation (Kaufmann and Krise, 2007).

Indirect assessment of lysosomal sequestration of basic drugs by disturbing the lysosome-cytosol pH gradient by alkalinising the lysosomes using basic compounds or chemical agents is the third commonly used method. Common approaches include incubation of basic compounds of interest in the presence of another lysosomotropic compound, allowing the assessment of competition for lysosomal accumulation (Ishizaki et al., 1996; Daniel and Wojcikowski, 1999b; Daniel and Wojcikowski, 1999a;
Ishizaki et al., 2000). Alternatively, sequestration of the drug of interest can be assessed in the presence of ammonium chloride (NH₄Cl), monensin or nigericin. The unionised fraction of NH₄Cl readily crosses lysosomal membrane and increases pH as a result of its protonation. Monensin and nigericin are ionophores and cause lysosomal alkalinisation by exchanging sodium and potassium ions with protons, respectively (Millot et al., 1997; Kazmi et al., 2013). In both cases, an indication of lysosomal accumulation of basic drugs is made by assessing the reduction in drug accumulation in the presence of these agents relative to control conditions (when agents are not present) (Daniel et al., 1995; Ishizaki et al., 1998a; Ishizaki et al., 2000; Kazmi et al., 2013). Although direct assessment of lysosomal and cytosolic concentrations of the drugs is not feasible with this approach, it is relatively easy to perform.

Another indirect approach for assessing lysosomal sequestration of amine drugs involves staining of cells with a fluorescent dye selective for lysosomes such as LysoTracker Red (LTR). The fluorescence of LTR in cells can be monitored when it is incubated in the presence of nonfluorescent compounds (Lemieux et al., 2004; Nadanaciva et al., 2011; Kazmi et al., 2013). If LTR fluorescence is reduced, the test compound is deemed to have accumulated in the lysosomes and raised the pH sufficiently to disrupt the retention of LTR in the lysosomes (Goldman et al., 2009). Although this approach is semi-quantitative and cannot indicate the mechanism by which pH is disturbed (e.g., membrane partitioning or inhibition of V-H⁺-ATPase), it is useful in ranking compounds by the extent they accumulate in lysosomes (e.g., comparison of the half maximum inhibitory concentration (IC₅₀) for LTR loss of fluorescence) and providing a qualitative assessment of compound accumulation in lysosomes.

A number of sophisticated analytical techniques have also been developed in assessing lysosomal drug sequestration. These involved use of capillary electrophoresis with laser-induced fluorescence dual channel detection (Chen et al., 2005) and magnetic chromatography followed by high performance liquid chromatography (HPLC) coupled with fluorescent detector (Duvvuri et al., 2004b). Although both techniques allowed determination of drug content in acidic organelles/lysosomes, some of the limitations related to procedures involving cell homogenisation, centrifugation or instrumentation still exist.
1.3.4.3 *In silico* models for assessing intracellular drug concentrations

In comparison to a number of studies which investigated lysosomal sequestration of drugs *in vitro*, the assessment of this process by modelling and simulation is still relatively limited. A number of modelling approaches to determine intracellular unbound drug concentrations have recently been reviewed (Chu et al., 2013). One of these approaches includes empirical models to predict the intracellular fraction unbound ($f_{u,cell}$) of non-transporter substrates in hepatocytes from the relationship with LogP (for basic and neutral drugs) and LogD$_{7.4}$ (for acidic drugs) (Austin et al., 2005; Kilford et al., 2008). More recently, a mechanistic two-compartment model has been developed that allows simultaneous assessment of transporter uptake, passive permeability and intracellular binding ($f_{u,cell}$) in plated hepatocytes (Ménochet et al., 2012). Consequently, empirical equations relating LogD$_{7.4}$ and $f_{u,cell}$ have been derived for acidic and neutral drugs which are hepatic transporter substrates (Yabe et al., 2011; Ménochet et al., 2012). In addition to the assessment of $f_{u,cell}$ in hepatocytes, a three-compartment model, accounting for the pH gradients between lysosome, cytosol and extracellular medium in the brain has been developed to predict drug partition coefficient of the cell and to explain the discrepancy observed in $f_{u,cell}$ obtained from brain slice and brain homogenate experiments (Friden et al., 2011). Moreover, three-compartment models representing apical, cellular and basolateral compartments in Madin-Darby canine kidney (MDCK) cells have been developed to evaluate the impact of P-gp efflux on intracellular drug concentrations (Korzekwa et al., 2012; Korzekwa and Nagar, 2014; Nagar et al., 2014). Studies focusing on efflux transporters have emphasized the necessity to incorporate membrane compartments in the *in silico* cell models in order to account for drug partitioning into membranes and improve prediction of intracellular concentrations (Korzekwa and Nagar, 2014; Nagar et al., 2014). Whilst some of the mentioned mechanistic models have the advantage of providing dynamic and physiological assessment of cellular processes including transporter uptake/efflux, intracellular binding and membrane partitioning, they often require large number of cells/data points for accurate definition of parameter estimates (e.g., $K_m$, $V_{max}$, $CL_{uptake}$).

In addition, they have been applied to specific cell systems including either hepatocytes (plated, suspended and sandwich-cultured) or cells forming monolayers (MDCK, Caco-2) (Zamek-Gliszczynski et al., 2013). Moreover, these models have not taken into account the subcellular distribution of drugs, as an important cellular process contributing to intracellular concentrations of certain drugs.
Mechanistic models which consider subcellular distribution of drugs:
A physiologically based liver model has previously been used to characterise hepatic extraction and retention of a number of drugs with different physicochemical properties by determining the contributions of ion-trapping (into lysosomes and mitochondria combined), intracellular binding (microsomal binding) and unbound drug distribution (Siebert et al., 2004). The model derived kinetic parameters for ion-trapping, intracellular binding and intrinsic metabolic clearance were compared to values obtained by fitting the in situ rat liver perfusion data (Siebert et al., 2004).

More recently, a mechanistic intestinal epithelial cell model comprising of apical, basolateral, cytosolic and mitochondrial compartments has been developed (Zhang et al., 2006); the approach was based on a previously published cell-based model studying the accumulation of lipophilic cations in human tumour cells (Trapp and Horobin, 2005). This model aimed to predict intestinal transcellular passive permeability and intracellular concentrations of drugs using a set of differential equations describing the physics of passive diffusion (based on Nernst-Planck and Fick equations) (Zhang et al., 2006). While the model considered passive diffusion of drugs, their interactions with membranes and concentration gradients across membranes, it did not account for transporter uptake, metabolism, binding to membranes and was limited to predicting the behaviour of non-zwitterionic, monocharged molecules with one ionisable group (Zhang et al., 2006). The principles applied in the mechanistic three compartment model (cytosol, mitochondria and medium) developed by Trapp and Horobin (2005) has also been adapted to generic human cell model that includes an additional lysosomal compartment for evaluating the intracellular accumulation of drugs based on passive permeation across membranes, subcellular pH gradients and electrostatic interactions with membranes (Trapp et al., 2008). One of the advantages of the model is that it allows the assessment of drug concentrations in any cell type and subcellular compartments at any time which can be simulated to represent the steady-state conditions. In addition, the model is physiologically-based, as it is parameterised by a range of cell and organelle specific input parameters in addition to drug specific physicochemical (LogP and pKa) data. Furthermore, an important feature of the model that has not been considered in other models (Friden et al., 2011; Korzekwa et al., 2012; Ménochet et al., 2012; Nagar et al., 2014) is that it recognises the permeability limitation of ionised drug species across membranes relative to neutral species (Trapp and Horobin, 2005; Zhang et al., 2006; Trapp et al., 2008). More recently, the model by
Trapp et al. (2008) has been extended to account for a number of feedback mechanisms such as increase in membrane permeability due to accumulation of drugs in membranes and increase in lysosomal pH due to basic drug accumulation in lysosomes (Kornhuber et al., 2010a).

1.3.4.4 Clinical importance of lysosomal trapping in alveolar macrophages

Lysosomal sequestration of amines has received a great deal of attention due to a number of important clinical implications. In cases where drug targets are contained within lysosomes, drug sequestration in the organelles is considered beneficial (Kaufmann and Krise, 2007; Logan et al., 2012). Such a case is seen with the antimalarial drug chloroquine. However, in many instances, drug targets are outside of lysosomes; therefore, sequestration in lysosomes limits the drug-target interactions and may reduce drug efficacy. Examples include anticancer drugs with cytosolic or nuclear targets such daunoburicin and vinblastine (Larsen et al., 2000). Furthermore, it has been indicated that some drug resistant cancer cells have larger cytosol-lysosome pH-gradient compared to drug sensitive cancer cells (Duvvuri and Krise, 2005a). As a result, these drug resistant cancer cells have more enhanced capacity to sequester anticancer drugs in their lysosomes and this has been proposed to be a potential contributor to their multidrug resistant phenotype (Duvvuri and Krise, 2005b; Chen and Rosania, 2006).

In addition to the potential effect on drug efficacy, lysosomal drug sequestration can have an influence on drug distribution in the body (MacIntyre and Cutler, 1988b). Most work in this area has been done by Daniel and co-workers. Their earlier work has shown extensive chloroquine accumulation in rat lung slices that contain a high number of alveolar macrophages which are abundant in lysosomes. Uptake of chloroquine by lung and other tissue slices including liver and kidney was significantly reduced in the presence of NH₄Cl and monensin (Appendix Table 7-3). In their later work, they have shown the contribution of lysosomal trapping to uptake of a number of psychotropic drugs by several tissues including lungs, liver, kidneys and brain. In these studies, they demonstrated that important drug-drug interactions (DDIs) may occur if such drugs are administered together, due to competition for lysosomal accumulation (Daniel and Wojcikowski, 1999b; Daniel and Wojcikowski, 1999a). As a consequence of this competition, a potential shift in the distribution of lysosomotropic drugs from organs rich in lysosomes to those with lower lysosomal abundance (e.g., heart and muscle) has been suggested to lead to toxicity in these tissues (Kaufmann and Krise, 2007), although
there is no clinical evidence to support this proposal. Nonetheless, there are no known clinical examples of lysosomal distribution related DDIs (Kazmi et al., 2013). The potential role for DDIs involving lysosomes has also been speculated more recently by a number of researchers (Funk and Krise, 2012; Logan et al., 2012; Logan et al., 2013). Such DDIs have been suggested to occur due to lysosomal sequestration of a secondarily administered drug being altered by another lysosomotropic drug. Drug-induced changes in lysosomal pH and/or volume as well as increase in lipid content have been proposed as mechanisms associated with these DDIs (Logan et al., 2012).

Drug-induced phospholipidosis, characterised by excessive accumulation of phospholipids and drugs within cells, is another important clinical implication of lysosomal drug sequestration (Hruban, 1976; Hruban, 1984; Reasor et al., 2006; Kaufmann and Krise, 2007). A number of mechanisms have been proposed for increased accumulation of phospholipids. The primary mechanism is thought to be the inhibition of phospholipase A1 activity (Reasor et al., 2006), whereas some reports also suggest a relatively minor role of enhanced proteolytic degradation of acid sphingomyelinase (hence loss of its activity) in the presence of CADs (Hurwitz et al., 1994; Kornhuber et al., 2010b). Two possible mechanisms have been put forward for the inhibition of phospholipase A1 activity; either by direct inhibition of the enzyme by CADs (Kubo and Hostetler, 1985; Reasor et al., 1996; Van Bambeke et al., 1996) or formation of drug-phospholipid complexes in the lysosomes which become indigestible by lysosomal phospholipases (Lüllmann et al., 1978).

At least 385 drugs in the US Food and Drug Administration (FDA) database have been identified to induce phospholipidosis and more than 290 of these have been reported to be CADs (FDA, 2010). Alveolar macrophages develop a foamy appearance in response to a number of conditions of which includes CAD accumulation in AMs, characterised by the presence of a large number of lysosomal lamellar bodies and drug particles (Reasor, 1981; Lewis et al., 2014). It has been reported that formation of foamy AMs is reversible and considered non-adverse in some instances, whereas in other cases this process may be irreversible and may lead to adverse events (e.g., fibrosis, emphysema) (Lewis et al., 2014). Better understanding of the implications and reversibility of this condition has been indicated as necessary for reducing the attrition in development of new respiratory drugs (Forbes et al., 2011).
1.4 Compounds selected for investigation

A set of 10 compounds have been selected for investigation of drug uptake in AMs (Table 1-4). The compounds range in terms of their physicochemical properties and therapeutic classes as these were considered as selection criteria. All selected basic drugs had a basic pKₐ range between 8.14 and 9.9, therefore were considered to accumulate in the lysosomes based on pH partitioning theory (de Duve et al., 1974; MacIntyre and Cutler, 1988b; Friden et al., 2011).

Furthermore, priority was given to respiratory drugs, given either by inhalation or orally. A priori literature information or reported in vitro data with regards to accumulation in AMs or in lungs were also considered in the selection of the drugset.

1.4.1 Respiratory antibiotics

1.4.1.1 Clarithromycin

Clarithromycin (6-O-methyl-erythromycin) is a 14-membered macrolide antibiotic with a basic dimethylamine (-N(CH₃)₂) group (Figure 1-5) and is used in treatment of various infections of respiratory tract (Nakagawa et al., 1992; Patel et al., 1996; Babić et al., 2007). It is a moderately lipophilic and basic compound with a LogP of 3.16 and basic pKₐ of 8.99 (Table 1-4). Clarithromycin is given orally and distributes to a variety of tissues, particularly the epithelial lining fluid and alveolar macrophages in the lung (Conte et al., 1995; Patel et al., 1996; Rodvold et al., 1997). Accumulation in alveolar macrophages has been associated with lysosomal sequestration based on studies performed in NR8383 cell line (Togami et al., 2013). Literature findings regarding clarithromycin transport by OATPs are conflicting. In vitro studies with transfected cells and in vivo rat studies have previously suggested the involvement of OATPs in clarithromycin intestinal absorption (Garver et al., 2008). However, more recent in vitro study in human hepatocytes and transfected cells has demonstrated that clarithromycin was not subject to cellular uptake by OATPs (Higgins et al., 2014).

Clarithromycin is primarily metabolised by CYP3A4 in the liver to an active metabolite, 14-hydroxyclarithromycin (Rodrigues et al., 1997; Rodvold, 1999). It is rapidly absorbed from the gastrointestinal (GI) tract and has oral bioavailability of approximately 55% (Neu, 1991; Rodvold, 1999). It has steady-state volume of distribution Vₐ,ss of 1.5 L/kg, plasma clearance (CL) of 7.3 ml/min/kg and elimination half-life (t₁/₂) of 2.8 h after intravenous (i.v.) administration (Davey, 1991; Chu et al.,
1992; Obach et al., 2008). Its plasma protein binding has been reported to range from 42 to 72% (Rodvold, 1999). Clarithromycin is a well-known inhibitor of CYP3A4 (mechanism based), OATPs and P-gp, and is associated with a number of clinically relevant DDIs (Gorski et al., 1998; Rengelshausen et al., 2003; Seithel et al., 2007; Quinney et al., 2008; Hughes and Crowe, 2010; Markert et al., 2014). Recently, the FDA has recommended the use of oral clarithromycin as a strong CYP3A inhibitor in clinical DDI studies (FDA, 2012; Ke et al., 2014).

Figure 1-5: Chemical structure of clarithromycin (Babić et al., 2007)

1.4.1.2 Rifampicin
Rifampicin is a bactericidal drug primarily used to treat tuberculosis and is active against both intracellular and extracellular bacteria (Ziglam et al., 2002). Rifampicin is administered orally as tablets or capsules (Agrawal and Panchagnula, 2005). It is a moderately lipophilic and zwitterionic compound with cLogP of 2.54 and acidic and basic pKₐ of 1.7 and 6.7, respectively (Table 1-4). Rifampicin has been demonstrated as an OATP1B1 and OATP1B3 substrate in OATP-expressing HEK293 cells and Xenopus laevis oocytes (Vavricka et al., 2002; Yamaguchi et al., 2011). It is metabolised in the liver mainly by deacetylation and hydrolysis to 25-O-desacetyl rifampicin (main metabolite) and 3-formylrifamycin, respectively (Kenny and Strates, 1981). The compound is known as a potent inducer of hepatic CYP2C and 3A enzymes and can cause clinically significant drug interactions (Jamis-Dow et al., 1997; Shou et al., 2008; Templeton et al., 2011). Rifampicin is readily absorbed from the GI tract with an absolute bioavailability of 95% after a single oral dose given to six adult patients (Loos
et al., 1985). Its reported $V_{dss}$ is 0.97 L/kg body weight, plasma clearance is 3.5 ml/min/kg body weight and has $t_{1/2}$ of 3.8 h (Obach et al., 2008). Plasma protein binding of rifampicin has been reported to be 80-91% (Becker et al., 2009). The molecular structure of rifampicin is shown in Figure 1-6.

![Figure 1-6: Chemical structure of rifampicin (Becker et al., 2009)](image)

1.4.1.3 Ciprofloxacin
Ciprofloxacin, (Figure 1-7), is a broad-spectrum fluoroquinolone antibiotic used in the treatment of respiratory tract infections (Ong et al., 2013). The interaction of the 6-fluoro and the 7-piperazine in ciprofloxacin has been indicated to exhibit an enhanced Gram-negative and Gram-positive antibacterial activity (Torniainen et al., 1996). Ciprofloxacin is a hydrophilic and zwitterionic compound with LogP of 0.28 and acidic and basic pK$_a$ of 6.3 and 8.6, respectively (Table 1-4). The compound has been shown to be subjected to efflux in J744 mouse macrophages through an MRP-related transporter (Michot et al., 2004). Furthermore, its transport through Calu-3 normal bronchial epithelial cells has been shown to occur primarily by transcellular passive diffusion (Cavet et al., 1997). In addition to passive diffusion, involvement of OCTs, OATPs and MRPs was demonstrated in the air interface Calu-3 cells for ciprofloxacin transport (Ong et al., 2013).

![Figure 1-7: Chemical structure of ciprofloxacin (Babić et al., 2007)](image)
The major route of elimination of ciprofloxacin is via the kidneys (Rohwedder et al., 1990; Cavet et al., 1997). After an oral and i.v. dose, the drug has been shown to be eliminated largely as unchanged by renal and extrarenal (transintestinal) routes (Bergan et al., 1988; Rohwedder et al., 1990). N-sulfation by SULT2A1 has been demonstrated as one metabolic pathway of ciprofloxacin. It has been reported that 10 to 20% of a single i.v. or oral dose of ciprofloxacin is eliminated by this route (Senggunprai et al., 2009). The oral bioavailability of ciprofloxacin was reported to be 70-85% (Bergan et al., 1988; Cullmann et al., 1993; Bergogne-Berezin, 2002). It shows low binding to plasma proteins (20-40%) (Bergan et al., 1988; Cullmann et al., 1993; Bergogne-Berezin, 2002). The Vd,ss of ciprofloxacin is 2.1 L/kg body weight and has plasma CL of 8.3 ml/min/kg body weight and t1/2 of 3.8 h (Ljungberg and Nilsson-Ehle, 1988; Lettieri et al., 1992; Jaehde et al., 1995).

1.4.2 Inhaled respiratory compounds
1.4.2.1 β2-adrenoceptor agonists

Three β2 agonists were selected for investigation of uptake in AMs, namely formoterol, fenoterol and terbutaline. The chemical structures of these compounds are shown in Figure 1-8. For all of these compounds, no in vitro uptake data available in alveolar macrophages were available.

Formoterol is a highly potent, selective long-acting β2-adrenergic receptor agonist used in the management of asthma and COPD by causing bronchodilation upon acting on smooth muscle cells in the airways (Lecaillon et al., 1999; Rosethorne et al., 2010). It is a moderately lipophilic and basic compound with cLogP of 1.99 and basic pKa of 8.14 (Table 1-4). Formoterol has been suggested to be a substrate for organic cation transporters OCTN1/2 and OCT 3 in human airway epithelial and smooth muscle cells (Horvath et al., 2007a; Horvath et al., 2007b). The major metabolic pathway and the main route of elimination of formoterol in human is direct glucuronidation. The specific UGT enzyme responsible for its glucuronidation has not been defined (Lecaillon et al., 1999). O-demethylation and deformylation of formoterol were also reported (Rosenborg et al., 1999) and four P450 enzymes have been indicated to be involved in these processes (Cheer and Scott, 2002). Although pharmacokinetics of inhaled formoterol has been studied extensively in clinical trials, published PK data are sparse and limited to maximum plasma concentration (Cmax), systemic area under the plasma concentration time curve (AUC) and elimination t1/2 (1.7-2.3 hours after inhalation) (Lecaillon et al., 1999; McGavin et al., 2001; Eklund et al., 2008; Tronde et al., 2008b). In vitro plasma
protein binding of formoterol has been reported to be 61-64% at concentrations 0.1-100 µg/L (Cheer and Scott, 2002).

![Figure 1-8: Chemical structure of formoterol, fenoterol and terbutaline (Brown et al., 2007; Reszka et al., 2009)](image)

Fenoterol is a short-acting β₂-adrenergic receptor agonist used in the management of asthma and preterm labour by causing bronchodilation and relaxation of the pregnant uterus, respectively (Gleiter, 1999). Fenoterol is a hydrophilic compound (Jeppsson et al., 1989) with cLogP of 1.09 and a basic pKₙ of 8.25 (Table 1-4). Brändle et al., (1992) demonstrated that fenoterol is an inhibitor of TEA⁺ uptake in cortical renal tubular cells (Brändle et al., 1992). Based on these data and considering cationic nature of TEA⁺, Ullrich (1999) speculated that fenoterol is a substrate for the organic cation transporters present in the basolateral side of renal proximal tubules (Ullrich, 1999). Sulphation of fenoterol has been reported as the major contributor to its first-pass elimination, although some glucuronidation was also evident (Hildebrandt et al., 1994). After i.v. administration, the Vₐss of fenoterol has been reported as 1.2 L/kg body weight while plasma clearance and half-life were 29 ml/min/kg body weight and 0.87 h, respectively (Obach et al., 2008).

Terbutaline is a selective short-acting β₂-adrenergic receptor used as a bronchodilator and tocolytic in the management of asthma and preterm labour (Boyle, 1995). It is a hydrophilic compound (Skolnik et al., 2010) with LogP of 0.9 and basic pKₙ of 9.9 (Table 1-4). Although terbutaline is generally accepted as a basic compound due to its amine group, a number of sources have cited it as a zwitterion (Takács-Novák et al., 1995; Avdeef, 2012). As fenoterol, terbutaline inhibited TEA⁺ uptake in renal tubular cells and has also been suggested to share the same organic cation transporters in these cells (Ullrich, 1999). Sulphate conjugate of terbutaline has been identified as the major metabolite in man both after oral and i.v. dosing (Nilsson et al., 1972; Davies et al.,
The oral bioavailability of terbutaline (racemate) was 14.2% based on data from six healthy subjects (Borgström et al., 1989). Its $V_{\text{d,ss}}$ has been reported as 1.8 L/kg body weight, plasma CL was 3.4 ml/min/kg body weight and $t_{1/2}$ was around 14 hours after i.v. dosing (Borgström et al., 1989; Obach et al., 2008). Low plasma protein binding (20%) has been reported for terbutaline (Borgström et al., 1989).

### 1.4.2.2 Budesonide

Budesonide, illustrated in Figure 1-9, is a potent glucocorticosteroid used in the treatment of asthma, allergic rhinitis and inflammatory bowel diseases (Ryrfeldt et al., 1989; Meloche et al., 2002). It is a neutral and moderately lipophilic compound with cLogP of 2.47 (Table 1-4). Budesonide has been identified as a P-gp substrate in L-MDR1 cells (porcine kidney epithelial cell line LLC-PK1 stably transfected with human multi drug resistance 1 complementary deoxyribonucleic acid (cDNA)) (Dilger et al., 2004). It has also been suggested to be a substrate for MRP1 in human bronchial epithelial cell line 16HBE14o- (van der Deen et al., 2008). Budesonide is metabolised predominantly by CYP3A4 in the liver and has high hepatic first pass inactivation, resulting in low systemic availability after oral dosing (Thorsson et al., 1994; Jönsson et al., 1995; Boobis, 1998; Borchard et al., 2002). In addition, fatty acid conjugates of budesonide have been identified in human lung and liver microsomes and human lung parenchymal cells (Tunek et al., 1997; Somers et al., 2007). Moreover, sulphation of budesonide has been reported by human cytosolic SULT2A1 (Meloche et al., 2002). The $V_{\text{d,ss}}$ of budesonide after i.v. administration has been reported to be 3.9 L/kg body weight. Its plasma clearance was 20 ml/min/kg and $t_{1/2}$ was 2.8 h (Ryrfeldt et al., 1989; Obach et al., 2008). Its oral bioavailability has been reported to be 11-13% (Thorsson et al., 1994; Baptist and Reddy, 2009). Budesonide is found to be highly bound to plasma proteins (88%) (Winkler et al., 2004).

![Chemical structure of budesonide](image)

**Figure 1-9: Chemical structure of budesonide (adapted from Boobis (1998))**
1.4.2.3 Muscarinic receptor antagonists

Two muscarinic receptor antagonists, ipratropium (short-acting) and tiotropium (long-acting) bromide (Figure 1-10) were selected for investigation of uptake in alveolar macrophages as there was no in vitro uptake data available as for the remaining inhaled compounds. Ipratropium and tiotropium bromide are permanently cationic hydrophilic compounds with cLogP of -1.20 and -1.23, respectively (Table 1-4). They are both used in the treatment of COPD and exert their effect through bronchodilation by blocking muscarinic receptors on airway smooth muscle (Prat et al., 2011). Both compounds have previously been shown to be a substrate primarily for OCTN2 and to a lesser extent for OCTN1 in human bronchial epithelial cells (Nakamura et al., 2010). In addition, they have been demonstrated to be substrates for both rat and human OCT1 and OCT2 in OCT-expressing HEK293 cells (Nakanishi et al., 2011).

![Tiotropium bromide](image1.png)  ![Ipratropium bromide](image2.png)

**Figure 1-10:** Chemical structure of tiotropium and ipratropium bromide (Prat et al., 2011)

Ipratropium bromide has been shown to form three inactive metabolites in humans from the enzymatic cleavage of the ester bond, or enzymatic loss of a H₂O or CH₃OH-group (Ensinger et al., 1987; Ensing et al., 1989; Wood et al., 1995). The oral bioavailability of ipratropium bromide in healthy volunteers after a single 2 mg dose was 2% compared to its bioavailability of 7% after inhalation (Ensing et al., 1989).

Tiotropium bromide has been reported to undergo minimal metabolism with 74% of an i.v. dose being excreted unchanged in kidneys in healthy subjects (Santus and Di Marco, 2009). A fraction of tiotropium is nonenzymatically hydrolysed in plasma to two inactive metabolites. In addition, in vitro studies with human liver microsomes and hepatocytes suggest a fraction of the remaining tiotropium is metabolised by CYP3A4 and CYP2D6 with subsequent glutathione conjugation to a variety of conjugates (Price et al., 2009; Santus and Di Marco, 2009). The oral bioavailability of tiotropium bromide is 2-3% compared to its bioavailability of 19.5% after inhalation. Its $V_{d,ss}$ is 32 L/kg and
72% of the drug is found plasma protein bound (Price et al., 2009; Santus and Di Marco, 2009).

1.4.3 Imipramine

Imipramine is the first generation tricyclic antidepressant used in treatment of depression. It is an inhibitor of neurotransmitters norepinephrine and serotonin uptake (Hollister, 1986). Structurally, it is a prototypical cationic amphiphile, containing a tricyclic ring with an alkyl amine substituent on the central ring (Figure 1-11) (Funk and Krise, 2012). Imipramine is lipophilic and basic drug with a LogP of 4.8 and basic pKₐ of 9.5 (Table 1-4). Its major metabolic pathway involves N-methylation to the active metabolite desipramine (involving CYP1A2 and CYP3A4) and aromatic hydroxylation to 2-hydroxyimipramine and 10-hydroxyimipramine (via CYP2D6) (Lemoine et al., 1993). Its oral bioavailability is approximately 43%, 85-93% of the drug is bound in the plasma (Nagy and Johansson, 1975; Abernethy et al., 1984; Abernethy et al., 1985; Obach et al., 2008), has V_d,ss of 12 L/kg, CL of 13 ml/min/kg and t₁/₂ of 16 h after i.v. administration (Abernethy et al., 1984; Abernethy et al., 1985; Brosen and Gram, 1988). Imipramine is a well-known lysosomotropic compound (Nadanaciva et al., 2011) and was therefore selected as a positive control for lysosomal accumulation experiments in the current project.

![Figure 1-11: Chemical structure of tricyclic antidepressant imipramine (White et al., 2008)](image-url)
Table 1-4: Physicochemical properties of 10 drugs selected for the investigation of drug accumulation in alveolar macrophages

<table>
<thead>
<tr>
<th>Drug</th>
<th>LogP</th>
<th>LogD&lt;sub&gt;7.4&lt;/sub&gt;</th>
<th>pK&lt;sub&gt;a,a&lt;/sub&gt;</th>
<th>pK&lt;sub&gt;a,b&lt;/sub&gt;</th>
<th>PSA (Å&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Acid-Base Property</th>
<th>References</th>
</tr>
</thead>
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<td>Clarithromycin</td>
<td>3.16</td>
<td>1.57</td>
<td>-</td>
<td>8.99</td>
<td>183</td>
<td>Base</td>
<td>McFarland et al., 1997</td>
</tr>
<tr>
<td>Imipramine</td>
<td>4.80</td>
<td>2.38</td>
<td>-</td>
<td>9.50</td>
<td>6.50</td>
<td>Base</td>
<td>Ishizaki et al., 2000; Austin et al., 2002; Volgyi et al., 2007</td>
</tr>
<tr>
<td>Formoterol</td>
<td>1.99&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.60</td>
<td>10.1, 11.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.14&lt;sup&gt;1&lt;/sup&gt;</td>
<td>90.8</td>
<td>Base</td>
<td>Jeppsson et al., 1989</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>1.09&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.74</td>
<td>9.40, 10.1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.25&lt;sup&gt;1&lt;/sup&gt;</td>
<td>93.0</td>
<td>Base</td>
<td>Jeppsson et al., 1989</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.90</td>
<td>-1.50&lt;sup&gt;2&lt;/sup&gt;</td>
<td>8.60, 11.0</td>
<td>9.90</td>
<td>72.7</td>
<td>Base</td>
<td>Takács-Novák et al., 1995; Winiwarter et al., 1998; Kerns et al., 2003</td>
</tr>
<tr>
<td>Budesonide</td>
<td>2.47&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.47&lt;sup&gt;1&lt;/sup&gt;</td>
<td>n/a</td>
<td>93.1</td>
<td>Neutral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>-1.23&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-1.49&lt;sup&gt;1&lt;/sup&gt;</td>
<td>n/a</td>
<td>59.1</td>
<td>Permanently cationic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>-1.20&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-1.75&lt;sup&gt;1&lt;/sup&gt;</td>
<td>n/a</td>
<td>46.5</td>
<td>Permanently cationic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2.54&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.3</td>
<td>1.7</td>
<td>6.70</td>
<td>217</td>
<td>Zwitterion</td>
<td>Assandri et al., 1977; Agrawal and Panchagnula, 2005</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.28</td>
<td>-1.11</td>
<td>6.30</td>
<td>8.60</td>
<td>74.6</td>
<td>Zwitterion</td>
<td>Takács-Novák et al., 1995; Volgyi et al., 2007</td>
</tr>
</tbody>
</table>

Physicochemical data were expressed as experimental values where available except indicated; <sup>1</sup> Predicted values from ADMET Predictor™ v7.0; <sup>2</sup> Average of two studies (Winiwarter et al., 1998; Kerns et al., 2003); pK<sub>a,a</sub>: acidic pK<sub>a</sub>; pK<sub>a,b</sub>: basic pK<sub>a</sub>
1.5 Aims

Drug clearance by alveolar macrophages is an important component of mucociliary clearance mechanism involving in drug removal from the airways. Therefore, these cells may reduce efficacy of inhaled drugs by preventing drug-receptor interactions. Furthermore, CADs can accumulate in lysosomes of AMs which can have therapeutic consequences and/or lead to adverse outcomes. The overall aim of this Thesis is to understand the accumulation behaviour (extent and mechanism) of a range of respiratory drugs in alveolar macrophages using in vitro and in silico approaches.

The first aim, as described in Chapter 2, was to develop an in vitro uptake method to investigate accumulation of the drugs selected in NR8383 cells. A plated uptake methodology was adapted from hepatocytes work (Ménochet et al., 2012) and optimised for NR8383 cells in terms of cell seeding densities, culturing times, incubation time points with substrates and determination of cell number involved in drug uptake. Following method optimisation, uptake of drugs in NR8383 was investigated both at single (for all drugs) and over a range of substrate concentration (for selected drugs) in order to delineate the role of active uptake and passive transport processes into AMs and kinetics of accumulation. Total uptake (CL_{uptake}) and passive diffusion clearances were determined and the contribution of active and passive processes was assessed for each compound. Total cell-to-medium concentration ratios (K_{p,NR8383}) were determined to evaluate the extent of accumulation of all compounds in NR8383 cell line. In addition, uptake kinetic estimates (K_{m}, V_{max} and CL_{active}) were determined for selected compounds in the NR8383 system for the first time.

The next aim, described in Chapter 3, was to assess the contribution of lysosomal sequestration of all compounds to their accumulation in NR8383 cells. A number of different approaches have been utilised in order to characterise lysosomal trapping behaviour of the compounds in NR8383 and visually assess lysosomes and amine accumulation. These include the use of NH_{4}Cl, monensin and nigericin (quantitative indirect assessment) and lysosomal fluorescent dye LysoTracker Red (qualitative assessment). CL_{uptake} and K_{p,NR8383} of compounds were determined in the absence and presence of the chemical agents and significant reduction in drug accumulation was attributed to lysosomal accumulation. The results were confirmed with LTR staining by assessing the confocal microscopic images of NR8383 and the reduction of LTR
accumulation in lysosomes in the presence of chemical agents and selected basic compounds.

Following from the studies in NR8383, the Thesis aimed to investigate accumulation of drugs in primary human alveolar macrophages isolated from the lung cancer patients. In addition, Chapter 4 aimed to provide a comparison of the trends seen in human cells to those observed in NR8383 cells. Furthermore, lysosomal accumulation of a number of selected drugs in human AMs was also assessed with the approaches previously used in NR8383 cells. The *in vitro* uptake methodology was optimised for human AMs in terms of cell seeding densities and culturing times. CL$_{\text{uptake}}$ and cell-to-medium concentration ratio of compounds in human AMs (K$_{p,hAM}$) were determined at single substrate concentration for all drugs with the exception of ciprofloxacin and results were compared to those obtained in NR8383. Likewise, the reduction in CL$_{\text{uptake}}$ and K$_{p,hAM}$ of imipramine and clarithromycin in the presence of the chemical agents was determined. The inter-individual variability in accumulation of these two compounds in AMs and in the extent of lysosomal trapping was assessed. The potential use of NR8383 cell line as a surrogate for human alveolar macrophages was evaluated.

The final aim, described in Chapter 5, was to develop an *in silico* mechanistic cell model specific for AMs and to use the model to predict drug concentrations in AM subcellular compartments including lysosomes. The cell model developed by Trapp et al. (2008) was adapted to alveolar macrophages by modifying the input cell related parameters; the model also accounted for drug physicochemical properties. The predictive performance of the cell model was evaluated by comparing the predicted K$_{p,\text{cell}}$ for each compound with experimentally determined K$_{p,NR8383}$ and K$_{p,hAM}$ values. In addition, the ability of the cell model to mimic the effect of lysosomal alkalinisation on lysosomal accumulation of drugs was assessed. Furthermore, the sensitivity of the predicted K$_{p}$ values to changes in some of the cell parameters (e.g., lysosomal pH or cell volume as reported in smokers) was also evaluated. The advantages and limitations of the cell model and the areas of improvement for its future application were discussed.
Chapter 2  *In vitro* assessment of drug accumulation in NR8383 cell line

2.1 Introduction

AMs are the most abundant cells in the alveoli, representing more than 95-98% of the free cell population. These cells represent the first line of defence against inhaled pathogens and insoluble drug molecules (Fels and Cohn, 1986; Oberdörster, 1988). Due to their phagocytic properties, they are also considered as one of the barriers to drug-receptor interactions and drug absorption in the lungs (Tronde et al., 2008a). A number of *in vitro* studies investigated the uptake of CADs (e.g., amiodarone, chlorphentermine, propranolol) and antibiotics (e.g., clindamycin, rifampicin, erythromycin) in primary human, rat and rabbit AMs and in NR8383 cells in an attempt to elucidate their mechanism of accumulation. These studies often involved investigation of the effect of temperature (37°C and 4°C), cell viability and metabolic inhibitors (e.g., ouabain, potassium cyanide, 2,4-dinitrophenol) to determine the contribution of energy-dependent processes to drug uptake (Hand and King-Thompson, 1982; Hand et al., 1983; Heyneman and Reasor, 1986; Antonini and Reasor, 1991; Togami et al., 2013). Some of these studies have also investigated drug uptake in the presence of hexoses, amino acids and nucleosides, as macrophages have been reported to have specific carrier-mediated transport systems for these nonelectrolytes (Hand and King-Thompson, 1982; Heyneman and Reasor, 1986). In addition, a number of studies have also investigated the uptake or accumulation of CADs (e.g., propranolol) and macrolides (e.g., clarithromycin) in AMs in the presence of other basic drugs (e.g., imipramine) or in the presence of lysosomotropic agents such as NH₄Cl (Vestal et al., 1980; Heyneman and Reasor, 1986; Togami et al., 2010; Togami et al., 2013). Although active transport mechanisms have been suggested to play a role in uptake of clindamycin, erythromycin and clarithromycin in AMs and isolated lung cells (Johnson et al., 1980; Hand et al., 1983; Kohno et al., 1990), their uptake has not been attributed to any particular transporter, largely because uptake transporters in AMs are still unknown (Table 1-2). A review on drug transporters in the lungs by Bosquillon (2010) indicated OCTN1 expression in AMs and referred to the study by Horvath et al. (2007b). However, Horvath et al. (2007b) did not detect OCTN1 in AMs specifically and rather speculated that the OCTN1-specific labelling they had seen was most likely
within inflammatory cells. More recently, Moreau et al. (2011) demonstrated low expression of OCTN1/2, OCT1 and high expression of OATP2B1 in monocyte derived human macrophages. As alveolar macrophages are also derived from blood monocytes, this system may closely represent the transporters expressed in AMs. However, no information currently exists whether and to what extent transporter expression in macrophages may differ depending on the cell differentiation process occurring in alveoli in vivo relative to the in vitro differentiation process induced by growth factors. In addition, differences in transporter expression levels may be a result of culturing conditions.

Uptake of a range of CADs and antibiotics has so far been studied in AMs at single substrate concentration, with minimal data available on the kinetics of uptake over a range of substrate concentrations in primary rat and rabbit AMs (Hand and King-Thompson, 1982; Heyneman and Reasor, 1986). While kinetic data have been reported for chlorphentermine and clindamycin, assessment of uptake kinetics of a larger number of compounds in AMs may facilitate better understanding of the involvement of active transporter systems and their relation to drug properties. In this regard, cell lines have the advantage of generating kinetic data that requires large number of cells which are difficult to obtain from in vivo species.

2.2 Aims

The aim of studies presented in this chapter is to characterise the uptake of a range of respiratory drugs in an AM cell line. Ten drugs were selected for the assessment of uptake in NR8383 cells, 9 of which were respiratory. In addition, imipramine was included in the assessment as a marker of lysosomal trapping for later studies. Uptake of selected drugs was investigated at 37°C and 4°C to determine the dependence of cell accumulation on active uptake and passive diffusion, respectively. Following initial assessment at a single substrate concentration, kinetics of accumulation was investigated for a subset of selected compounds.
2.3 Methods

2.3.1 Chemicals and Reagents

1-Aminobenzotriazole (ABT), Ciprofoxacin, Clarithromycin, Dimethyl sulfoxide (DMSO), Imipramine, Lactate dehydrogenase (LDH) assay kit, Trypan blue 0.4% and Verapamil hydrochloride were all from Sigma Aldrich Ltd., Dorset, UK. Budesonide, Ipratropium Bromide, Fenoterol, Formoterol, Terbutaline and Tiotropium Bromide were all kind gifts from GlaxoSmithKline, UK. Further chemicals include Diazepam (Tocris Bioscience, Bristol, UK), Methanol (VWR, UK), Midazolam (Hoffman La Roche, Switzerland) and Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Loughborough, UK). Reagents include Bovine Serum Albumin (BSA) and Penicillin-Streptomycin (Sigma Aldrich Ltd., Dorset, UK), Dulbecco’s Phosphate Buffered Saline (DPBS), heat-inactivated Foetal Bovine Serum (FBS) and trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25%) with phenol red (Life Technologies, Paisley, UK) and Kaighn’s modification of Ham’s F12 (Ham’s F12K) medium (American Tissue Culture Collection (ATCC), Mannasas, VA, USA).

2.3.2 NR8383 cell culture and maintenance

The NR8383 cell line (CRL-2192) was purchased from ATCC, Mannasas, VA, USA. Cells were grown in Ham’s F12K medium with 2 mM glutamine and 1.5 g/L sodium bicarbonate which was further supplemented with 15% heat-inactivated foetal bovine serum and 100 units/mL penicillin-100 µg/mL streptomycin to make complete growth medium (CGM). Cells, which were present as both floating and attached cell populations, were routinely maintained in T75 cm$^2$ or T175 cm$^2$ tissue culture flasks. CGM was changed twice per week and involved removal of the floating cells from flasks, rapid addition of fresh CGM into the flasks to maintain adherent cells in medium and re-suspension of floating cells in CGM to be returned into flasks. The latter was performed following centrifugation of the cells at 1200 rpm for 5 minutes (Eppendorf Centrifuge 5804, Cambridge, UK) at room temperature. Upon ~70% confluency, cells were passaged by gentle scraping of the attached cells into the existing culture medium, followed by centrifugation at the aforementioned conditions and seeding into new T75 cm$^2$ flasks at ~2x10$^5$ cells per mL or T175 cm$^2$ flasks at ~3.5x10$^5$ cells per mL. Following centrifugation and before seeding into flasks, the cell pellet was gently re-suspended in 1 mL CGM previously maintained at 37°C, and cell viability was assessed.
with the trypan blue exclusion method using a haemocytometer under the light microscope (Olympus CK2, Olympus Optical Co., Japan). Cells were maintained at 37°C, 5% CO₂ in a humidified atmosphere (CO₂ incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK). Only cells until passage number 25 were used to ensure consistency in the cell line morphology.

2.3.3 Optimisation of in vitro uptake conditions
Drug uptake conditions were optimised in terms of cell detachment method from culture flasks, type of coating (extracellular matrix) in 24-well plates, seeding density per well, culturing time of the cells, drug incubation time points and measurement of cell number for normalisation of uptake clearance and cell-to-medium concentration ratio. While optimisation of in vitro uptake conditions was done mainly by qualitative analysis (i.e., microscopic examination), drug uptake was also assessed where necessary to support qualitative results. In all optimisation experiments, clarithromycin was used as a test compound.

2.3.3.1 Detachment of NR8383 from cell culture flasks
Gentle scraping versus Mg²⁺-Ca²⁺ free DPBS treatment were tested for their effects on cell viability, attachment of the cells to 24-well plates, cell confluency following 4 hours culture and drug uptake clearance. Following microscopic examination, the cells were detached from the flasks using either of the two methods investigated. When cells were treated with Mg²⁺-Ca²⁺ free DPBS, floating cells were first aspirated and transferred into a sterile falcon tube. The attached cells were then treated with appropriate volume of Mg²⁺-Ca²⁺ free DPBS (1-2 mL for each 25 cm² area) on an orbital incubator (Orbital Incubator SI50, Stuart Scientific, UK) at 37°C for 15 minutes. The cells were viewed under the microscope to observe any detachment of the cells. Following cell detachment, cells were plated into non-coated polystyrene 24-well plates (Greiner Bio-One Ltd., Stonehouse, UK) at 0.36 x 10⁶ cells/well and cultured for 4 hours at 37°C, 5% CO₂. Uptake of 1 µM clarithromycin was then assessed up to 5 minutes at 37°C to determine the effect of these two detachment methods on uptake clearance. Upon completion of the incubation, plates were examined visually under the microscope to observe the confluency of remaining cells.
2.3.3.2 Culture time in different types of 24-well plates
The effect of culture time prior to uptake and the coating type of 24-well plates on cell attachment, distribution in the wells and detachment with DPBS washes were investigated. Cells were seeded into non-coated, rat-tail collagen-I and poly-D-lysine coated 24-well plates at 0.36 $\times$ 10$^6$ cells/well and cultured for 4, 6, 16, 20 and 24 hours at 37°C, 5% CO$_2$. Uptake of 1 µM clarithromycin was then assessed up to 5 minutes at 37°C. After each culture time, microscopic examination was performed to assess attachment, confluency and distribution of the cells before uptake and cell loss after uptake.

2.3.3.3 Seeding density of NR8383 in 24-well culture plates
Based on the observations of cell loss during previous optimisation experiments, the effect of using higher cell seeding densities on cell confluency, plating efficiency, attachment and detachment following DPBS washes was tested. Cells were plated at 0.36 and 0.72 $\times$ 10$^6$ cells/well into collagen-coated plates and cultured for 16 hours at 37°C, 5% CO$_2$. After 16 hours, plating efficiency was determined by measuring the number of cells in wells (by BCA protein assay as described in Section 2.3.3.5) following media removal. After removing the media, the cells were washed three times with DPBS to remove any contaminating protein from CGM. The number of cells in the DPBS used to wash the cells was determined by the BCA protein assay. This was combined with the number of cells remaining in wells to determine the total number of cells that were initially attached. Uptake of clarithromycin was also assessed up to 5 minutes at 37°C. Cell loss following uptake was also assessed under the microscope.

2.3.3.4 Drug incubation time
The uptake experiments performed as part of method optimisation were used to guide the optimal drug incubation times. The incubation times used in these experiments were 0.5, 1, 2 and 5 minutes. These early time points were initially selected to allow accurate determination of the initial uptake rates. The intracellular drug concentrations measured at these time points, their accuracy of quantitation and the linearity of the increase in concentration over time were considered in determining the optimal drug incubation times. Furthermore, incubation times generally used in previous uptake studies in NR8383 were also considered in guiding the decision.
2.3.3.5 Determination of cell number and protein content

During method optimisation, a number of methods were assessed to determine the cell number in wells of collagen-coated 24-well plates. Initially, cell loss was assessed qualitatively by microscopic examination of the confluency of the cells remaining in wells after several DPBS washing steps, which are present in the uptake experimental protocol. The confluency of washed and unwashed cells was compared to provide a qualitative assessment of the proportion of the cells remaining.

The next method to assess cell number involved treatment of cells with 0.25% trypsin-EDTA in an attempt to enzymatically detach cells. In these experiments, cells were plated into collagen-coated 24-well plates, cultured for 16 hours and washed with DPBS equivalent to the number of times in an uptake experimental protocol. Cells were then treated with 0.25% trypsin-EDTA for 15 minutes at 37°C followed by inactivation of trypsin with CGM. Cells were dislodged by pipetting and counted under the microscope.

In the third method, the cell number was determined indirectly by measuring the amount of protein per well by BCA protein assay. Four wells per plate were allocated as representative of experimental samples. Cells were washed with DPBS in the same manner and number of times as the drug treated cells. They were then lysed in distilled water and the amount of protein was measured against a standard curve plotted for known concentrations of BSA standards against absorbance. All absorbances were measured at 562 nm using Boeco UV-Visible spectrophotometer (Boeco S22, Hamburg, Germany). In addition, cell suspensions with a range of cell concentration (0.031-1x10^6/mL) were prepared in distilled water and the protein concentrations in each suspension was measured to construct cell number versus protein concentration plot. This plot was then used to determine the cell number in experimental samples from their measured protein concentration. When plotting the curve for absorbance versus protein concentration, only absorbance values up to 1 were considered in order to maintain linearity of the curve. Furthermore, the reproducibility of the method was assessed by determining the cell number in wells before and after DPBS washes (n=20 for both conditions) following 16 hours in culture. This way, the degree of cell loss during the experiment could also be quantitatively determined.
2.3.4 *In vitro* drug uptake in NR8383

2.3.4.1 Investigation of drug uptake at single concentration in NR8383

Initially, uptake experiments were carried out at single substrate concentration of 5 µM at 37 and 4°C in order to delineate active uptake and passive diffusion processes. The reliability of the 4°C approach to accurately determine passive diffusion is often questioned due to the reduced fluidity of the cellular membrane under these conditions (Frezard and Garnier-Suillerot, 1998; Poirier et al., 2008). However, in the case of NR8383, the 4°C method was used since membrane uptake transporters are not yet characterised in this cell line, eliminating the possibility to assess this process using specific transporter inhibitors. Substrate solutions were prepared by dilution of the stock solutions in DPBS resulting in final DMSO content < 1% (v/v). In addition, substrate solutions contained 1 mM ABT, a nonspecific P450 inhibitor, for 6 out of 10 compounds, excluding terbutaline, fenoterol, rifampicin and ipratropium bromide due to literature evidence of non-P450 mediated metabolism (Section 1.4). Therefore, ABT was present both in pre-incubation and incubation medium for the remaining 6 compounds in order to avoid any potential impact of Phase I metabolism. In the case of rifampicin, which has been reported to undergo ester hydrolysis (Kenny and Strates, 1981), a preliminary experiment performed in the absence and presence of a nonspecific esterase inhibitor sodium fluoride (5 mM) did not indicate a significant difference in measured cell concentrations. This finding was in agreement with literature indication for the lack of rifampicin metabolite in NR8383 cells (Onoshita et al., 2010). Therefore, incubations were performed in DPBS buffer only.

Following microscopic examination of the cells, the CGM was removed, cells were washed twice with 800 µL of either pre-warmed or ice-cold DPBS (for experiments at 37 and 4°C, respectively) and pre-incubation with 500 µL of pre-warmed or ice-cold ABT/DPBS was carried out for 20 minutes. Where ABT pre-treatment was not necessary, cell monolayers were treated with 500 µL of DPBS only. Once pre-incubation buffer was removed from the cell monolayers, incubations were started by addition of 400 µL of substrate solution (pre-warmed or ice-cold). Each substrate was incubated with the cells for 1, 2, 5 and 10 minutes in duplicate at 37°C and in single at 4°C. For 37°C experiments, incubation was carried out on a dry heater block maintained at 37°C throughout the experiment (Tecam Dri-block DB-3, Tecam, Princeton, NJ) and for 4°C experiments, the procedure was carried out on ice. Incubation with substrate was ceased by removal of the substrate solution and rinsing the monolayers three times.
with 800 µL ice-cold DPBS. The cells were lysed by addition of 200 µL ice-cold deionised water to the wells. The removed incubation media was retained for the measurement of drug concentrations and subsequent \( K_d \) determination. Cell lysates were kept at -20°C overnight, until they were further processed the next day for analysis by LC-MS/MS. Experiments were performed on three or more separate occasions for each drug investigated. The performance of each experiment was assessed by measuring the uptake of 5 µM clarithromycin in each experiment, providing a control of the activity of the cells. The amount of protein per well was measured to determine the cell number as described in Section 2.3.3.5.

2.3.4.2 Investigation of uptake kinetics of selected drugs over a range of concentration

Following initial analysis, a subset of drugs were selected for further investigation to determine their uptake kinetics over a range of substrate concentrations over 10 minutes at 37 and 4°C. The selection included imipramine, clarithromycin, rifampicin, formoterol and tiotropium bromide in an attempt to cover a range of properties in terms of the amount of drug accumulating in NR8383 and physicochemical data. The range of substrate concentrations used for the compounds are given in Table 2-1. The maximum concentration used for imipramine was 30 µM due to preliminary experiments suggesting saturation at low concentrations. For the rest of the drugs, this wide range was chosen as the affinity of these drugs towards uptake transporters in rat alveolar macrophages were unknown.

**Table 2-1: List of compounds used in assessment of uptake kinetics and the range substrate concentrations considered in NR8383 cells.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Substrate concentrations investigated (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>1, 3, 5, 10, 20, 30, 50, 60, 80, 100</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1, 3, 5, 10, 20, 30, 50, 60, 80, 100</td>
</tr>
<tr>
<td>Formoterol</td>
<td>1, 3, 5, 10, 20, 30, 50, 60, 80, 100</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>3, 5, 10, 20, 30, 40, 50, 60, 80, 100</td>
</tr>
<tr>
<td>Imipramine</td>
<td>0.1, 0.5, 1, 3, 5, 10, 20, 30</td>
</tr>
</tbody>
</table>
In all experiments, the incubation media was collected at the end of incubation and the concentrations were measured over time in order to determine the extent of nonspecific binding ($f_{\text{u}_{\text{med}}}$: fraction unbound in the media) which includes binding of drug to cell membrane and/or experimental support upon exposure of the cells to incubation media. Therefore, the concentration of the drug in media at time zero ($t_0$) represents the free fraction remaining after nonspecific binding and drug flux into cells. The presence of proteins in the media was assumed to be negligible because the cells were rinsed thoroughly between removal of CGM and start of incubation. In addition, only a very small number of cells were likely to present floating during the incubation. The unbound concentration can therefore be expressed as the intercept of the linear regression of the measured media concentration with time plot. The $f_{\text{u}_{\text{med}}}$ was calculated from the slope of the linear regression of the unbound concentration extrapolated at $t_0$ vs. the nominal incubation concentration plot. Nominal media concentrations were then corrected for $f_{\text{u}_{\text{med}}}$ when plotting kinetic profiles (Ménochet (2012)).

### 2.3.4.3 Cytotoxicity assessment

LDH assay kit (Sigma-Aldrich, Dorset, UK) was used to measure the cytotoxicity of the compounds used in uptake kinetic experiments at their 50 and 100 µM concentrations. In addition, the LDH activity of drug untreated cells was also determined in order to assess the cell viability in the absence of compounds. The incubation medium (50 µL) was transferred into a clear flat bottom Cellstar 96-well plate (Greiner bio-one, Gloucestershire, UK) and the reaction was started by addition of assay’s 50 µL Master Reaction Mix, consisting LDH assay buffer and LDH Substrate Mix. The assay also consisted of reduced nicotinamide adenine dinucleotide (NADH) calibration standards (0-12.5 nmol) to determine the amount of NADH in experimental samples which was then used in LDH activity calculations. The LDH activity of the experimental samples was compared against the 100% activity of the LDH positive control included in the assay kit. All samples and standards were run in duplicate and absorbance values were measured using a plate reader (Tecan Safire, A-5082, Reading, UK). Data acquisition was performed using Magellan software (version 7.1, Tecan Group Ltd., Austria).

### 2.3.4.4 Sample preparation and mass spectrometry analysis

All cell lysates and media samples from kinetic experiments were thawed and quenched with methanol containing internal standard. The internal standard concentration was 1
µM for all drugs except for budesonide and rifampicin for which 0.1 µM was used. For cell lysates, equal volume of methanol/internal standard mixture (200 µL) was used. The same was also applied for the media samples, except for those with substrate concentrations of 10 µM and above which were diluted down to 2 µM with DPBS before quenching with the same volume of methanol/internal standard mixture (200 µL). This initial dilution was performed to avoid saturation of ionisation in HPLC coupled with tandem mass spectrometry (MS/MS) at high concentrations. Samples were placed at -20°C for at least an hour to precipitate the proteins, followed by their centrifugation at 2500 rpm for 10 minutes (Eppendorf Centrifuge 5804, Cambridge, UK). The supernatant (10-20 µL) was analysed by LC-MS/MS as detailed below. Samples were analysed on a Waters Alliance 2795 HPLC system coupled to a Micromass Quattro Ultima mass spectrometer (Waters, Watford, UK) using electrospray positive ionisation mode. All compounds and their internal standards were separated on Luna C18 column (3 µm, 50 x 4.6 mm) (Phenomenex, Macclesfield, UK), except for fenoterol, terbutaline, ipratropium and tiotropium bromide which were separated on Luna Phenyl Hexyl column (3 µm, 50 x 4.6 mm) (Phenomenex, Macclesfield, UK). The mobile phases used were: Solvent A, 90% water, 10% methanol, and 0.05% formic acid; Solvent B, 10% water, 90% methanol, and 0.05% formic acid; Solvent C, 90% water, 10% methanol, and 1 mM ammonium acetate; and Solvent D, 10% water, 90% methanol, and 1 mM ammonium acetate. The gradient of mobile phases varied for each drug. The flow rate through HPLC was 1 mL/min, which was then split to 0.25 mL/min before entry into the mass spectrometer. For the Waters Ultima, the capillary voltage was 3.5 kV; desolvation and source temperatures were 350 and 125°C, respectively; desolvation gas and cone gas flow rates were 600 and 150 L/hr, respectively. LC-MS/MS conditions for each individual drug and their corresponding internal standards are detailed in Table 2-2. A calibration standard, containing the compound of investigation at a concentration range covering that of the experimental samples with an additional zero blank, was prepared in the same matrix of the experimental samples. The standards were analysed twice (before and after the experimental samples) during each run, in order to confirm compound stability, consistent peak area for internal standard and any potential compound carry over. Samples were quantified against the standard curve using MassLynx v.4.1 (Waters Inc., Milford, MA). During analysis, only standards which were measured within 30% of the nominal concentration were included in the standard curve.
Table 2-2: Summary of LC-MS/MS conditions used for assessing uptake of 10 drugs in NR8383 cell line

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mass transition (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision voltage (eV)</th>
<th>Retention time (min)</th>
<th>LLOQ (µM)</th>
<th>Internal Standard</th>
<th>Mass transition (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision voltage (eV)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>748.4&gt;158.2</td>
<td>70</td>
<td>31</td>
<td>2.70</td>
<td>0.001</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>3.00</td>
</tr>
<tr>
<td>Imipramine</td>
<td>281.3&gt;86.2</td>
<td>60</td>
<td>21</td>
<td>2.40</td>
<td>0.002</td>
<td>Diazepam</td>
<td>285.1&gt;257.1</td>
<td>60</td>
<td>21</td>
<td>2.87</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>821.5&gt;789.3</td>
<td>50</td>
<td>15</td>
<td>2.70</td>
<td>0.010</td>
<td>Verapamil</td>
<td>455.3&gt;165.1</td>
<td>60</td>
<td>30</td>
<td>2.50</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>332.1&gt;314.3</td>
<td>80</td>
<td>20</td>
<td>2.30</td>
<td>0.010</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>2.70</td>
</tr>
<tr>
<td>Formoterol</td>
<td>345.3&gt;149.1</td>
<td>25</td>
<td>20</td>
<td>2.35</td>
<td>0.002</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>2.80</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>304.2&gt;107.1</td>
<td>50</td>
<td>30</td>
<td>2.56</td>
<td>0.002</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>3.13</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>226.3&gt;152.2</td>
<td>60</td>
<td>15</td>
<td>2.80</td>
<td>0.002</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>3.00</td>
</tr>
<tr>
<td>Budesonide</td>
<td>431.35&gt;413.35</td>
<td>70</td>
<td>8</td>
<td>3.48</td>
<td>0.010</td>
<td>Verapamil</td>
<td>455.3&gt;165.1</td>
<td>60</td>
<td>30</td>
<td>2.95</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>332.35&gt;166.2</td>
<td>85</td>
<td>25</td>
<td>2.80</td>
<td>0.002</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>3.10</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>392.3&gt;152.15</td>
<td>75</td>
<td>25</td>
<td>2.90</td>
<td>0.002</td>
<td>Midazolam</td>
<td>326.0&gt;291.2</td>
<td>70</td>
<td>25</td>
<td>3.12</td>
</tr>
</tbody>
</table>

1 0.1 µM; internal standard concentration 1 µM; LLOQ, Lower limit of quantification
2.3.4.5 Data analysis

2.3.4.5.1 Determination of total uptake and passive diffusion clearances

Total uptake (CL\textsubscript{uptake}) and passive diffusion (CL\textsubscript{diff}) clearances were estimated at single substrate concentration for each compound from the 37 and 4°C data, respectively. Uptake rate was determined from the slope of the linear regression of the drug cell concentration vs. time plot and normalised by the cell number determined by BCA protein assay. Uptake clearance was then estimated from the ratio of the uptake rate against the substrate concentration in the incubation medium. Where the linear regression of the drug cell concentration vs. time plot did not produce a positive slope using all the experimental incubation time points at 4°C, an attempt was made to estimate the rate using a relatively narrow range of time points that would yield an increase in cell concentration (e.g., from 2 to 10 minutes). Where it was not feasible to estimate the rate with this approach, cell concentration at time 0 was assumed to be zero and the linear regression was plotted between time 0 and the final incubation time point. Both CL\textsubscript{uptake} and CL\textsubscript{diff} were determined for each compound on a minimum three separate occasions. Active uptake clearance (CL\textsubscript{active}) was determined from the difference between CL\textsubscript{uptake} and CL\textsubscript{diff} for each drug. Maximal % contribution of passive diffusion and active process to the total uptake were calculated for each drug. In addition, the relative importance of active uptake compared to passive diffusion was expressed as the ratio of CL\textsubscript{active} against CL\textsubscript{diff}.

2.3.4.5.2 Determination of cell-to-medium partition coefficients (K\textsubscript{p}) of drugs in NR8383 cells

Accumulation of drugs in NR8383 was determined by calculating K\textsubscript{p,NR8383} of all compounds at 5 µM drug concentration at 10 minutes (final incubation time point). K\textsubscript{p,NR8383} was determined by Equation 2-1 as reported previously (Jones and Houston, 2004; Hallifax and Houston, 2006);

\[ K_p = \frac{C_{cell}}{C_{medium}} \]

where \( C_{cell} \) and \( C_{medium} \) represent concentration of the drug in the cell and medium, respectively. Both represent the measurements made at final incubation time point (10 minutes). Here, the \( K_p \) represents partition into total cell and reflects intracellular binding, subcellular distribution and active and passive uptake processes. \( C_{cell} \) was calculated by multiplying the measured lysate concentration with lysate volume (200
µL) and dividing this amount by the cell volume (1.04 µL/10⁶ cells, CV=24%) and number measured per well. Cell volume was calculated by multiplying the reported NR8383 volume of 4.2 µL/mg protein (Togami et al., 2010) with the amount of protein measured experimentally (0.25 ± 0.06 mg/10⁶ cells). Cₘₐₜₜ is the measured total medium concentration, not corrected for nonspecific binding to experimental support (fuₘₐₜ). This correction was not performed because fuₘₐₜ could not be determined for half of the drugs investigated. For 5 drugs which it was determined (Table 2-1), fuₘₐₜ was >0.83 (Tables 2-6 and 3-6), therefore it was assumed that binding to experimental support was not significant. Both Cₜₜ and Cₘₐₜ were measured in duplicates in each experiment. Kₚ,NR8383 was determined as a mean of at least 3 experiments done on separate occasions.

While Kₚ,NR8383 was determined at 5 µM for all drugs, the accumulation of 5 drugs including imipramine, clarithromycin, formoterol, rifampicin and tiotropium bromide was characterised over a concentration range (Table 2-1) at 10 minutes of incubation. In the case of imipramine, a wider concentration range (0.1-100 µM) relative to that used in uptake kinetic parameter estimation was used to determine Kₚ,NR8383.

The concentration dependence of drug accumulation was investigated by fitting the Kₚ,NR8383 and concentration data by nonlinear regression to a two-site model incorporating a saturable site and a linear function for unsaturated binding (Equation 2-2) in Grafit v6.0.6 software (Erithacus Software Limited, Horley, UK) (Hallifax and Houston, 2007).

Equation 2-2  

\[ K_p = K_{p,U1,max} \frac{K_{p,U1,max} C_{medium}}{K_{U1} + C_{medium}} + K_{p,min} \]

where Kₚ,U1,max is maximum Kₚ for saturable uptake, Kₚ,min is Kₚ for nonsaturable uptake and Kₚ,U1 is apparent saturable uptake equilibrium constant (drug concentration at which saturable uptake is half of its maximum). Kₚ for maximum total uptake was calculated as the sum of Kₚ,U1,max and Kₚ,min (Hallifax and Houston, 2007).

2.3.4.5.3 Determination of uptake kinetic parameters

The estimation of uptake kinetic parameters was performed using the conventional two-step method (Poirier et al., 2008; Ménochet et al., 2012). First, uptake rates were calculated for each substrate concentration studied as described in Section 2.3.4.5.1. Both the nominal substrate concentrations corrected for nonspecific binding and the uptake rates were put into Grafit v6.0.6 (Erithacus Software Limited, Horley, UK) for
the estimation of active uptake kinetic parameters. CL\textsubscript{diff}, corrected for nonspecific binding, was estimated from the slope of the linear regression of the substrate concentration vs. uptake rates estimated at 4°C and used as a constant parameter in Equation 2-3. Nonlinear regression was used to estimate kinetic parameters $K_m$ and $V_{max}$.

Equation 2-3

$$v = \frac{V_{\text{max}} \times S_{\text{med}}}{K_m + S_{\text{med}}} + CL_{\text{diff}} \times S_{\text{med}}$$

where $v$ is the uptake rate and $V_{max}$ is the maximum uptake rate, both expressed in pmol/min/10\textsuperscript{6} cells. $K_m$ is the Michaelis-Menten constant. $S_{\text{med}}$ is the nominal media concentration corrected for $f_u_{\text{med}}$, both are expressed in $\mu$M. CL\textsubscript{active} was estimated from the ratio of $V_{max}$ over $K_m$. CL\textsubscript{uptake} represents the sum of CL\textsubscript{active} and CL\textsubscript{diff}. All clearances were expressed as $\mu$L/min/10\textsuperscript{6} cells.

2.3.4.6 Statistical analysis

The arithmetic mean, standard error and coefficient of variation (CV) were calculated for uptake clearances (CL\textsubscript{uptake} and CL\textsubscript{diff}) and $K_{p,NR8383}$ which were determined on at least three different occasions. A box plot was generated for clarithromycin which was used as a control in each experiment to observe the distribution of the clearance across experiments.
2.4 Results

2.4.1 Optimal in vitro conditions for drug uptake

Several in vitro conditions were assessed for optimisation of uptake methodology. These conditions included cell detachment method from culture flasks, coating type of 24-well plates, cell seeding density per well, culturing time of the cells, drug incubation time points and determination of the cell number in wells. The overall scheme of method optimisation is shown in Figure 2-1. For cell detachment, no difference was observed between gentle scraping and Mg²⁺-Ca²⁺ free DPBS treatment in terms of cell viability, attachment to 24-well plates, confluency following culture and clarithromycin uptake clearance. Among the three types of 24-well plates tested, collagen coated plates resulted in significantly better cell attachment compared to non-coated plates. There was no significant difference in cell attachment, distribution and drug uptake between collagen and poly-D-lysine coated plates; however, qualitative assessment of the cells showed slightly less cell loss in collagen coated plates.

Extended culture times also did not show much improvement in attachment in non-coated plates, with up to 30% of the cells remained floating in wells. In contrast, 16 hour culturing resulted in almost complete adherence and >80% confluency of cells with even distribution in the wells in collagen-coated plates, which was not significantly improved over longer culture times. Therefore, 16 hours was selected as an optimal culture period, allowing the experiments to be commenced and completed within reasonable times of the day.

In order to determine the optimal cell seeding density per well, the plating efficiency was determined by measuring the number of attached cells in wells after 16 hour culturing. Plating efficiency was similar (~70%) at both seeding densities used (0.36 and 0.72 x 10⁶ cells/well). A representative example of the plating efficiency obtained at 0.72 x 10⁶ cells/well seeding density is illustrated in Figure 2-2. Almost 75% of the cells initially seeded were removed with DPBS washings. This was also clear from the microscopic examination of the wells following clarithromycin uptake. Higher confluency was observed in wells at higher seeding density, however, slightly more cells were observed to become detached with PBS washings. Therefore, 0.5 x 10⁶ cells/well was selected as the optimal seeding density to provide larger number of cells to start with and to minimise extensive cell loss from 24-well plates. Nonetheless, cell loss with washings was observed to be unavoidable and consistent later across
experiments at this seeding density as well, with as much as 95% of the cells (0.01 x 10^6 cells measured with CV < 10%) being removed in some instances.

**Figure 2-1:** Schematic of optimisation of drug uptake conditions for NR8383 cells.

*LLOQ: limit of quantification*
Three different methods were tested to determine the cell number in wells. Qualitative microscopic examination of the plates was subjective and did not provide definite measurements. Second method, trypsinisation, was inefficient in detaching all the cells to be counted later. Furthermore, this method resulted in very few cells to be counted in the mm² area of the haemocytometer, compromising the accuracy of the cell count. Definitive and more accurate measurement of cell number was achieved with protein measurement using BCA protein assay. The reproducibility of the assay involving measurement of absorbance in BSA and NR8383 cell standards, and relation of BSA concentration to NR8383 cell number was illustrated in Appendix Figures 7-1, 7-2 and 7-3, respectively. The assay showed good reproducibility in absorbance measurement of BSA samples with CV values less than 20% at most protein concentrations, except for the lowest BSA concentration (25 µg/ml) which had CV of 41% (Appendix Figure 7-1). The absorbance measurement of NR8383 cell standards also showed reasonable reproducibility at most cell numbers (CV<31%) except for the lowest cell number (0.031 x 10⁶ cells) which showed 40% variation (Appendix Figure 7-2). Similar trends were also observed when relating measured protein concentration to cell number. The CV was less than 35% when converting BSA concentration to NR8383 cell number at all times except for the conversion to the lowest cell number which had a CV of 51% (Appendix Figure 7-3). The measurement of protein in wells of experimental plates was also reproducible (CV ~20%) across a large number of repeated measurements (n=20). However, in some instances (usually when measuring cell number < 0.1 x 10⁶ cells) CV
of 4 wells was more than 30% within an experiment, compromising the accuracy of cell number determination.

During method optimisation, clarithromycin was used as a control compound at 1 µM concentration with incubation times from 0.5 to 5 minutes. Despite linearity in uptake, the measured intracellular concentrations were very low and close to its limit of quantification, particularly at the earliest time point. In addition, there was no increase in observed intracellular concentration from 0.5 to 1 minute. Therefore, drug uptake was further optimised in terms of substrate concentration (including clarithromycin as control) and incubation time points as 5 µM and 1, 2, 5 and 10 minutes, respectively.

2.4.2 Determination of uptake clearances of selected drugs in NR8383 cells

Uptake of 10 drugs was assessed in NR8383 at single substrate concentration (5 µM) at 37 and 4°C in order to delineate active uptake and passive diffusion processes. Estimates of total uptake and passive diffusion clearances of all drugs investigated are shown in Table 2-3. The trends in Cl\textsubscript{active} and Cl\textsubscript{diff} are also presented in Figure 2-3. More than a 400-fold range in Cl\textsubscript{uptake} in NR8383 was observed for the current dataset. Imipramine showed the highest uptake clearance (16.7 µL/min/10\textsuperscript{6} cells) in NR8383 with maximal contribution of the passive process to the total uptake of 30.2%. Its Cl\textsubscript{active} was 11.7 µL/min/10\textsuperscript{6} cells. The second highest uptake clearance was observed for clarithromycin with 6 µL/min/10\textsuperscript{6} cells, whereas Cl\textsubscript{uptake} ranged between 0.04 – 2 µL/min/10\textsuperscript{6} cells for the remaining 8 drugs.
Table 2-3: Total uptake (CL<sub>uptake</sub>) and passive diffusion (CL<sub>diff</sub>) clearances and % contribution of passive process (CL<sub>diff</sub>/CL<sub>uptake</sub>) estimated at 5 μM substrate concentration in NR8383. Data represent mean of at least 3 experiments ± SD

<table>
<thead>
<tr>
<th>Drug</th>
<th>CL&lt;sub&gt;uptake&lt;/sub&gt; (µL/min/10&lt;sup&gt;6&lt;/sup&gt; cells)</th>
<th>CL&lt;sub&gt;diff&lt;/sub&gt; (µL/min/10&lt;sup&gt;6&lt;/sup&gt; cells)</th>
<th>CL&lt;sub&gt;diff&lt;/sub&gt;/CL&lt;sub&gt;uptake&lt;/sub&gt; (%)</th>
<th>CL&lt;sub&gt;active&lt;/sub&gt;/CL&lt;sub&gt;diff&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>16.7 ± 4.30</td>
<td>5.06 ± 1.70</td>
<td>30.2</td>
<td>2.31</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>6.00 ± 3.66</td>
<td>0.03 ± 0.03</td>
<td>0.53</td>
<td>188</td>
</tr>
<tr>
<td>Formoterol</td>
<td>2.02 ± 0.73</td>
<td>0.08 ± 0.08</td>
<td>3.71</td>
<td>26.0</td>
</tr>
<tr>
<td>Budesonide</td>
<td>1.50 ± 0.74</td>
<td>1.46 ± 0.90</td>
<td>97.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1.47 ± 0.44</td>
<td>0.03 ± 0.01</td>
<td>1.87</td>
<td>52.4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1.33 ± 0.43</td>
<td>0.30 ± 0.20</td>
<td>22.3</td>
<td>3.48</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>0.36 ± 0.31</td>
<td>0.06 ± 0.04</td>
<td>16.3</td>
<td>5.15</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>0.32 ± 0.17</td>
<td>0.03 ± 0.01</td>
<td>8.03</td>
<td>11.5</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>0.24 ± 0.19</td>
<td>0.01 ± 0.01</td>
<td>5.19</td>
<td>18.3</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>47.6</td>
<td>1.10</td>
</tr>
</tbody>
</table>
Figure 2-3: Active uptake (CL\textsubscript{active}, ■) and passive diffusion (CL\textsubscript{diff}, □) clearances of 10 drugs in NR8383. Data represent mean ± SD of at least 3 separate experiments.

A large variation in clarithromycin CL\textsubscript{uptake} used as a control was observed across a large number of experiments (n=59, CV=54\%) (Figure 2-4). Over 100-fold difference between estimated CL\textsubscript{active} (5.9 µL/min/10\textsuperscript{6} cells, CV=43\%) and CL\textsubscript{diff} (0.03 µL/min/10\textsuperscript{6} cells) was observed for clarithromycin, suggesting a dominant contribution of an active process to its accumulation in NR8383 at the concentration investigated. Difference in 37°C and 4°C data indicated that the active process was the major contributor to the accumulation of the majority of the drugs investigated (Figure 2-3). The exception was budesonide where contribution of passive diffusion to accumulation in NR8383 was ≥90\% (CL\textsubscript{active}/CL\textsubscript{diff} of <1). Moderate contribution of passive diffusion was observed
for terbutaline and imipramine (30-48%). Formoterol showed an uptake clearance of 2 µL/min/10^6 cells and similar to clarithromycin, its accumulation appears to be driven by active process (with CL_{active} of 1.94 µL/min/10^6 cells and CL_{active}/CL_{diff} of 26-fold). Budesonide, rifampicin and ciprofloxacin showed comparable CL_{uptake} in NR8383 (1.3-1.5 µL/min/10^6 cells), but a marked difference was seen in the contribution of passive diffusion to their uptake, ranging from 1.87% in the case of rifampicin to almost solely passive uptake observed in the case of budesonide. The remaining respiratory drugs, terbutaline, fenoterol, ipratropium and tiotropium bromide were marginally uptaken in NR8383 cells with a CL_{uptake} of < 1 µL/min/10^6 cells.

**Figure 2-4**: Box plot of the total uptake clearance (µL/min/10^6 cells) of 5 µM clarithromycin in NR8383. The data were obtained from 59 experiments in total. — represents the median, ◇ is the mean, box is for the 25th and 75th percentiles, whiskers are for 5th and 95th percentiles.

**2.4.3 Determination of K_{p,NR8383} of all drugs in NR8383 at 5 µM**

Drug concentrations in NR8383 were measured for 10 compounds at 5 µM substrate concentration up to 10 minutes at 37°C. Concentration vs. time profiles of all compounds are illustrated in Figure 2-5. The cellular concentrations showed large variability for all drugs, partially as a reflection of different number of cells remaining in wells following washing steps between different experiments. In addition, at time 0, cell concentrations were not zero for most drugs.
Figure 2-5: Concentration-time profiles of 10 drugs investigated at 5 µM substrate concentration in NR8383 cells. Data represent mean ± SD of at least 3 separate experiments.
This was most likely due to some of the drug binding to the cell surface which was not removed during the washing steps and being released when methanol was added to the plates during sample preparation for LC-MS/MS analysis. For all drugs, the amount of drug estimated to be present at time zero (from the intercept of the concentration-time curve) was less than 2.5% of the amount of drug present in the incubation, except for imipramine for which it was 6.5%.

Measured cell concentrations of all drugs at 10 minutes were used to determine cell-to-medium concentration ratios. The results are shown in Table 2-4. There was almost a 570-fold range in observed $K_{p, NR8383}$ between compounds. The most extensive accumulation in NR8383 was observed for imipramine which showed $K_{p, NR8383}$ of 391. The second highest accumulation was observed for clarithromycin for which $K_{p, NR8383}$ was 81.6. Ciprofloxacin, rifampicin, budesonide and formoterol accumulated in NR8383 to a similar extent with their $K_{p, NR8383}$ values ranging between 24.5 and 43. For the remaining compounds, relatively less partitioning into cells was observed ($K_{p, NR8383} <10$). Tiotropium and ipratropium bromide and fenoterol achieved a cell-to-medium concentration ratio between 5 and 9. In the case of terbutaline, the media concentration of the compound remained higher than its cell concentration throughout the incubation period, leading to a $K_{p, NR8383}$ of 0.69 at 10 minutes.

**Table 2-4:** Cell-to-medium concentration ratios of 10 drugs in NR8383 cells at 5 µM drug concentration. Data represent mean ± SD of at least 3 separate experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{p, NR8383}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>391 ± 108</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>81.6 ± 22.8</td>
</tr>
<tr>
<td>Formoterol</td>
<td>24.5 ± 8.71</td>
</tr>
<tr>
<td>Budesonide</td>
<td>37.6 ± 11.4</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>38.6 ± 18.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>43.0 ± 21.1</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>8.74 ± 7.24</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>5.69 ± 0.98</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>5.42 ± 3.38</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.69 ± 0.16</td>
</tr>
</tbody>
</table>
2.4.4 Determination of $K_{p, NR8383}$ of selected drugs over a concentration range

The cellular drug concentrations at the final incubation time point (10 min) obtained in kinetic studies were used to determine the $K_{p, NR8383}$ of 5 drugs. This incubation time did not represent equilibrium between intracellular and extracellular drug concentration for investigated drugs although for imipramine, the cellular concentrations observed at 5 minutes were close to that at 10 minutes at concentrations above 30 µM, indicating an attainment of equilibrium at high concentrations. A concentration dependent accumulation was observed for all investigated drugs (Figure 2-6). Fitting of the two-site binding model to the experimental data was performed for 3 drugs (imipramine, clarithromycin and formoterol) where uptake parameters $K_{p,U1,max}$, $K_{p,min}$ and $K_{U1}$ were estimated. For rifampicin and tiotropium bromide, uptake parameter estimates could not be determined accurately using the two-site binding model. For these two drugs, the uptake profile was hyperbolic and the second binding site was lacking, indicating the two-site binding model was not appropriate. Therefore, for these two drugs, fitting was not performed. Consequently, the data presented in Table 2-5 represent estimated values for imipramine, clarithromycin and formoterol, and the actual data points for rifampicin and tiotropium bromide. The mean maximum fitted $K_{p, NR8383}$ was 1166 for imipramine and 69 for clarithromycin and formoterol at concentrations below 0.1 and 1 µM, respectively. These values decreased to an estimated minimum of 141, 24 and 10 for imipramine, clarithromycin and formoterol, respectively. The apparent saturable uptake equilibrium constants were between 0.9 and 1.5 µM for these three drugs. The fold difference between $K_{p, NR8383,max}$ and $K_{p, NR8383,min}$ of these drugs ranged between 2.9 and 8.3. For rifampicin and tiotropium bromide, the observed $K_{p,max}$ values were 35 and 1.7, respectively. The data were highly variable for rifampicin (CV >50%). The minimum accumulation values observed for rifampicin and tiotropium bromide were 13 and 0.56, respectively. The $K_{p,max}/K_{p,min}$ ratios were close to 3 for both drugs.
Figure 2-6: Cell-to-medium concentration ratios of 5 drugs investigated over a range of concentration in NR8383 cells. Data represent mean ± SD of 3 experiments except for tiotropium (n=2) and the first two data points of imipramine (n=1). The fitting was obtained for 3 drugs using a two-site binding model (Equation 2-1) in Grafit v6.0.6. A) imipramine (■); B) clarithromycin (●); C) formoterol (▲); D) rifampicin (●); E) tiotropium bromide (▼).
Table 2-5: Uptake parameter values for five drugs investigated in NR8383 over a concentration range. Data represent mean ± SD of 3 experiments except for tiotropium bromide for which data are from 2 experiments. $K_{p,U1,max}$, $K_{p,min}$ and $K_{U1}$ data are estimated for imipramine, clarithromycin and formoterol whereas data for rifampicin and tiotropium bromide represent the actual data.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{p,U1,max}$</th>
<th>$K_{p,min}$</th>
<th>$K_{U1}$ (µM)</th>
<th>$K_{p,max}$</th>
<th>$K_{p,max}/K_{p,min}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>1025 ± 99.6</td>
<td>141 ± 30.6</td>
<td>0.9 ± 0.3</td>
<td>1166</td>
<td>8.3</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>44.9 ± 16.6</td>
<td>24.2 ± 2.06</td>
<td>1.18 ± 1.45</td>
<td>69</td>
<td>2.9</td>
</tr>
<tr>
<td>Formoterol</td>
<td>59.2 ± 8.77</td>
<td>10.0 ± 0.92</td>
<td>1.54 ± 0.49</td>
<td>69</td>
<td>6.9</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>ND</td>
<td>13.0 ± 3.34</td>
<td>ND</td>
<td>35.2 ± 22.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>ND</td>
<td>0.56 ± 0.06</td>
<td>ND</td>
<td>1.66 ± 0.24</td>
<td>3.0</td>
</tr>
</tbody>
</table>

ND: not determined; Actual data point at highest (⁎) and lowest (⁎) substrate concentration
2.4.5 Estimation of uptake kinetic parameters of selected drugs in NR8383 cells

Following the assessment of drug uptake at a single concentration, kinetics of drug uptake was investigated for five drugs including imipramine, clarithromycin, rifampicin, formoterol and tiotropium bromide. Uptake kinetic parameters were estimated using the conventional two-step method for 4 out of 5 drugs (Table 2-6) and the concentration-rate profiles obtained for all drugs are shown in Figure 2-7. All drugs investigated showed saturable uptake under 37°C conditions, with the exception of clarithromycin and formoterol for which concentration-rate profile showed a linear relationship up to 100 µM (Figure 2-7D and E, respectively). The highest $K_m$ was obtained for clarithromycin (>500 µM) for which both kinetic parameters could not be determined accurately under these conditions (CV for $K_m$ and $V_{max}$ was >100%). A 129-fold range in $K_m$ was observed among the compounds excluding clarithromycin.

The second highest $K_m$ was obtained for formoterol (172 µM). In the case of tiotropium bromide and rifampicin, $K_m$ was comparable (19.4 and 17.2 µM, respectively). Imipramine showed the lowest $K_m$ with a value of 1.3 µM although large variability was associated with the value (CV>100%). While the $K_m$ of imipramine was in agreement with its estimated $K_{U1}$ (Table 2-5), in the case of formoterol and clarithromycin, a large discrepancy was observed between the values, possibly reflecting differences in fitted models. Compared to the observed range in $K_m$, the fold range in $V_{max}$ was less pronounced (50-fold) between the investigated drugs excluding clarithromycin. The $V_{max}$ ranged from 4.21 to 213 pmol/min/10$^6$ cells for tiotropium bromide and formoterol, respectively. $CL_{diff}$ ranged by at least 100-fold, from 0.03 to 5.5 µL/min/10$^6$ cells for tiotropium bromide and imipramine, respectively.

$CL_{active}$, which was defined from the ratio of $V_{max}$ and $K_m$, showed the highest fold range (276-fold) among imipramine, rifampicin, formoterol and tiotropium bromide. The parameter was as low as 0.22 µL/min/10$^6$ cells for tiotropium bromide and highest for imipramine (60.8 µL/min/10$^6$ cells). These estimates were comparable with the active uptake clearance values obtained at the single concentration data (5 µM) for rifampicin, formoterol and tiotropium bromide. Likewise, their $CL_{uptake}$ was in agreement with their $CL_{uptake}$ estimated at 5 µM. In contrast, imipramine $CL_{active}$ was almost 5-fold higher than the value obtained at 5 µM (11.6 µL/min/10$^6$ cells); this discrepancy can be rationalised by the fact that single concentration used exceeded the $K_m$ of imipramine and therefore, clearance might have been measured at saturating rather than linear conditions.
<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/10^6 cells)</th>
<th>$CL_{diff}$ (µL/min/10^6 cells)</th>
<th>$CL_{active}$ (µL/min/10^6 cells)</th>
<th>$CL_{uptake}$ (µL/min/10^6 cells)</th>
<th>$fu_{med}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>1.33 ± 1.53</td>
<td>80.8 ± 22.4</td>
<td>5.51 ± 0.20</td>
<td>60.8</td>
<td>66.3</td>
<td>0.83 ± 0.05</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>17.2 ± 7.59</td>
<td>53.1 ± 7.38</td>
<td>0.06 ± 0.01</td>
<td>3.08</td>
<td>3.14</td>
<td>0.88 ± 0.08</td>
</tr>
<tr>
<td>Formoterol</td>
<td>172 ± 88.2</td>
<td>213 ± 80.5</td>
<td>0.04 ± 0.01</td>
<td>1.24</td>
<td>1.28</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>19.4 ± 12.9</td>
<td>4.21 ± 0.91</td>
<td>0.03 ± 0.01</td>
<td>0.22</td>
<td>0.25</td>
<td>1.00 ± 0.10</td>
</tr>
</tbody>
</table>

All parameters have been corrected for nonspecific binding.
Figure 2-7: Uptake kinetic profiles of five selected drugs in NR8383 cells. Data points are the uptake rates measured in duplicate over 10 minutes. Solid and dashed lines represent total uptake and passive diffusion, respectively. Data represent mean ± SD of 3 experiments.

(A) Total uptake at 37°C; (B) Passive diffusion at 4°C. A) rifampicin; B) tiotropium bromide; C) imipramine; D) clarithromycin; E) formoterol.
2.4.6 Assessment of cytotoxicity of high drug concentrations

Cytotoxicity of the compounds used in uptake kinetic studies (clarithromycin, imipramine, formoterol, rifampicin and tiotropium bromide) was assessed. While 100 µM was the highest substrate concentration used in the studies, 50 µM was also included in the assessment to determine the effect of lower concentrations if any cytotoxic effect would be encountered at 100 µM. Minimal cytotoxicity was observed for all compounds at both substrate concentrations; LDH activity was less than 5% compared to 100% positive control, except for 100 µM clarithromycin for which LDH activity was 10% that of the control.
2.5 Discussion

2.5.1 Optimal in vitro conditions for drug uptake in NR8383

In this chapter, the rat alveolar macrophage cell line NR8383 was used as an in vitro system to characterise uptake of 10 drugs which were mainly respiratory. This cell line was originally derived from primary alveolar macrophages of Sprague-Dawley rats which attained characteristics of a continuous cell line over long term culture (Helmke et al., 1987). The NR8383 cells have previously been shown to exhibit several characteristics and functions of freshly isolated rat alveolar macrophages as described in Section 1.3.2.2 (Helmke et al., 1987; Helmke et al., 1989). Cell lines provide the advantage of the use of a continuous system, allowing large number of experiments to be performed in the same system. This approach can also reduce the large variability in data commonly associated with the use of primary cells obtained from different animals or humans. However, it is expected that cell lines may not represent the in vivo conditions due to changes in cell physiological characteristics over long term culture (Pan et al., 2009). Therefore, it is important to compare cell lines with their primary counterparts to establish any differences or similarities between the systems. With this regard, the work presented here for NR8383 provides a scope for future comparison of the two systems in terms of drug accumulation.

One other factor that needs to be taken into account in the use of cell lines is their appropriate maintenance in culture. A number of well-known issues are cross-contamination and over-subculturing of the cell lines which lead to wrong and misleading results as summarised previously by Hughes et al. (2007). In their review, they pointed out that cell lines behave differently with increased passage number. The effects of over-passaging cell lines on cell line morphology, development and gene expression have been well documented on a number of cell lines including Caco-2 cells (Sambuy et al., 2005). Therefore, cell passage number is considered to be an important factor for cell line related/associated variability which can lead to uncertainty in results. In the current work the passage number of NR8383 cells was limited to 25 to ensure the morphological and functional properties of the cell line are maintained. This passage number is in good agreement with maximum passage numbers used for this cell line in other studies (Riley et al., 2005; Stokes and Surprenant, 2009; Bhattacharjee et al., 2010). Nevertheless, large variability in in vitro data was still evident which may be
associated with different passage numbers. The impact of different passage numbers on NR8383 behaviour in culture and gene expression is currently unknown.

The plated uptake methodology used to study drug uptake was adapted from Ménochet et al. (2012) and optimised for the NR8383 cell line. One of the biggest challenges in the adaptation was the semi-adherent nature of the NR8383 cells in culture which required a number of modifications in the original method. These modifications included increase in cell seeding density in the collagen-coated 24-well plates and culture time of 16 hours prior to uptake experiments to allow adherence of the cells to the wells. In addition, the method to determine the cell number in each experiment had to be modified. While long term culture allowed almost complete adherence of the cells to collagen-coated wells prior to uptake studies, cell loss during the experiments was unavoidable. In resolving this issue, determining the cell number indirectly from protein measurement provided a reproducible and more accurate approach compared to qualitative methods such as microscopic examination. One assumption made using this approach was that the wells allocated in the 24-well plates to measure cellular protein and the estimates obtained were representative of all experimental wells. Although the cell number can vary to some extent between different wells, this variation was less than 20% in most cases. Therefore, it was reasonable to assume that when all the wells are treated in the same manner, the representative wells would reflect the experimental wells in terms of cell number.

2.5.2 Determination of uptake clearances at single concentration in NR8383 cells

In this chapter, total uptake and passive diffusion clearances of 10 drugs were measured in NR8383 cells at 5 µM substrate concentration. The analysis of drug uptake at 37°C showed a wide range of accumulation of the investigated respiratory drugs in NR8383, as over a 400-fold range was observed in CL_{uptake} of compounds investigated. The highest total uptake clearance was observed for imipramine (16.7 µL/min/10^6 cells) and clarithromycin (6 µL/min/10^6 cells). For budesonide, rifampicin, formoterol and ciprofloxacin, total uptake clearance ranged between 1-2 µL/min/10^6 cells whereas for the remaining drugs, CL_{uptake} was less than 1 µL/min/10^6 cells.

The delineation of active uptake and passive diffusion processes was performed by carrying out experiments at 4°C in parallel to 37°C incubations. Currently, this is still a widely used approach to estimate passive diffusion process in uptake studies, as transporter activity is reduced to a minimum at this temperature. Indeed, this approach
has often been used when assessing passive diffusion of a number of drugs in AMs (Hand and King-Thompson, 1982; Heyneman and Reasor, 1986; Togami et al., 2013). There was over a 500-fold range in CL$_{diff}$ of 10 compounds investigated in NR8383. Out of all compounds, only imipramine and budesonide which are both lipophilic (Appendix Table 1-4) seem to be taken up by passive diffusion to a considerable level. While passive diffusion was only a moderate contributor to imipramine uptake, it was almost the only process occurring for budesonide. Previously, budesonide was also shown to be transported through Calu-3 cells by passive diffusion (Borchard et al., 2002). Its reported apparent permeability ($P_{app}$) in Caco-2 cell line was $>$100 nm/s (Appendix Table 7-1). Even though clarithromycin is also a moderately lipophilic compound (Table 1-4), CL$_{diff}$ of this compound was very low (0.5% of CL$_{uptake}$) compared to imipramine and budesonide. Recently, Togami et al. (2013) has reported a 90% reduction in $K_p$ of clarithromycin at 4°C ($K_p$ of 1.5) compared to its value at 37°C ($K_p$ of 15.7) in NR8383. Moreover, the uptake of this compound in isolated lung cells (likely to include AMs) was also found to be reduced by 90% at this low temperature relative to its uptake at 37°C (Kohno et al., 1990). Therefore, these studies provide further evidence for the contribution of the active process to the uptake of clarithromycin in alveolar macrophages. One reason for the low passive diffusion of this compound, despite its moderate lipophilicity, could be its relatively larger molecular size and polar surface area (Table 4-1) compared to imipramine and budesonide which can limit its permeation through membranes at low temperature. The same rationale can also be applied for rifampicin findings as it is a relatively larger molecule than clarithromycin. While the passive diffusion clearance or its contribution to total uptake was determined with higher confidence for compounds such as imipramine, budesonide and ciprofloxacin, the confidence in determining this process was relatively low for a number of compounds such as terbutaline, fenoterol, ipratropium and tiotropium bromide. This was due to the large variability observed across experiments and the analytical challenges in quantifying low drug concentrations at 4°C (applicable to most drugs in the current dataset). In addition, for the latter compounds, concentrations measured at 37 and 4°C were similar, therefore not indicating a clear difference between the two conditions (particularly evident in the case of terbutaline). Nonetheless, some literature regarding permeability characteristics of some of these compounds have been reported. For instance, measured terbutaline and fenoterol $P_{app}$ was $<$ 10 nm/s in most studies performed in Caco-2 and was comparable to $P_{app}$ of mannitol which is a
paracellular marker (Appendix Table 7-1). Indeed, these two hydrophilic compounds have been reported to be transported paracellularly although some transcellular diffusion and contribution of active transport have also been indicated (Avdeef et al., 2005). Poor membrane permeability was also indicated for hydrophilic and permanently cationic ipratropium and tiotropium bromide (Nakamura et al., 2010). For instance, $P_{\text{app}}$ of ipratropium has been reported to be less than 1 nm/s in porcine bronchial epithelial tissue (van Zyl et al., 2008). Therefore, for these mentioned cationic compounds, uptake into NR8383 may occur either by transporters or endocytosis as suggested for some cationic hydrophilic drugs (Fischer et al., 2012). In the case of moderately lipophilic formoterol and rifampicin, some passive diffusion would be expected from their relatively higher apparent permeability data obtained in Caco-2 cells (Appendix Table 7-1), although this mechanism is not likely to be the predominant process for uptake in NR8383 cells. In fact, active transport mechanisms have been proposed for compounds other than clarithromycin including formoterol, ipratropium and tiotropium bromide, rifampicin and ciprofloxacin in different cell systems. For instance, formoterol has been suggested to be a substrate for OCT3 and OCTN1/2 in human bronchial smooth muscle cells and airway epithelial cells, respectively (Horvath et al., 2007a; Horvath et al., 2007b). Similarly, both ipratropium and tiotropium bromide have been shown to be transported mainly by OCTN2 and to a lesser extent by OCTN1 in OCTN-expressing human embryonic kidney 293 cells (Nakamura et al., 2010). In addition, experiments using OCT-expressing HEK293 cells have demonstrated these two compounds as substrates for human and rat OCT1 and OCT2 (Nakanishi et al., 2011). The reported affinity values of ipratropium bromide for these transporters are given in Appendix Table 7-2. In the case of rifampicin, it was shown to be a substrate for OATP1B1 and OATP1B3 in OATP-expressing HEK293 cells (Yamaguchi et al., 2011) and Xenopus laevis oocytes in which it showed high affinity ($K_m$ 2.3-13 µM) for these two transporters (Appendix Table 7-2). For ciprofloxacin transport, involvement of OCTs, OATP2B1 and OATP1A2 was demonstrated in Calu-3 cells and OATP-expressing Xenopus laevis oocytes (Maeda et al., 2007; Ong et al., 2013). Moreover, uptake of ciprofloxacin was found to be reduced by 77% in J774 mouse macrophages when incubated at 4°C (Michot et al., 2005). However, contradictory results also exist with regards to uptake mechanisms of ciprofloxacin and rifampicin. For instance, $K_p$ of these two drugs was shown to be independent of cell viability at 37°C when previously assessed in either mouse peritoneal or human alveolar macrophages (Johnson et al.,
1980; Easmon and Crane, 1985). Consequently, the authors suggested passive diffusion as the main mechanism for their uptakes in macrophages. However, when Johnson et al. (1980) measured rifampicin uptake in viable human AMs at 4°C, they observed a significant reduction in its cellular uptake compared to its accumulation at 37°C as in agreement with the results presented here (Johnson et al., 1980). Consequently, this observation made the authors suggest that the membrane permeability of this compound was altered at low temperature.

Unlike for formoterol, there is no evidence whether OCTs or OCTNs are involved in transport of other cationic beta-2 agonists fenoterol and terbutaline, although these have been shown to be potent inhibitors of uptake of prototype substrate tetraethylammonium in renal proximal tubules in rats, with an apparent inhibition constant $K_i$ of 0.24 and 0.29, respectively (Brändle et al., 1992). Therefore, considering with the previously mentioned permeability characteristics of these cationic compounds, they are also likely to be taken up by endocytosis or organic cation transporters which remain to be investigated.

2.5.3 Determination of $K_{p, NR8383}$ of compounds in NR8383 cells

The accumulation of compounds in NR8383 cells was assessed by determining their cell-to-medium concentration ratios at 5 µM at 10 minutes of incubation at 37°C. At this substrate concentration, the cellular accumulation of all drugs was linear over time, indicating equilibrium with external medium was not attained. Consequently, $K_{p, NR8383}$ values do not truly reflect the steady-state condition for all investigated compounds. Similar to the wide range in $C_{L,uptake}$ observed, there was a 550-fold range in $K_{p, NR8383}$ for all compounds investigated at 5 µM. Imipramine and clarithromycin, the two most lipophilic basic drugs in the set, showed the highest accumulation in NR8383 with $K_{p, NR8383}$ of 391 and 81.6, respectively. The high accumulation of these two compounds in NR8383 is in line with previous studies which showed high accumulation of CADs (e.g., chlorphentermine) and macrolides (e.g., clarithromycin) in AMs in vitro (Heyneman and Reasor, 1986; Togami et al., 2013) (Appendix Tables 7-6 and 7-7). A number of studies have also reported substantial imipramine accumulation in isolated perfused lungs of rats ($K_p$ 17-44 depending on concentration), or other cell systems including rat hepatocytes ($K_{p,max}$ 360) relative to external medium (Junod, 1972; Drew et al., 1981; Hallifax and Houston, 2006). For budesonide, rifampicin, formoterol and ciprofloxacin, $K_{p, NR8383}$ was in the range of 25-43. For the remaining drugs, much less
accumulation was observed; $K_{p,NR8383}$ ranged between 0.5-10. For terbutaline, $K_{p,NR8383}$ was 0.69, suggesting the incubation time was not long enough for it to accumulate in the cells to a greater extent than the incubation medium, considering its low lipophilicity (Table 1-4) and high ionisation (93.6% at pH 7.4). Recently, Togami et al. (2013) has reported $K_p$ of clarithromycin in NR8383 cells as 15.7 at 10 minutes at 37°C which is about 5-fold lower than our observed $K_{p,NR8383}$ value at this time point. This can be explained by the use of higher initial clarithromycin concentration (50 µM) in the incubation in that study compared to what we used (5 µM).

Accumulation of 5 selected drugs was further characterised by determining $K_{p,NR8383}$ over a range of substrate concentration. Such analysis in NR8383 has been performed for the first time. Concentration-dependent accumulation was demonstrated for all investigated drugs, although the magnitude of saturable uptake varied between drugs. Comparable extent of saturable uptake was observed for clarithromycin, rifampicin and tiotropium bromide (almost 3-fold difference between $K_{p,max}$ and $K_{p,min}$) whereas the magnitude of this uptake was much greater for imipramine and formoterol ($K_{p,max}/K_{p,min}$ ratio of 6.9-8.3). Accumulation of imipramine, clarithromycin and formoterol could be described by a two-site process; one that is saturable and another that is linear representing nonsaturable binding. This finding for imipramine is in agreement with Hallifax and Houston (2006) who also described imipramine accumulation in rat hepatocytes as a two-site process including a high affinity, low capacity process (saturable) and a low affinity, high capacity process (nonsaturable). A two-site binding process for imipramine has also been previously demonstrated in lysosomes isolated from rat liver (Ishizaki et al., 1996). The authors showed that the high affinity-low capacity site disappeared in the presence of NH₄Cl, and suggested lysosomes as the high affinity-low capacity binding site (Ishizaki et al., 1996).

The $K_{p,min}$ of compounds at the highest substrate concentration can be assumed to represent membrane partitioning upon saturation of active processes at this concentration. However, under the current experimental conditions, for clarithromycin and formoterol, $K_{p,min}$ obtained may represent both membrane partitioning and active processes, as the latter has not been saturated at the highest concentration (Figure 2-7). Nonetheless, $K_{p,min}$ of the compounds ranged a great deal between drugs (0.6-141) in agreement with the order of their lipophilicities (Table 1-4). Imipramine, a cationic amphiphilic molecule well known to interact with membrane acidic phospholipids (Joshi et al., 1988; Fisar et al., 2004), showed the highest membrane partitioning ($K_{p,min}$
of 141), as opposed to hydrophilic tiotropium bromide for which concentration in external medium exceeded its cellular concentration ($K_{p,min}$ of 0.6).

### 2.5.4 Assessment of drug uptake kinetics in NR8383 cells

Upon completion of the assessment of drug uptake at single concentration, an attempt was made to describe the uptake kinetics of a number of selected drugs in NR8383 cell line. The drive for kinetic assessment of drug uptake was to provide uptake kinetic parameter estimates for a number of drugs in this cell line for the first time. While the uptake kinetic assessment was focused on the respiratory drugs, imipramine was included in the analysis due to its relatively high $CL_{uptake}$ and $CL_{diff}$ in NR8383 compared to other drugs. All investigated drugs showed saturable uptake with the exception of clarithromycin and formoterol for which uptake was linear across the wide concentration range. In the case of clarithromycin, linearity observed did not allow accurate estimation of uptake kinetic parameters. Previously, a kinetic analysis of clarithromycin uptake in isolated rat lung cells demonstrated a $K_m$ of 230 µM and $V_{max}$ of 834 pmol/min/10⁶ cells (Kohno et al., 1990). It could be that clarithromycin shows low affinity for transporters involved in its uptake by lung cells including AMs. Another possibility is that there are other mechanisms within cells that prevent saturation of clarithromycin uptake including lysosomal sequestration. Indeed, clarithromycin has previously shown to distribute in granules fraction (containing lysosomes) of NR8383 cells (Togami et al., 2013). In the case of imipramine, the kinetic profile suggests the presence of both saturable and unsaturable processes which is in agreement with $K_{p,NR8383}$ vs. imipramine concentration profile, as discussed in the previous section. The saturable process may be suggestive of a transporter mediated uptake and/or lysosomal sequestration, as discussed earlier. Although imipramine was previously indicated as a substrate for OCTs (Nakanishi et al., 2011), its uptake by OCT-expression systems have not been demonstrated, despite its ability to inhibit the uptake of a number of OCT and OCTN substrates (Wu et al., 2000a; Wu et al., 2000b; Fujita et al., 2006). In NR8383, it is unclear whether any transporters are involved in imipramine uptake as transporters in this cell line have not been characterised to date. Previous uptake study of chlorphentermine, another cationic lipophilic drug, in primary rat AMs, has shown similar kinetic profile to that of imipramine (Heyneman and Reasor, 1986). In the study, imipramine inhibited chlorphentermine accumulation in AMs and the authors have suggested that this drug is taken up by AMs by a carrier-mediated process at low
concentrations and shares a common mechanism for uptake with imipramine. Although imipramine uptake may involve membrane transporters, the contribution of this process would not be likely to be substantial, as highlighted by the work of Hallifax and Houston (2006) as imipramine is considered to be a class 1 compound (i.e., highly permeable) in the Biopharmaceutical Drug Disposition Classification System (BDDCS) (Wu and Benet, 2005). Basic lipophilic compounds show extensive tissue binding (Rodgers et al., 2005a) and therefore, imipramine binding to acidic phospholipids on the membrane can be an important contributing process to its high accumulation observed in NR8383.

The importance of active saturable uptake in the accumulation of five selected drugs was assessed by both determining their $K_m$ using the Michaelis-Menten model and the $K_U$ using the two-site model incorporating both saturable and nonsaturable processes. While there was an agreement between the $K_m$ (1.3 µM) and $K_U$ (0.9 µM) of imipramine, in the case of formoterol and clarithromycin, a large discrepancy was observed between the values (the $K_U$ values being at least a hundred-fold lower than the $K_m$). The reason for this observed discrepancy is currently unclear. While the large variability associated with the $K_U$ (CV>30%) and the $K_m$ (CV>50%) of both drugs affects the accurate interpretation of the values, this variation is possibly not sufficient alone in explaining the observed discrepancy. One plausible explanation is the use of two different models and transformation of the data (i.e., using initial uptake rates vs. cell-to-medium concentration ratios) in the assessment of the concentration dependency of drug uptake.

Similar to clarithromycin, uptake of the remaining compounds rifampicin, formoterol and tiotropium bromide was driven by an active process. The uptake of these drugs into NR8383 may involve uptake transporters with varying affinities towards these compounds. As outlined previously here, these compounds are substrates for OCTs, OCTNs and OATPs which may be expressed in NR8383. The study by Moreau et al. (2011) indicates low expression of OCTN1/2 and OCT1 and either very low or no expression of OATP1B1, OATP1B3 and OCT2 in monocyte derived human macrophages. As AMs are primarily differentiated from blood monocytes, the cell system used in this study may closely represent AMs and the transporters expressed in them. However, differentiation of blood monocytes to AMs in the alveoli may up- or down-regulate transporters differently than the situation when such differentiation is
induced in vitro. Moreover, it is unknown whether possible expression of transporters has been remained in AMs during their transformation to NR8383 cell line.

2.5.5 Conclusion

In conclusion, this chapter presented first time characterisation of the accumulation of a range of respiratory drugs in the NR8383 cell line. A plated uptake methodology was optimised for NR8383 cells in terms of the type of 24-well plates, culture time, cell seeding density, drug incubation time points and measurement of cell number to normalise uptake data. A wide range in drug accumulation was observed from assessment of both total uptake clearances (0.04 - 16.7 µL/min/10⁶ cells) and cell-to-medium concentration ratios (0.69 – 391) at 5 µM. Delineation of active and passive uptake processes showed that for most compounds, their accumulation is driven by an active process in NR8383. Assessment of \( K_{p,NR8383} \) showed that most compounds accumulated in NR8383 at a much greater magnitude relative to the external medium concentration. A number of processes can contribute to observed accumulation including transporter-mediated uptake or endocytosis, passive diffusion and intracellular distribution (i.e., lysosomal trapping and membrane partitioning). Investigation of drug uptake kinetics showed that uptake was saturable for a number of compounds (e.g., rifampicin, tiotropium bromide) which may be attributed to membrane transporters or endocytosis. For drugs where uptake was not saturated (e.g., clarithromycin, formoterol), the involvement of low affinity transporters and/or other mechanisms such as lysosomal accumulation may be considered. The latter active process can be investigated by a number of well-established in vitro methods and this may increase the understanding of the processes that are involved in accumulation of these drugs in AMs (Chapter 3).
Chapter 3  *In vitro* assessment of lysosomal accumulation of drugs in NR8383 cell line

3.1 Introduction

Drug accumulation in AMs is desired for efficacy when targeting intracellular bacteria (e.g., in the case of macrolide antibiotics). However, for drugs targeting receptors in the respiratory airways, the uptake by AMs and subsequent removal from the airways can lead to their reduced efficacy. In addition, pronounced accumulation of CADs in lysosomes of AMs represents a safety concern, as it may lead to drug induced phospholipidosis (Reasor et al., 2006). The process of CAD sequestration in lysosomes is known as lysosomal trapping and was first described by de Duve et al. (1974). Lysosomes are acidic organelles (pH=4.7-4.8) with important functions in the degradation and recycling of macromolecules (e.g., phospholipids, peptides) and other waste products (e.g., bacteria, viruses) delivered by endocytosis, phagocytosis and autophagy (Cuervo et al., 2005; Appelqvist et al., 2013). Lysosomes are abundant in alveolar macrophages, comprising up to 7.8% of the total cell volume in primary rat alveolar macrophages (Davies et al., 1977; Davies et al., 1978; Castranova et al., 1979; Reasor et al., 1979; Crapo et al., 1982; Lum et al., 1983; Chang et al., 1986; Kradin et al., 1986; Gladue et al., 1989; Sebring and Lehnert, 1992; Krombach et al., 1997). The weakly basic drugs with a pKₘ near 8 are present largely (>60%) in their ionised form at cytosolic pH (pH=7.2). The unionised form of basic drugs can rapidly diffuse into lysosomes where they become largely ionised due to the acidic milieu of lysosomes (De Duve et al., 1974). This pH difference between cytosol and lysosome is the mechanistic basis for the trapping of the ionised drug as a result of its limited permeability across the lysosomal membrane back into cytosol. It has been reported that ionisation and permeability (linked to lipophilicity) properties of amine drugs are the two key determinants of lysosomal trapping potential of basic compounds (de Duve et al., 1974; MacIntyre and Cutler, 1988b; Duvvuri et al., 2004b; Nadanaciva et al., 2011). As a result of the trapping process, basic drugs can attain significantly higher intracellular concentrations which may have important therapeutic and toxicological consequences (Friden et al., 2011; Logan et al., 2012; Chu et al., 2013; Logan et al., 2013). The extent of lysosomal sequestration, hence the availability of the drug to bind with intended intracellular targets, can influence the drug activity. Moreover, lysosomotropic drugs
typically have unique pharmacokinetic properties, often having a very large volume of distribution and half-life due to extensive binding to tissue phospholipids (MacIntyre and Cutler, 1988b; Marceau et al., 2012). In addition, recent studies have highlighted a potential clinical implication of drug-drug interactions when two lysosomotrophic drugs are co-administered (Funk and Krise, 2012; Logan et al., 2012; Logan et al., 2013).

A number of methods can be employed to investigate lysosomal trapping of drugs in vitro including direct approaches such as measurement of drug concentration in isolated lysosomes following homogenisation (Chen et al., 2005; Duvvuri and Krise, 2005a; Togami et al., 2013) and indirect approaches based on the use of chemical agents that reduce lysosomal trapping (Daniel et al., 1995; Ishizaki et al., 1996; Togami et al., 2010; Kazmi et al., 2013) and lysosome specific basic fluorescent probe LysoTracker® Red (Duvvuri et al., 2004a; Duvvuri et al., 2004b; Lemieux et al., 2004; Kazmi et al., 2013). These agents have been extensively used to assess lysosomal trapping of drugs in rat tissue slices, isolated rat liver lysosomes and a number of cell systems including immortalised human hepatocytes (Fa2N-4 cells) and NR8383 cells, as summarised in the Appendix Table 7-3. However, the number of such studies in alveolar macrophages is limited. To date, there are only 2 studies investigating lysosomal accumulation of a number of antibiotics including clarithromycin, azithromycin and telithromycin in NR8383 cells, highlighting the need for more extensive studies that would cover a wider range of respiratory compounds (Togami et al., 2010; Togami et al., 2013).

### 3.2 Aims

The aim of the studies presented in this chapter is to assess lysosomal trapping of 10 selected compounds in NR8383 cells. The compounds selected covered a range of physicochemical properties (Table 1-4) and extent of accumulation in this cell line (Chapter 2). The compound list included imipramine as a marker for lysosomal trapping. Quantitative assessment of lysosomal trapping of drugs was investigated at 37°C and single substrate concentration in the presence of chemical agents NH₄Cl, monensin and nigericin. The latter two agents were used in the assessment of selected drugs to confirm positive or negative results as well as any uncertain results from the initial assessment with NH₄Cl. The influence of lysosomal trapping on uptake kinetics was explored using clarithromycin as a representative compound. Cell-to-medium concentration ratios (K_p,NR8383) and total uptake clearances (CL_uptake) were calculated in
the presence and absence of these agents to determine the extent of lysosomal trapping of drugs investigated. Localisation of lysosomes and semi-quantitative assessment of the effect of the chemical agents and the lysosomal trapping of a number of basic drugs was performed using LysoTracker® Red. The physicochemical properties of the basic drugs were correlated to their experimentally obtained extent of lysosomal trapping to determine the presence of any trends.
3.3 Methods

3.3.1 Chemical and Reagents

In addition to those used in Chapter 2, the chemicals and reagents used were as follows; Ammonium chloride, monensin and nigericin sodium salts and acetic acid from Sigma Aldrich Ltd., Dorset, UK, chloroform and formaldehyde 37-41% from Fisher Scientific, Loughborough, UK, Lysotracker® Red DND-99 from Life Technologies, Paisley, UK and Collagen Type I rat tail from BD Biosciences, Oxford, UK.

3.3.2 Optimisation of in vitro conditions for lysosomal trapping

The in vitro conditions used to assess lysosomal trapping of drugs with NH₄Cl, monensin and nigericin in NR8383 were optimised in terms of the concentration of the agents used, the incubation conditions and time points.

3.3.2.1 Optimisation of lysosomal trapping studies with ammonium chloride

The concentration of ammonium chloride used in lysosomal trapping studies was determined based on preliminary experiments. Previous literature studies used NH₄Cl within 10-50 mM concentration range (Ohkuma and Poole, 1981; Daniel and Wojcikowski, 1997; Funk and Krise, 2012; Kazmi et al., 2013; Togami et al., 2013) and these concentrations were selected for initial investigation of imipramine and clarithromycin uptake. Imipramine was included in the drug dataset as a positive marker for lysosomal trapping (Orton et al., 1973; Vestal et al., 1980; Ishizaki et al., 1996; Daniel and Wojcikowski, 1997; Kazmi et al., 2013). The uptake experiments were performed as described in Section 2.3.4.1. Both compounds (at 5 and 20 µM) were incubated in the absence and presence of NH₄Cl at each concentration of the agent for up to 10 minutes at 37°C. Previous studies have reported that lysosomal pH rises by 1.5 units within 5 minutes of incubation in the presence of NH₄Cl in mouse peritoneal macrophages (Ohkuma and Poole, 1978; Ohkuma and Poole, 1981). This increased pH was reported to be maintained for 20 minutes of incubation in the presence of NH₄Cl (Poole and Ohkuma, 1981).

The CL_uptake of the compounds at control and treatment conditions was calculated up to 10 minutes. The CL_uptake at control condition was considered 100% and the values under treatment conditions were determined as % relative to their control. Subsequently, the
% reduction in CL\textsubscript{uptake} of both compounds under treatment conditions was determined by subtracting their clearance from 100% control.

A previous study by Kazmi et al. (2013) has investigated propranolol accumulation in Fa2N-4 cells in the presence of NH\textsubscript{4}Cl under different incubation conditions consisting of co-incubation, pre-incubation and both co- and pre-incubation of the agent with propranolol. In the current study, the effect of these different incubation conditions on drug uptake was assessed in a preliminary experiment with clarithromycin and imipramine. Following optimisation of the NH\textsubscript{4}Cl concentration, uptake of 5 and 20 µM clarithromycin and imipramine was performed in the absence and presence of 20 mM NH\textsubscript{4}Cl which was either i) pre-incubated, ii) co-incubated, or iii) both pre- and co-incubated with the cells at 37°C. In the pre-incubation method, the NR8383 cells which were cultured for 16 hours in 24-well collagen-coated plates were washed twice with 800 µL of pre-warmed DPBS and pre-incubated with 20 mM NH\textsubscript{4}Cl-1 mM ABT mixture for 20 minutes at 37°C (CO\textsubscript{2} incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK) prior to incubation of the cells with 5 and 20 µM of imipramine or clarithromycin, as described in Section 2.3.4.1. In co-incubation, the cells were pre-incubated with 1 mM ABT/DPBS only, followed by the incubation of the cells with the drug solution containing 20 mM NH\textsubscript{4}Cl. In pre- and co-incubation, 20 mM NH\textsubscript{4}Cl was present both in ABT/DPBS pre-incubation solution and in the drug incubation media. The % reduction in CL\textsubscript{uptake} of both compounds under treatment conditions was determined as described above.

3.3.2.2 Optimisation of lysosomal trapping studies with monensin and nigericin
The concentrations of both ionophores used in this study were optimised based on previous studies (Daniel and Wojcikowski, 1997; Funk and Krise, 2012; Kazmi et al., 2013) and in house solubility observations. In contrast to NH\textsubscript{4}Cl, these ionophores have been used in micromolar concentrations in drug uptake and sub-cellular distribution studies (Appendix Table 7-3). The selected concentrations of monensin and nigericin to be used in assessment of lysosomal trapping were 5 and 10 µM, respectively. For consistency, the optimal incubation condition (i.e., co-incubation with compound) previously used in NH\textsubscript{4}Cl experiments was also applied with these ionophores. Nigericin concentration of 2 µg/ml (equivalent of ~2.8 µM) has been previously shown to increase lysosomal pH by almost 1.5 units (to pH 6.16) within 3 minutes of incubation with mouse peritoneal macrophages (Ohkuma and Poole, 1978). However,
Unlike NH₄Cl, this pH was observed to be maintained until 5 minutes after which it decreased slightly to a level (~pH 6.10) that was maintained until 20 minutes (Ohkuma and Poole, 1978). The preliminary experiments with clarithromycin (5 µM) incubated with 10 µM nigericin showed a steady decrease in clarithromycin cell concentration up to 5 minutes after which a slightly higher concentration of the drug was measured at 10 minutes. Therefore, the incubation of the cells with ionophores was performed only up to 5 minutes (single incubation time point). Consequently, the $C_{\text{L}}$uptake of the compounds at control and ionophore treatment conditions was calculated up to 5 minutes. The $C_{\text{L}}$uptake at control condition was considered 100% and the values under treatment conditions were determined as % relative to the control. Subsequently, the % reduction in $C_{\text{L}}$uptake of both compounds under treatment conditions was determined by subtracting their clearance from 100% control.

### 3.3.3 In vitro lysosomal trapping in NR8383

#### 3.3.3.1 Assessment of lysosomal trapping in NR8383 cells using Lysotracker® Red

Semi-quantitative assessment of lysosomal trapping was made using a lysosome specific basic fluorescent probe LysoTracker® Red (Life Technologies, Paisley, UK). The protocol used for staining of the cells with LTR was adapted from a previous study (Cantrill et al., 2012). Clear flat bottom Ibidi 8-well chamber slides (Thistle Scientific, Glasgow, UK) were previously coated with 50 µg/mL collagen-I solution (4.05 mg/mL Collagen type I rat tail stock was diluted in 0.02N acetic acid). NR8383 cells, which were checked for at least 80% viability using the trypan blue exclusion method, were plated (250,000 cells/well) in the slides and cultured for 16 hours to 70-80% confluency and to ensure attachment. Stock solutions of NH₄Cl, monensin and nigericin were prepared by diluting them in serum-free Ham’s F12K medium resulting in final incubation concentrations of 20 mM, 5 µM and 10 µM, respectively. In addition, stock solutions of clarithromycin, imipramine and formoterol were diluted in fresh serum-free Ham’s F12K medium containing 1 mM ABT to achieve final incubation concentrations of 5 µM. LTR was added into the solutions of the chemical agents and the drugs to achieve a final concentration of 200 nM as used by Kazmi et al. (2013). A control solution of LTR at the same concentration was also prepared by dilution of the original DMSO stock in serum-free Ham’s F12K medium. Upon confluency, cells were examined under the microscope (Olympus CK2, Olympus Optical Co., Japan), the CGM was removed and the cells were pre-treated with 300 µL warm DPBS containing...
1 mM ABT for 20 minutes at 37°C in the presence of 5% CO₂ (CO₂ incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK). Following this, the cells were treated with 300 µL of serum-free Ham’s F12K medium containing either LTR alone (i.e., control) or LTR with each of the chemical agents or selected drugs (i.e., LTR with NH₄Cl or clarithromycin) for 1 hour at 37°C in the CO₂ incubator and protected from light. Following the incubation, the cells were washed three times with 300 µL ice-cold DPBS and immediately fixed with 4% (v/v) formaldehyde in DPBS for 10 minutes at room temperature, protected from light. The cells were then treated with 50 mM NH₄Cl in DPBS to quench the residual formaldehyde fluorescence for 5 minutes at room temperature. Three subsequent rinses with ice-cold DPBS were performed before visualisation of the LTR with a confocal laser scanning microscope (LSM) (Zeiss LSM 510, Jena, Germany). Image J v1.47 software was used for the quantification of the fluorescence intensity of LTR in the absence or presence of chemical agents and basic drugs (Image J, National Institutes for Health, US). The corrected total cell fluorescence (CTCF) was calculated as integrated density – (area of selected cell x mean fluorescence of background reading) (Cappell et al., 2012). The CTCF of treated cells was compared to 100% control (i.e., LTR only).

3.3.3.2 Investigation of lysosomal trapping of drugs in NR8383 using chemical agents

Lysosomal trapping was assessed using the uptake methodology described in Section 2.3.4.1. The cells were maintained in cell culture flasks containing CGM at 37°C 5% CO₂ (CO₂ incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK) before the experiments. Drug uptake into NR8383 cells was performed in the absence and presence of NH₄Cl, monensin and nigericin. The concentration of these chemical agents and the incubation conditions were optimised as described in Section 3.3.2. Upon optimisation of the experimental conditions, lysosomal accumulation of all compounds was investigated using 20 mM NH₄Cl co-incubated with the compounds up to 10 minutes at 37°C. Following the initial assessment with NH₄Cl, confirmation of lysosomal trapping for a number of selected drugs including imipramine, clarithromycin, formoterol and fenoterol was further carried out using 5 µM monensin and 10 µM nigericin co-incubated with the selected drugs. The concentrations of both ionophores used in this study were also optimised as described in Section 3.3.2. Following the initial washing of the cell monolayers with DPBS and incubation with
ABT/DPBS for 20 minutes where appropriate, the cells were treated with the compounds which were either in the absence (i.e., control) or presence of NH₄Cl, monensin and nigericin at 37°C. All drug solutions were pre-warmed to 37°C in water bath (Stuart Shakebath SB30, Bibby Scientific Limited, Stone, UK). Stock solutions of NH₄Cl, monensin and nigericin were in deionised water, methanol and chloroform respectively. For incubations with NH₄Cl, all compounds were prepared at 5 and 20 µM concentrations, with the exception of ciprofloxacin (5 µM) due to solubility issues at higher concentrations. The incubation time points used were 1, 2, 5 and 10 minutes. For incubations with monensin and nigericin, all compounds were prepared at 5 µM concentration and the incubations were carried out for 5 minutes only. Following the treatment of the cell monolayers with drug alone or drug with a chemical agent, incubations were stopped by rinsing of the cells with 800 µL ice-cold DPBS three times, followed by cell lysis in 200 µL deionised water. The removed incubation media was retained for the measurement of drug concentrations and subsequent Kₚ,NR8383 determination. Furthermore, the pH of incubation media containing NH₄Cl was monitored for any changes in pH using a pH meter (Mettler-Toledo 340, Beaumont Leys, UK). All cell lysates and media samples were kept -20°C overnight before analysis by LC-MS/MS. The amount of protein per well was measured using BCA protein assay (Thermo Scientific, Loughborough, UK) to determine the cell number in each well as described in Section 2.3.3.5

3.3.3.3 Investigation of uptake kinetics of clarithromycin in the presence of NH₄Cl

In order to investigate the impact of lysosomal sequestration on kinetics of drug uptake, uptake of clarithromycin in NR8383 was assessed in the absence and presence of 20 mM NH₄Cl over a range of concentrations. Clarithromycin was selected as a representative compound for investigation based on the findings from the assessment of lysosomal trapping of drugs at single substrate concentration (Section 3.4.3). These experiments were performed in the same manner as detailed in Section 2.3.4.2. Briefly, following pre-incubation of cells with 1 mM ABT/DPBS for 20 minutes at 37°C, the pre-incubation buffer was removed and cells were treated with clarithromycin in the absence and presence of 20 mM NH₄Cl at a concentration range 1-100 µM over 10 minutes at 37°C. The range of concentrations used was the same as shown in Table 2-1 (Chapter 2). The incubation media was collected at the end of incubation and the concentrations were measured over time in order to determine the extent of nonspecific
binding and $K_{p, NR8383}$. Both control and treatment conditions were assessed in duplicate within the same experiment. All kinetic experiments were performed on at least three separate occasions.

### 3.3.3.4 Assessment of cytotoxicity of chemical agents for lysosomal trapping

LDH assay kit (Sigma-Aldrich, Dorset, UK) was used to measure the cytotoxicity of the chemical agents used to assess lysosomal trapping. When measuring the cytotoxicity of the chemical agents, four types of samples were included in the analysis. The incubation medium consisting of DPBS alone (i.e., negative control), drug alone (i.e., clarithromycin, imipramine), chemical agents alone (i.e., $\text{NH}_4\text{Cl}$, monensin and nigericin), and chemical agent and drug combination (e.g., $\text{NH}_4\text{Cl} + \text{clarithromycin}$) used in lysosomal trapping experiments were all analysed in order to assess the viability of the untreated cells and the effect of the individual drugs and chemical agents, as well as the effect of their combinational use. The concentrations used for the assessment of the cytotoxic effect of individual chemical agents on NR8383 were the same concentrations in the lysosomal trapping studies. The cytotoxicity in above samples was measured on three separate occasions. The experiments were performed and the LDH activity of experimental samples was determined in the same manner as described in Section 2.3.4.3.

### 3.3.3.5 Correlation of physicochemical properties with lysosomal trapping of basic drugs

Theoretical assessment of the accumulation ratio between lysosomes and cytosol or extracellular medium was performed for basic drugs. This analysis was based on drug partitioning due to the pH differences between the mentioned compartments (i.e., pH partitioning theory) (de Duve et al., 1974; MacIntyre and Cutler, 1988b), as done previously (Hallifax and Houston, 2007; Friden et al., 2011). Lysosomal and extracellular medium pH of 4.75 and 7.4, respectively were used, resulting in lysosome-to-medium concentration ratios between 315 and 443 for terbutaline and imipramine, respectively (Table 3-1). Therefore, all the basic drugs were expected to show lysosomal accumulation. In order to determine the relationship between physicochemical properties and lysosomal trapping of basic drugs in the dataset in NR8383 cells, the % reduction in $\text{CL}_{uptake}$ and $K_{p, NR8383}$ were correlated to LogP and $pK_a$ of the drugs investigated. For imipramine, clarithromycin and terbutaline, the reported
experimental values for LogP and pKₐ were used (Table 1-4). For formoterol and fenoterol, the physicochemical data were predicted by ADMET Predictor™ v7.0 (SimulationsPlus, Lancaster, US).

**Table 3-1:** Lysosome-to-medium concentration ratio of basic drugs in the dataset calculated based on the pH partitioning theory at lysosomal and external medium pH of 4.75 and 7.4, respectively.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Basic pKₐ</th>
<th>Calculated Kₚ,lysosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>9.50</td>
<td>443</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>8.99</td>
<td>435</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>8.28</td>
<td>391</td>
</tr>
<tr>
<td>Formoterol</td>
<td>8.14</td>
<td>378</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>9.90</td>
<td>315</td>
</tr>
</tbody>
</table>

pKₐ data were reproduced from Table 1-4

3.3.3.6 Sample preparation and mass spectrometry analysis

The preparation of cell lysates and media samples from lysosomal trapping experiments was performed in the same manner as described in Section 2.3.4.4. Samples were analysed on a Waters Alliance 2795 HPLC system coupled to a Micromass Quattro Ultima mass spectrometer (Waters, Watford, UK). The LC-MS/MS conditions used for each individual drug and their internal standards are given in Table 2-2. The details of the LC-MS/MS system and procedures for sample quantification were also the same as detailed in Section 2.3.4.4.

3.3.3.7 Confocal microscopy

The visualisation of LTR in fixed cells was performed using Zeiss LSM (Zeiss LSM 510, Jena, Germany) equipped with a C-Apochromat 40x/1.2 NA (Numerical Aperture) water-immersion objective. The LTR labelled cells were excited using the helium/neon laser at 543 nm and the emitted light was collected at 560 nm following passage through a bandpass filter (BP 560-615 nm). Images were captured with a resolution of at least 512 x 512 pixels. Images were collected and processed using Combi LSM-FCS v.3.2 and LSM Image Browser v4.2 softwares (Jena, Germany).
3.3.3.8 Determination of the extent of lysosomal trapping of drugs in NR8383 cells

The magnitude of lysosomal trapping of drugs in NR8383 cells was assessed indirectly by calculating $CL_{uptake}$ and $K_{p, NR8383}$ for each compound in the absence (control) and presence of NH$_4$Cl, monensin and nigericin used to reduce lysosomal trapping. $CL_{uptake}$ was calculated as described in Section 2.3.4.5.1. $K_{p, NR8383}$ was calculated as described in Section 2.3.4.5.2 and was determined as a mean of at least 3 lysosomal trapping experiments performed on separate occasions. Therefore, control $K_{p, NR8383}$ data presented here differs slightly from the $K_{p, NR8383}$ data presented in Chapter 2 in which the parameter was determined from experiments performed at single concentration without involvement of NH$_4$Cl. Furthermore, both $CL_{uptake}$ and $K_{p, NR8383}$ of drugs were determined using 1-10 minute incubation times when evaluating the effect of NH$_4$Cl, whereas for monensin and nigericin, the data were determined up to 5 minutes (0-5 minutes), as a single incubation time was used. In order to confirm whether the data from both conditions were comparable, NH$_4$Cl data from representative experiments (n=3) were re-evaluated up to 5 minutes and compared to those obtained using 1-10 minute incubation times. Although $CL_{uptake}$ was higher when estimated using a single incubation time point (up to 5 minute), there was no significant difference in the % reduction in $CL_{uptake}$ obtained when the clearance was estimated either up to 5 minutes or using 4 incubation time points (1-10 minutes) (% reduction in $CL_{uptake}$ was 85% and 77% using 4 and single incubation time points, respectively with $p=0.096$ revealed by Student’s t-test).

Assessment of lysosomal trapping was made by the comparison of both $CL_{uptake}$ and $K_{p, NR8383}$ determined under control and chemical agent treatment conditions. The reduction in $CL_{uptake}$ and $K_{p, NR8383}$ of each compound in the presence of the chemical agents was expressed as a percentage relative to control (100%). A value of 50% reduction in both $CL_{uptake}$ and $K_{p, NR8383}$ was set as a cut-off for lysosomal trapping, while also taking into account the physicochemical properties of the drugs (Table 1-4) and the variability associated with the experimental data.

The concentration dependence of clarithromycin accumulation both in the absence and presence of NH$_4$Cl was investigated using a two-site binding model that incorporates a saturable site and a linear component for unsaturated binding by nonlinear regression analysis in Grafit v6.0.6 software (Erithacus Software Limited, Horley, UK) (Section 2.3.4.5.2).
3.3.3.9 Determination of uptake kinetic parameters for clarithromycin in NR8383 cells

Uptake kinetic parameters for clarithromycin were determined using the conventional two-step method (Poirier et al., 2008; Ménochet et al., 2012) as described in Section 2.3.4.5.3. When estimating the uptake kinetic parameters for clarithromycin in the presence of NH₄Cl, CL_diff estimated at single substrate concentration was used as a constant in Equation 2-1 as the experiments were performed only at 37°C. Kinetic parameters $K_m$ and $V_{max}$ were determined by nonlinear regression using Grafit v6.0.6 software.

3.3.3.10 Statistical analysis

The arithmetic mean, standard error and CV were calculated for uptake clearance $CL_{uptake}$ and $K_{p,NR8383}$ calculated on at least three different occasions. When assessing the extent of lysosomal trapping, the control and chemical agent treated cells were compared using the two-tailed, paired Student’s t-test in order to determine the existence of a statistically significant difference between the two conditions. Values were reported as significant when $p < 0.05$. 
3.4 Results

3.4.1 Optimisation of lysosomal trapping experiments with NH₄Cl

Uptake of 5 and 20 µM imipramine and clarithromycin in NR8383 was assessed in preliminary experiments where 10, 20 or 50 mM NH₄Cl was co-incubated with the investigated drug for 10 minutes at 37°C. Table 3-2 shows CL uptake of both compounds obtained in the absence and presence of each individual concentration of NH₄Cl. For both imipramine and clarithromycin, the CL uptake decreased with increasing concentration of NH₄Cl, regardless of the substrate concentration used. The reduction in CL uptake of clarithromycin in the presence of 10 mM NH₄Cl was 85%, whereas use of 50 mM NH₄Cl resulted in up to 94% reduction in clarithromycin uptake at both substrate concentrations. In the case of imipramine, the reduction in CL uptake was less pronounced, as 20-40% of the CL uptake was unaffected depending on the conditions investigated. A maximum of 80% reduction was observed in CL uptake of imipramine at 5 µM and at the highest NH₄Cl concentration. Based on this preliminary set of data, 20 mM concentration was selected to be used for later lysosomal trapping experiments for a number of reasons. First, this concentration of NH₄Cl is one of the most widely used in previous studies investigating sub-cellular distribution of drugs in either cells or tissues (Appendix Table 7-3). While 10 mM NH₄Cl has also been commonly used, the reduction of lysosomal trapping was more pronounced (at least 70%) at 20 mM for both imipramine and clarithromycin.

Table 3-2: Percent reduction in uptake clearance of 5 and 20 µM imipramine and clarithromycin in the presence of 10, 20 and 50 mM NH₄Cl

<table>
<thead>
<tr>
<th>Drug</th>
<th>Reduction in uptake clearance (%)</th>
<th>5 µM</th>
<th>20 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>58</td>
<td>71</td>
</tr>
<tr>
<td>Imipramine</td>
<td>+ 10 mM NH₄Cl</td>
<td>58</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>+ 20 mM NH₄Cl</td>
<td>72</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>+ 50 mM NH₄Cl</td>
<td>80</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>+ 10 mM NH₄Cl</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>+ 20 mM NH₄Cl</td>
<td>93</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>+ 50 mM NH₄Cl</td>
<td>94</td>
<td>93</td>
</tr>
</tbody>
</table>
Secondly, although slightly higher reduction of lysosomal trapping was observed at 50 mM NH₄Cl, 20 mM was selected in order to minimise any potential cytotoxic effect of such high concentrations on NR8383.

Upon optimisation of the NH₄Cl concentration to be used in NR8383, different incubation conditions including pre-incubation, co-incubation and both pre- and co-incubation of NH₄Cl with NR8383 and imipramine or clarithromycin were investigated as described in Section 3.3.2.1. The CL\textsubscript{uptake} of both compounds resulting from these incubation conditions are shown in Figure 3-1. For imipramine, all three incubation conditions caused a reduction in CL\textsubscript{uptake}. This reduction was similar at both substrate concentrations when the agent was co-incubated with imipramine (71 and 62% reduction in CL\textsubscript{uptake} at 5 and 20 µM, respectively) and when it was pre-incubated with the cells followed by co-incubation with imipramine (74 and 61% reduction in CL\textsubscript{uptake} at 5 and 20 µM, respectively). Use of pre-incubation step only was not as effective compared to two other incubation conditions, resulting in reduction of CL\textsubscript{uptake} of approximately 55% at both concentrations. In the case of clarithromycin, the largest reduction in CL\textsubscript{uptake} was observed when NH₄Cl was only co-incubated with clarithromycin at both substrate concentrations (78 and 69% reduction in CL\textsubscript{uptake} at 5 and 20 µM, respectively). Performing both pre- and co-incubation did not lead to more extensive reduction in CL\textsubscript{uptake} (71 and 60% at 5 and 20 µM, respectively). Similar to imipramine, pre-incubation with NH₄Cl in isolation did not have any additional contribution to the reduction of lysosomal trapping. In fact, when the chemical agent was pre-incubated with the cells and was not present in the drug incubation media, the effect of NH₄Cl was completely reversed, resulting in either minimal (5% at 5 µM) or no reduction (at 20 µM) in uptake clearance. Therefore, based on these preliminary data, co-incubation of NH₄Cl with compounds only was seen sufficiently effective in reducing CL\textsubscript{uptake} of lysosomotropic compounds in NR8383 cells.
3.4.2 Assessment of lysosomal trapping in NR8383 using LysoTracker® Red

In addition to the quantitative assessment of lysosomal trapping, confocal microscopic examination of NR8383 cells was performed in order to localise functional lysosomes in NR8383 and assess the effect of the chemical agents on LTR accumulation in lysosomes. In addition, the lysosomal targeting of selected drugs from the current dataset was investigated. A differential contrast image was taken every time prior to excitation of the cells for LTR detection. A contrast image of an NR8383 cell is shown in Figure 3-2A demonstrating the presence of cytoplasmic vesicular structures likely to represent lysosomes. The corresponding fluorescent image of the same cell is illustrated in Figure 3-2B where LTR was localised to lysosomes, confirming the presence and abundance of these organelles in NR8383 cell line. Treatment of NR8383 with LTR in the presence of the chemical agents NH₄Cl (20 mM), monensin (5 µM) and nigericin (10 µM) showed reduced fluorescent signal by almost 85% for all three agents, indicating less accumulation of LTR in lysosomes of NR8383 (Figure 3-2 C, E, G). Such effect of the agents towards LTR accumulation confirmed lysosomal targeting of this prototypic basic probe in NR8383. Assuming the competition of basic drugs for lysosomal trapping, LTR was co-incubated with 5 µM of clarithromycin, imipramine and formoterol. Clarithromycin and imipramine decreased LTR accumulation (Figure 3-
2 D, F) by 86 and 72%, respectively, indicating indirectly the lysosomotropic properties of these two drugs. In contrast, the localisation of LTR was still evident in the presence of formoterol (Figure 3-2H) which caused only 25% reduction in LTR accumulation, suggesting its nontargeting of NR8383 lysosomes. The observations from the imaging studies provided a confirmation to the results outlined in Section 3.4.3 with regards to both the effectiveness of the chemical agents and the lysosomal accumulation of clarithromycin, imipramine and formoterol.
**Figure 3-2:** Confocal microscopic images of NR8383 treated with LysoTracker® Red (LTR) in the absence and presence of chemical agents and basic drugs. (A) A contrast image of a single NR8383 cell treated with 200 nM LTR; (B) the same cell being excited to detect LTR localised in lysosomes under control conditions; the localisation of LTR in the lysosomes of NR8383 was reduced in the presence of 20 mM NH$_4$Cl (C), 5 µM monensin (E), 10 µM nigericin (G), 5 µM clarithromycin (D) and 5 µM imipramine (F), and was relatively unaffected in the presence of 5 µM formoterol (H).
3.4.3 **Assessment of lysosomal trapping in NR8383 by chemical agents**

Lysosomal trapping of 10 drugs was assessed in NR8383 cells using three different chemical agents. The analysis was performed initially with 20 mM NH₄Cl co-incubated with each compound at 37°C for 10 minutes. The pH of the incubation media showed minimal changes in the presence of NH₄Cl (reduction of up to 0.06 unit) relative to control media solutions. The effect of this agent on CL$_{\text{uptake}}$ of the drugs at 5 and 20 µM substrate concentrations is shown in Figure 3-3 and Figure 3-4, respectively. Of all drugs investigated, a significant reduction in CL$_{\text{uptake}}$ by NH₄Cl was only observed in the case of imipramine and clarithromycin (>59 and 75%, respectively, Table 3-3). The presence of NH₄Cl in the incubation did not significantly alter the uptake clearance of the remaining drugs at both 5 and 20 µM substrate concentrations. Furthermore, the degree of reduction in CL$_{\text{uptake}}$ for most drugs was comparable at both substrate concentrations. Even though CL$_{\text{uptake}}$ was reduced considerably for some drugs in the presence of NH₄Cl (e.g., by 45% for ipratropium bromide at 5 µM), the large variability associated with the data (both control and NH₄Cl conditions) resulted in no significant difference between the treatment and the control conditions.
Figure 3-3: CL_{uptake} estimated in NR8383 in the absence (■) (control) and presence (□) of 20 mM ammonium chloride (NH\textsubscript{4}Cl) at 5 µM concentration of (A) imipramine, clarithromycin, formoterol, rifampicin, budesonide, ciprofloxacin, and (B) ipratropium bromide, fenoterol, tiotropium bromide and terbutaline. Data represent mean ± SD of at least 3 experiments carried out at separate occasions (*, p < 0.05; **, p < 0.01 by Student’s t-test).
**Figure 3-4:** CL\textsubscript{uptake} estimated in NR8383 in the absence (■) (control) and presence (□) of 20 mM ammonium chloride (NH\textsubscript{4}Cl) at 20 µM concentration of (A) imipramine, clarithromycin, formoterol, rifampicin and budesonide, and (B) ipratropium bromide, fenoterol, tiotropium bromide and terbutaline. Data represent mean ± SD of at least 3 experiments carried out at separate occasions (*, p < 0.05 by Student’s t-test).
Table 3-3: Estimated reduction of $\text{CL}_{\text{uptake}}$ of investigated drugs by ammonium chloride (NH$_4$Cl), monensin and nigericin in NR8383. Lysosomal trapping was assessed at 5 and 20 µM drug concentration using NH$_4$Cl and at 5 µM only using monensin and nigericin. Data represent mean ± SD of at least 3 experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>NH$_4$Cl At 5 µM</th>
<th>NH$_4$Cl At 20 µM</th>
<th>Monensin At 5 µM</th>
<th>Monensin At 10 µM</th>
<th>Nigericin At 5 µM</th>
<th>Nigericin At 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>68 ± 6.1$^*$</td>
<td>59 ± 12</td>
<td>72 ± 10</td>
<td>62 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>85 ± 7.5$^*$</td>
<td>78 ± 9.0$^*$</td>
<td>78 ± 8.0$^{**}$</td>
<td>84 ± 9.2$^{**}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formoterol</td>
<td>25 ± 14</td>
<td>27 ± 13</td>
<td>41 ± 5.6</td>
<td>19 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fenoterol</td>
<td>36 ± 32</td>
<td>22 ± 28</td>
<td>29 ± 21</td>
<td>25 ± 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terbutaline</td>
<td>15 ± 15</td>
<td>36 ± 23</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Budesonide</td>
<td>18 ± 15</td>
<td>NR</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NR</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>NR</td>
<td>NR</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>45 ± 21</td>
<td>33 ± 16</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>15 $^#$</td>
<td>28 $^#$</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n/a, not available; NR, no reduction in $\text{CL}_{\text{uptake}}$; $^*$ Data represent mean of 2 experiments; *, $p < 0.05$; **, $p < 0.01$ by Student’s t-test

Lysosomal trapping of imipramine, clarithromycin, formoterol and fenoterol was investigated further in the presence of monensin and nigericin; total uptake clearance of these drugs in the absence and presence of the agents are illustrated in Figure 3-5. $\text{CL}_{\text{uptake}}$ of imipramine was significantly reduced in the presence of both monensin (by 72%) and nigericin (by 62%), in agreement with the effect of NH$_4$Cl. The reduction of clarithromycin uptake clearance was more pronounced than that of imipramine (78% and 84% by monensin and nigericin, respectively), confirming the rank order for lysosomal trapping seen from NH$_4$Cl data. In contrast, formoterol and fenoterol uptake clearance was not affected substantially in the presence of these agents. Less than 40% decrease in $\text{CL}_{\text{uptake}}$ was observed for fenoterol in the presence of both agents (Table 3-3). For formoterol, <20% decrease in $\text{CL}_{\text{uptake}}$ was also observed in the presence of nigericin, while monensin caused maximum 41% reduction in $\text{CL}_{\text{uptake}}$ of this drug in
NR8383. These findings are in agreement with the data obtained from NH₄Cl experiments.

**Figure 3-5:** CL\textsubscript{uptake} estimated in NR8383 in the absence (■) (control) and presence of (A) 10 µM nigericin (■) (B) 5 µM monensin (■) at 5 µM concentration of imipramine, clarithromycin, formoterol and fenoterol. Data represent mean ± SD of at least 3 experiments (*, p < 0.05; **, p < 0.01 by Student’s t-test).
In addition, $K_{p, NR8383}$ of each drug was estimated in order to assess the effect of NH$_4$Cl and other agents on the total cellular accumulation of the drugs investigated. Values obtained in both conditions are listed in Table 3-4. The highest CL$_{uptake}$ of imipramine in NR8383 was also reflected in its $K_{p, NR8383}$ of 347 (at 5 µM) under control conditions which was reduced by 3.4-fold in the presence of NH$_4$Cl. A similar fold change in its $K_{p, NR8383}$ in the presence of NH$_4$Cl was also observed at an imipramine concentration of 20 µM. The fold change in cellular accumulation was even more pronounced for clarithromycin, showing almost 6-fold reduction in $K_{p, NR8383}$ at 5 µM in the presence of NH$_4$Cl when compared to its value under control conditions (decrease in $K_{p, NR8383}$ from 82.7 to 13.3). The % reduction in $K_{p, NR8383}$ of majority of drugs in the presence of NH$_4$Cl was in good agreement (within 20%) at both substrate concentrations and with their % reduction in CL$_{uptake}$ at 5 µM. The effect of monensin and nigericin on $K_{p, NR8383}$ of clarithromycin and imipramine (Table 3-5) was comparable to the effect observed on CL$_{uptake}$ (Table 3-3), ranging from 65-75% for imipramine to 76-82% for clarithromycin. In the case of formoterol and fenoterol, the negative findings from studies with NH$_4$Cl were also confirmed by monensin and nigericin. The effect of chemical agents on these drugs and terbutaline (all basic) was less evident with <50% reduction in $K_{p, NR8383}$ and CL$_{uptake}$. In the case of neutral, permanently cationic and zwitterionic compounds, all three agents had either no or insignificant effects on their $K_{p, NR8383}$ and CL$_{uptake}$. 
Table 3-4: $K_{p,NR8383}$ estimated under control and NH$_4$Cl treatment conditions for 10 drugs investigated at 5 and 20 µM in NR8383 and % reduction in $K_{p,NR8383}$ following NH$_4$Cl (20 mM) treatment as a measure of the degree of lysosomal trapping. Data represent mean ± SD of at least 3 experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{p,NR8383}$ Control</th>
<th>$K_{p,NR8383}$ + NH$_4$Cl</th>
<th>Reduction by NH$_4$Cl (%)</th>
<th>$K_{p,NR8383}$ Control</th>
<th>$K_{p,NR8383}$ + NH$_4$Cl</th>
<th>Reduction by NH$_4$Cl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>347 ± 59.8</td>
<td>102 ± 17.1</td>
<td>71</td>
<td>238 ± 29.3</td>
<td>81.3 ± 6.50</td>
<td>66</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>82.7 ± 27.3</td>
<td>13.3 ± 6.50</td>
<td>84</td>
<td>58.2 ± 11.6</td>
<td>14.2 ± 4.44</td>
<td>76</td>
</tr>
<tr>
<td>Formoterol</td>
<td>20.8 ± 3.60</td>
<td>15.5 ± 1.16</td>
<td>26</td>
<td>26.5 ± 6.54</td>
<td>19.2 ± 4.13</td>
<td>28</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>4.25 ± 2.64</td>
<td>2.91 ± 2.11</td>
<td>32</td>
<td>1.50 ± 0.93</td>
<td>0.96 ± 0.33</td>
<td>36</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.92 ± 0.39</td>
<td>0.72 ± 0.31</td>
<td>22</td>
<td>0.79 ± 0.46</td>
<td>0.56 ± 0.29</td>
<td>29</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>27.9 ± 3.54</td>
<td>33.2 ± 11.9</td>
<td>NR</td>
<td>34.6 ± 12.1</td>
<td>33.3 ± 12.2</td>
<td>4</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>4.39 ± 2.90</td>
<td>3.87 ± 2.61</td>
<td>12</td>
<td>2.40 ± 1.00</td>
<td>2.00 ± 0.64</td>
<td>17</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>4.94 ± 0.30</td>
<td>2.69 ± 1.09</td>
<td>46</td>
<td>3.71 ± 2.54</td>
<td>2.16 ± 1.37</td>
<td>42</td>
</tr>
<tr>
<td>Budesonide</td>
<td>31.5 ± 8.46</td>
<td>28.1 ± 7.12</td>
<td>11</td>
<td>33.9 ± 12.5</td>
<td>31.0 ± 12.0</td>
<td>9</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>43.0 ± 21.1</td>
<td>37.6 ± 20.5</td>
<td>13</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a, not available; NR, no reduction in $K_{p,NR8383}$
Table 3-5: $K_{p, NR8383}$ estimated under control and monensin (5 µM) or nigericin (10 µM) treatment conditions for 4 drugs investigated at 5 µM in NR8383 and % reduction in $K_{p, NR8383}$ following treatment with ionophore agents monensin and nigericin. Data represent mean ± SD of at least 3 experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{p, NR8383}$ Control</th>
<th>$K_{p, NR8383}$ + Monensin</th>
<th>$K_{p, NR8383}$ + Nigericin</th>
<th>% Reduction in $K_{p, NR8383}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>339 ± 83.0</td>
<td>85.7 ± 48.0</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>277 ± 54.0</td>
<td>-</td>
<td>96 ± 43.0</td>
<td>65</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>117 ± 22.0</td>
<td>27.8 ± 13.0</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>94.5 ± 30.0</td>
<td>-</td>
<td>17.4 ± 12.3</td>
<td>82</td>
</tr>
<tr>
<td>Formoterol</td>
<td>30.2 ± 16.3</td>
<td>17.7 ± 8.20</td>
<td>-</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>35.7 ± 23.6</td>
<td>-</td>
<td>25.7 ± 16.4</td>
<td>28</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>5.93 ± 1.38</td>
<td>4.54 ± 2.56</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>7.61 ± 4.36</td>
<td>-</td>
<td>6.29 ± 4.20</td>
<td>17</td>
</tr>
</tbody>
</table>

3.4.4 Characterisation of uptake kinetics and accumulation of clarithromycin in NR8383 cells in the presence of NH₄Cl

Assessment of uptake kinetics of clarithromycin previously showed non-saturating conditions over a wide concentration range (1-100 µM). Under these conditions, kinetic parameters could not be determined for clarithromycin (Chapter 2). In order to assess whether lysosomal trapping could be the reason for the non-saturable uptake of clarithromycin in NR8383 cells, the uptake kinetics of this drug was further investigated in the presence of NH₄Cl. The concentration vs. rate profile of clarithromycin in the absence and presence of NH₄Cl is shown in Figure 3-6A and the profile in the presence of NH₄Cl in isolation is shown in Figure 3-6B. The uptake rates of clarithromycin were reduced markedly at all substrate concentrations compared to the rates under control conditions. The kinetic profile in the presence of NH₄Cl suggested saturable uptake although uptake rates were highly variable (CV>30% at most concentrations). In the presence of NH₄Cl, $K_m$ and $V_{max}$ of clarithromycin were estimated as 28.5 µM and 12.4 pmol/min/10⁶ cells, respectively (Table 3-6). Large variability was associated with both parameters, particularly with $K_m$ (CV>30%). CL_{active} was determined from the ratio of
$V_{\text{max}}$ and $K_{\text{m}}$ as 0.43 $\mu$L/min/10$^6$ cells. $\text{CL}_{\text{diff}}$ was 0.03 $\mu$L/min/10$^6$ cells and obtained at 4°C at 5 $\mu$M. Consequently, $\text{CL}_{\text{uptake}}$ was the sum of $\text{CL}_{\text{active}}$ and $\text{CL}_{\text{diff}}$ and was 0.46 $\mu$L/min/10$^6$ cells.

The accumulation of clarithromycin in NR8383 in the absence and presence of NH$_4$Cl was characterised by determining $K_{p,NR8383}$ over the range of drug concentrations. Similar to the uptake rates, a marked reduction in $K_{p,NR8383}$ of clarithromycin was observed at all substrate concentrations (Figure 3-7). Accumulation of clarithromycin showed concentration dependence under control conditions (Figure 2-6B, Chapter 2), whereas in the presence of NH$_4$Cl, minimal concentration dependence was observed. The fitting of the cellular accumulation of clarithromycin under control conditions by nonlinear regression to a two-site model resulted in estimated $K_{p,U1,\max}$ and $K_{p,\min}$ of 45 and 24, respectively (Table 2-5, Chapter 2). Therefore, the mean maximum fitted $K_{p,NR8383}$ was 69 under control conditions. In the presence of NH$_4$Cl, $K_{p,NR8383}$ of clarithromycin was less dependent on the initial drug concentration and markedly reduced at both low and high concentrations compared to that observed under control conditions. Estimated $K_{p,U1,\max}$ and $K_{p,\min}$ values in the presence of NH$_4$Cl were 9.7 and 4.7, respectively, whereas the mean maximum fitted $K_{p,NR8383}$ was 14.4.
Figure 3-6: Uptake kinetic profile of clarithromycin in NR8383 cells. Data points are the uptake rates measured in duplicate over 10 minutes from the cell concentrations. Solid line represents total uptake at 37°C. (●) Total uptake under control conditions at 37°C; (□) Total uptake in the presence of 20 mM NH₄Cl at 37°C. Data represent mean ± SD of 3 experiments. A) Uptake rates in the absence and presence of NH₄Cl; B) Uptake rates in the presence of NH₄Cl.
Table 3-6: Uptake kinetic parameters estimated for clarithromycin in NR8383 cells in the presence of 20 mM NH$_4$Cl. Data represent mean ± SD of 3 experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (µM)</td>
<td>28.5 ± 21.7</td>
</tr>
<tr>
<td>Vmax (pmol/min/10$^6$ cells)</td>
<td>12.4 ± 3.76</td>
</tr>
<tr>
<td>CL$_{diff}$ (µL/min/10$^6$ cells)</td>
<td>0.03 ± 0.03$^1$</td>
</tr>
<tr>
<td>CL$_{active}$</td>
<td>0.43</td>
</tr>
<tr>
<td>CL$_{uptake}$</td>
<td>0.46</td>
</tr>
</tbody>
</table>

$^1$CL$_{diff}$ estimated at 5 µM at 4°C and used as a constant in Equation 2-1 (Chapter 2) to estimate K$_m$ and V$_{max}$. The parameters have been corrected for nonspecific binding (fu$_{med}$ = 0.93 ± 0.03).

Figure 3-7: K$_{p,NR8383}$ of clarithromycin over the range of drug concentration in the absence (●) and presence (□) of 20 mM NH$_4$Cl at 37°C. Data represent mean ± SD of 3 experiments.
3.4.5 Assessment of cytotoxicity of the chemical agents used in lysosomal trapping studies

The cytotoxicity of all three agents either on their own or when co-incubated with clarithromycin or imipramine was assessed. When the agents were tested on their own, their concentrations were the same as used in lysosomal trapping studies (20 mM for \( \text{NH}_3\text{Cl} \), 5 \( \mu \text{M} \) for monensin and 10 \( \mu \text{M} \) for nigericin). The concentration of clarithromycin and imipramine was 5 \( \mu \text{M} \). Cytotoxic effect of the agents was minimal at the concentrations they were used (alone or with a drug); the LDH activity was less than 2% compared to 100% of the positive control of the assay kit. At all times, the viability of the control (i.e., untreated) cells were more than 95%.

3.4.6 Correlation of physicochemical properties of basic drugs to their lysosomal trapping

Physicochemical properties of five basic drugs (LogP and basic pK\(_a\)) were correlated to the % reduction in CL\(_{\text{uptake}}\) as a measure of their lysosomal trapping in NR8383. A positive correlation (\( R^2 = 0.67 \)) was found between LogP and % reduction in CL\(_{\text{uptake}}\) (Figure 3-8), indicating increased lysosomal trapping of basic drugs with increasing lipophilicity. This correlation was not found to be significant (\( p=0.09 \)), most likely due to a small number of drugs in the dataset. This dataset was considered representative as it covered a wide range in LogP (from 0.9 to 4.8 for terbutaline and imipramine, respectively) and % reduction in CL\(_{\text{uptake}}\) (ranging from 15 to 85% for terbutaline and clarithromycin, respectively). In contrast to logP, no correlation was evident between % reduction in CL\(_{\text{uptake}}\) and the basic pK\(_a\) of the drugs investigated (\( R^2 = 0.02 \), Figure 3-9). The significance of the correlation was lower (\( p=0.82 \)) compared to that observed between LogP and % reduction in CL\(_{\text{uptake}}\). In contrast to the wide range in LogP, range in pK\(_a\) was relatively low, spanning from 8.14 for formoterol to 9.9 for terbutaline.
Figure 3-8: Correlation between LogP and % reduction in CL_{uptake} measured at 5 µM of the basic drugs in NR8383. The CL_{uptake} data were obtained from at least three separate experiments. LogP data were from Table 1-4. (■) imipramine; (■) clarithromycin; (■) formoterol; (■) fenoterol; (■) terbutaline.

Figure 3-9: Correlation between basic pKa and % reduction of CL_{uptake} measured at 5 µM of basic drugs in NR8383. The CL_{uptake} data were obtained from at least three separate experiments. Basic pK_a data were from Table 1-4. (■) imipramine; (■) clarithromycin; (■) formoterol; (■) fenoterol; (■) terbutaline.
3.5 Discussion

3.5.1 Assessment of lysosomal trapping by use of chemical agents

In the present study, the quantitative analysis of lysosomal trapping was performed using a number of chemical agents reported to reduce lysosomal trapping such as NH₄Cl, monensin and nigericin (Appendix Table 7-3). The CLuptake and Kᵢ,NR8383 were determined in the absence and presence of these agents and the reduction observed in their values was attributed to lysosomal distribution. This approach provided an indirect way of evaluating lysosomal sequestration of drugs, as it did not involve direct measurement of drug concentrations in lysosomes. Although direct measurement is preferable and more informative about the extent of drug sequestration, it also has a number of practical difficulties and disadvantages. It takes considerable time and effort to isolate individual organelles through homogenisation and fractionation which can often result in cross-contamination of isolated fractions (Yoshida et al., 1987). A major problem associated with homogenisation and/or fractionation is the poor recovery of the drug in isolated fractions. Significant drug loss can occur during the isolation of lysosomes which involves many steps and transfers. Furthermore, the disruption of lysosomes during the procedure may result in the redistribution of the compounds to other cellular fractions. Moreover, possible diffusion of the drugs out of the lysosomes during sample preparation remains to be a major limitation (MacIntyre and Cutler, 1988b; Duvvuri et al., 2004a; Duvvuri and Krise, 2005a).

The initial investigation of the extent of lysosomal trapping presented in this chapter showed that clarithromycin and imipramine substantially accumulated in the lysosomes of NR8383, as illustrated by the reduction of their CLuptake and Kᵢ,NR8383 in presence of NH₄Cl (>59%, Table 3-3 and Table 3-4). This reduction was consistently greater for clarithromycin by approximately 10-20% compared to imipramine. While this suggests a greater degree of lysosomal trapping of clarithromycin, the smaller % reduction observed for imipramine may also be a reflection of its higher accumulation (4-fold higher Kᵢ,NR8383 under control conditions) due to a greater degree of binding to membrane phospholipids. Therefore, the results suggest an important contribution of both lysosomal trapping and membrane binding to the observed accumulation of these two drugs in rat AM cell line. The demonstration of the lysosomal trapping of clarithromycin in NR8383 is in good agreement with the recently published studies in the same cell line (Togami et al., 2013). Togami and colleagues have shown that
treatment of NR8383 with NH₄Cl reduced the amount of clarithromycin accumulated in the granule fraction (containing lysosomes) by 88% (Appendix Table 7-3). Therefore, our observation of at least 75% reduction in CLuptake or Kp,NR8383 of clarithromycin by NH₄Cl further supports these findings.

The reduction of cellular accumulation of imipramine in NR8383 cells in the presence of NH₄Cl is in agreement with previous studies showing reduced accumulation of the drug in the lung (by 35-59%) and liver (by 57-62%) tissues of rats by this agent (Appendix Table 7-3). In addition, accumulation of imipramine in rat hepatocytes or tissues (particularly lungs, liver and kidneys) has been shown to be reduced by other lysosomotropic lipophilic amine compounds (Daniel and Wojcikowski, 1999a; Daniel and Wojcikowski, 1999b; Hallifax and Houston, 2006). Furthermore, imipramine has previously been shown to accumulate in lysosomes isolated from rat liver and this accumulation was reduced by NH₄Cl by 30-70% depending on NH₄Cl concentration (Ishizaki et al., 1996; Ishizaki et al., 1998b). For the remaining basic drugs in the dataset including formoterol, fenoterol and terbutaline, no significant lysosomal trapping was observed even though they were predicted to accumulate in the lysosomes (Table 3-1).

Following the initial assessment of lysosomal trapping of all the compounds in the dataset with NH₄Cl, monensin and nigericin were used to confirm some of the previous findings with NH₄Cl and to compare the potencies of the three agents in assessing the extent of lysosomal trapping indirectly. The data obtained with ionophores were in agreement with those obtained with NH₄Cl for the drugs selected for detailed investigation. Significant reduction in CLuptake and Kp,NR8383 of clarithromycin and imipramine was also seen using both ionophores (>76 for clarithromycin and >62% for imipramine), whereas this was not evident for formoterol and fenoterol (Table 3-3 and Table 3-5). Reduced accumulation of imipramine by monensin (by 50%) has previously been reported in rat lung slices (Daniel and Wojcikowski, 1997). Both monensin and nigericin generally caused similar level of reduction in CLuptake and Kp,NR8383 of all four compounds compared to NH₄Cl. Moreover, both caused a greater reduction in CLuptake and Kp,NR8383 of clarithromycin compared to imipramine, which is in further agreement with NH₄Cl data. Therefore, these findings suggest that the effectiveness of the three agents in reducing lysosomal trapping of basic drugs was comparable at the concentrations used. This was in agreement with the findings of Daniel and Wojcikowski (1997) who demonstrated a similar extent of reduction in imipramine
accumulation \((K_p)\) in rat lung and liver slices by NH$_4$Cl and monensin (Daniel and Wojcikowski, 1997).

### 3.5.2 Assessment of lysosomal trapping by use of LysoTracker® Red

In an effort to localise lysosomes in NR8383 and further assess lysosomal trapping in the cell line, experiments were performed using a lysosome specific fluorescent dye LysoTracker® Red and the cells were visualised following treatment with confocal laser scanning microscopy. The use of LTR is considered as an indirect and semi-quantitative approach for the evaluation of the lysosomal sequestration compounds that are not fluorescent (Goldman et al., 2009; Nadanaciva et al., 2011). The advantages of this approach are its simplicity, no requirement for cell fractionation, and that it enables the assessment of non-fluorescent compounds (Lemieux et al., 2004). The confocal imaging of NR8383 treated with LTR provided localisation of lysosomes which were found to be abundant in the cells. The accumulation of LTR in the lysosomes was evident and much reduced in the presence of NH$_4$Cl, monensin and nigericin at the concentrations used in the quantitative studies. The reduction of LTR staining of lysosomes by 85% in the presence of all agents confirmed their inhibitory effect on lysosomal trapping of basic drugs in NR8383 cells. Furthermore, the treatment of the cells with LTR in the presence of the selected basic drugs further provided a confirmation for the accumulation of these drugs in NR8383 cells. In the case of clarithromycin and imipramine, accumulation of LTR in the lysosomes was reduced by 86 and 72%, respectively, which resulted from the competition of these basic drugs with LTR. In contrast, LTR accumulation remained relatively intact in the presence of formoterol which caused only 25% reduction in measured LTR fluorescence intensity, suggesting no significant competition of this drug with LTR for lysosomal accumulation. These studies demonstrated for the first time localisation of lysosomes and assessment of amine accumulation in the presence of chemical agents and other basic drugs in NR8383 using the LTR staining method.

### 3.5.3 Assessment of uptake kinetics of clarithromycin in the presence of NH$_4$Cl

Assessment of clarithromycin uptake in NR8383 over a wide concentration range under control conditions at 37°C showed that the uptake of this drug was not saturable (Section 2.4.5). Having demonstrated that clarithromycin undergoes lysosomal trapping in NR8383, its uptake was re-evaluated in the presence of NH$_4$Cl in order to determine
whether the mentioned process could impact the kinetics of clarithromycin uptake in NR8383. A reduction in the extent of lysosomal trapping in the presence of NH₄Cl resulted in the markedly reduced uptake rates. Under this condition, estimates of Vₘₐₓ and Kₘ could be obtained in contrast to the control condition. However, the variation associated with Vₘₐₓ and Kₘ was 30% and 76%, respectively. Although it is tempting to suggest that clarithromycin showed uptake saturation kinetics in the presence of NH₄Cl, the large variability associated with the rate data at most substrate concentrations (Figure 3-7B) makes it difficult to reach this conclusion. Another factor compromising the reliability of the estimates is that CLₐ₅ were estimated from 4°C experiments (0.03 μL/min/10⁶ cells, CV=100%) at single concentration and fixed in the equation (Equation 2-1, Chapter 2) used to derive the kinetic parameters. Therefore, the unreliability of the CLₐ₅ estimate obtained at 4°C may lead to an error in the Vₘₐₓ and Kₘ estimates. CL₂ uptake, obtained from the sum of CLactive and CLdiff, was 0.46 μL/min/10⁶ cells and was comparable to the value obtained at 5 μM in the presence of NH₄Cl (0.71 ± 0.28 μL/min/10⁶ cells, Figure 3-3).

While it is clear that uptake of clarithromycin in NR8383 is largely driven by lysosomal sequestration, an involvement of a low affinity transporter may also explain the so-called saturation. However, clarithromycin has not been demonstrated to be a substrate for any particular transporter, although it has been suggested to be a substrate for hepatic uptake transporters (Yabe et al., 2011). A recent study using OATP (OATP1B1, OATP1B3 and OATP2B1) and OCT1 transfected HEK293 cells showed that clarithromycin was not a substrate for these transporters (Higgins et al., 2014). These results were further supported by demonstrating that clarithromycin uptake was not impaired in cryopreserved human hepatocytes by inhibitors of these transporters in vitro (Higgins et al., 2014). A more plausible explanation of the present data could be that lysosomal pH may have increased with increasing clarithromycin concentration, leading to saturation. Although lysosomal trapping of clarithromycin was reduced to a large extent in the presence of NH₄Cl, this process may not be eliminated completely if lysosomal pH was not equilibrated with cytosolic pH. Therefore, some contribution of ion trapping that is saturable in combination with its interaction with lysosomal and plasma membrane phospholipids (Kosol et al., 2012) can lead to observed accumulation.
3.5.4 Assessment of clarithromycin accumulation over a concentration range in the presence of NH₄Cl

Accumulation of clarithromycin ($K_{p,NR8383}$) in NR8383 was shown to be concentration dependent in the absence of NH₄Cl (Section 2.4.4). The use of a two-site binding model was explored in describing the accumulation of clarithromycin in NR8383 cells. The estimated maximum $K_p$ of clarithromycin was 69 under these conditions. While this indicates a considerable accumulation of the drug in NR8383 cells, its accumulation was less substantial compared to imipramine ($K_{p,max}$ of 1166, Table 2-5). This may be a reflection of relatively less binding interactions of clarithromycin with membranes relative to imipramine, a prototypical cationic amphiphilic amine, due to differences in their lipophilicity and amphiphilicity. The minimum $K_{p,NR8383}$ of clarithromycin in the absence of NH₄Cl was 24. If saturation at high concentration was assumed, this baseline accumulation would reflect the extent of nonspecific binding of clarithromycin to phospholipids in plasma and lysosomal membranes. In contrast, binding of imipramine to membrane lipids is more extensive as reflected in its approximately 6-fold higher estimated $K_{p,min}$ of 141. The maximum uptake of clarithromycin was reduced by 4.8-fold in the presence of NH₄Cl to an estimated $K_{p,max}$ of 14.4, demonstrating a substantial role of an active process which can be attributed to its lysosomal trapping in NR8383. This active process seems to be still present as concentration dependent accumulation was also observed in the presence of NH₄Cl (3-fold difference between $K_{p,max}$ and $K_{p,min}$). An incomplete reduction of lysosomal trapping of clarithromycin by NH₄Cl and/or involvement of another active process (i.e., transporter uptake) that was not affected by NH₄Cl may explain the observed saturable component of clarithromycin uptake in the presence of the agent. Therefore, the extent of accumulation estimated in the presence of NH₄Cl seems to reflect both saturable and nonsaturable uptake processes (diffusion and subsequent binding to phospholipids of lysosomal and plasma membranes). The $K_{p,min}$ in the presence of NH₄Cl was 4.7, indicating almost a 5-fold difference in $K_{p,min}$ compared to control conditions. This may indicate the contribution of lysosomal trapping to clarithromycin uptake in the absence of NH₄Cl even at high concentrations, suggesting incomplete saturation of this active process at 100 µM. Therefore, the true $K_{p,min}$ of clarithromycin is likely to be closer to the estimate in the presence of NH₄Cl.
3.5.5 Correlation of the extent of lysosomal trapping with physicochemical properties of the drugs investigated

Compounds which are sequestered in lysosomes are typically basic lipophilic or CADs (Nadanaciva et al., 2011; Kazmi et al., 2013). As LogP and basic pK\textsubscript{a} have often been related to the lysosomal trapping potential of basic lipophilic compounds, a correlation between these properties and % reduction in CL\textsubscript{uptake} was sought for the basic drugs in the dataset. A positive correlation was found between LogP and % reduction in CL\textsubscript{uptake} of the compounds. The two most lipophilic compounds imipramine and clarithromycin (LogP>3, Table 1-4) showed lysosomal sequestration in NR8383, whereas the remaining basic drugs formoterol, fenoterol and terbutaline (LogP<2, Table 1-4) did not accumulate in the lysosomes. While terbutaline and fenoterol are hydrophilic, formoterol is moderately but less lipophilic than clarithromycin which may explain their non-targeting of lysosomes.

However, no relationship could be established between basic pK\textsubscript{a} in isolation and the % reduction in CL\textsubscript{uptake} of the basic drugs investigated (R\textsuperscript{2}=0.02). Previously, lysosomal accumulation relative to cytosol of a number of weakly basic drugs (pK\textsubscript{a} < 9) has been shown to increase with increasing pK\textsubscript{a} of the compounds (Duvvuri et al., 2005). However, the pK\textsubscript{a} range in that dataset was wider in comparison to the current study, with all the compounds having comparable lipophilicity (LogP between 1.3-2.1) (Duvvuri et al., 2005).

More recently, a number of studies have evaluated lysosomal sequestration of a range of therapeutic compounds with a wide range of physicochemical properties in different cell systems (Nadanaciva et al., 2011; Kazmi et al., 2013). In both studies, authors indicated that both LogP>2 and basic pK\textsubscript{a} between 6.5-10.5 were required for lysosomal sequestration of drugs investigated and having either of the physicochemical properties alone was not sufficient for lysosomal accumulation. Therefore, the authors highlighted the importance of coexisting sufficient lipophilicity and ionisation for basic drugs for lysosomal sequestration. For instance, the dataset in Nadanaciva et al. (2011) included both imipramine and ciprofloxacin; imipramine was shown to be a potent lysosomotropic compound (IC\textsubscript{50}:10.7 µM) and this was supported by its cLogP: 5.04 and basic pK\textsubscript{a}: 9.49. In contrast, ciprofloxacin was not found to decrease LTR accumulation (IC\textsubscript{50}>150 µM) and this could be rationalised by its inadequate lipophilicity (cLogP: -0.73), despite a basic pK\textsubscript{a} of 8.34. These findings are in agreement with the results presented here for these drugs. Despite the physicochemical
space mentioned that is needed for lysosomal trapping of basic, lipophilic drugs, a number of exceptional drugs have also been indicated in both studies by having LogP>2 and pK_a 6.5-10.5 (e.g., chlorpheniramine, raclopride) but not showing lysosomal sequestration (Nadanaciva et al., 2011; Kazmi et al., 2013). Although these two studies were informative as they were covering a large dataset with a wide range of physicochemical properties, they generalised that drugs with LogP>2.0 and pK_a>6.5 will undergo lysosomal sequestration. While there were exceptional drugs in these studies as mentioned above, an earlier study by Duvvuri et al. (2004b) has shown that a number of weakly basic compounds with LogP>2.0 and basic pK_a near neutrality (6.9-8.0) showed mitochondrial accumulation. In that study, they evaluated the permeability characteristics of weakly basic compounds with similar basic pK_a and LogP values and how it influenced their sub-cellular distribution. They have shown that compounds with a low permeability ratio of the ionised form to the unionised form (i.e., near zero) specifically accumulated in the lysosomes compared to those with the ratio near unity which were specifically sequestered in mitochondria, allowing to distinguish between lysosomal and mitochondrial accumulation of investigated weakly basic compounds. Therefore, this study highlighted the importance of the permeability ratio, other than LogP and pK_a, which should also be considered when evaluating intracellular distribution of weakly basic compounds.

3.5.6 Mechanisms driving lysosomal accumulation of drugs in NR8383 cells
While pH partitioning is the predominant driving force for lysosomal sequestration of basic and lipophilic drugs, a number of other mechanisms have also been proposed to potentially contribute to their overall accumulation in the lysosomes (Ishizaki et al., 2000; Duvvuri and Krise, 2005a). The reason behind this proposal was down to the observations that experimentally determined lysosome-to-medium concentration ratios of the basic, lipophilic drugs were much higher than the theoretically determined ratios based solely on pH-partitioning. For instance, Ishizaki et al. (2000) reported a 2 orders of magnitude difference between experimentally and theoretically determined lysosomal K_p of imipramine. They suggested that in addition to pH-gradient dependent uptake, binding (partition or adsorption) of the protonated form to the lysosomal membrane and/or its aggregation (dimerization or self-association) in the lumen of the lysosomes possibly contribute to its accumulation. More recently, a similar suggestion has also been made by Duvvuri and Krise (2005b) who observed higher concentrations of
quanacrine and LTR in isolated lysosomes than their predicted concentrations based solely on pH-partitioning. Although such comparison cannot be performed here as lysosomal accumulation was not measured directly, observed cellular accumulation of imipramine ($K_{p,NR8383}$: 347 at 5 µM) can be compared with its predicted cellular accumulation based on the pH-partitioning theory ($K_p$ (cytosol-to-medium concentration ratio): 1.58). This comparison also shows that imipramine accumulates in the cell to a much greater extent than the predicted accumulation based solely on pH-partition calculation which does not consider the more pronounced pH-gradient between lysosomes and cytosol, in addition to lysosomal and intracellular binding processes.

In the current study, imipramine uptake in NR8383 was reduced by at least 60-75% in the presence of chemical agents which abolished lysosome-cytosol pH gradient. This suggests that much of its accumulation in NR8383 is due to this pH-gradient dependent mechanism while the remaining accumulation that was not affected is possibly due to binding to cell and lysosomal membranes. Another possible contributor to the observed remaining accumulation, which can be assumed to be minimal compared to binding, is ion trapping in the lysosomes if lysosomal pH did not equilibrate with cytosolic pH. In the case of clarithromycin, the larger reduction of its uptake (by 76-85%) in the presence of the agents indicates greater pH-gradient dependence of this compound for lysosomal accumulation than imipramine. A lesser reducing effect of NH$_4$Cl towards imipramine uptake compared to clarithromycin may be due to higher lipophilicity of imipramine which resulted in greater intracellular binding. Drug lipophilicity has previously been correlated with the % reduction in lysosomal accumulation of basic drugs by NH$_4$Cl. The % reduction in basic drug uptake by NH$_4$Cl was shown to decrease with increasing lipophilicity (Ishizaki et al., 1998b).

3.5.7 Conclusion

In conclusion, the results presented in this chapter provide information with regards to intracellular distribution of respiratory drugs in AMs. Lysosomal sequestration of a range of respiratory drugs was assessed in NR8383 cells using a number of different techniques including the use of chemical agents known to reduce lysosomal trapping of basic drugs (NH$_4$Cl, monensin and nigericin) and LTR used to localise lysosomes in NR8383. Lysosomal trapping of drugs was assessed indirectly from the % reduction in $CL_{uptake}$ or $K_{p,NR8383}$ of the drugs in the presence of the agents relative to control conditions. The % reduction in $K_{p,NR8383}$ of drugs showed a wide range from no
reduction in the case of rifampicin to 84% reduction seen for clarithromycin. Among the respiratory drugs investigated, clarithromycin was shown to accumulate in the lysosomes of NR8383 cells and for the remaining drugs there was no indication of significant lysosomal accumulation. The work presented here also demonstrated that lysosomal sequestration of basic, lipophilic drugs can influence their uptake kinetics which is an important consideration when scaling these parameters to \textit{in vivo}. The assessment of the accumulation and lysosomal trapping of drugs in NR8383 at a single substrate concentration will be extended next to human alveolar macrophages and comparison of the two systems will be provided (Chapter 4).
Chapter 4  *In vitro* investigation of uptake and lysosomal trapping of drugs in human alveolar macrophages

4.1 Introduction

Although investigation of drug accumulation in rat and rabbit AMs has been performed (Hand and King-Thompson, 1982; Heyneman and Reasor, 1986; Antonini and Reasor, 1991; Togami et al., 2009; Togami et al., 2010; Togami et al., 2011; Togami et al., 2013), corresponding studies in human AMs are still relatively limited. Over the last three decades, there have been only a few published studies characterising uptake of respiratory drugs in human AMs. In one of the first studies, uptake of a range of antibiotics (n=12) including rifampicin, clindamycin and erythromycin at a single substrate concentration was investigated in AMs from healthy non-smokers and varying levels of accumulation were reported (Hand et al., 1984). In addition, the involvement of transporter mediated drug uptake was investigated by evaluating the dependence of uptake on cell viability and temperature. The energy requirement of drug uptake was further evaluated by using inhibitors of metabolic processes (such as mitochondrial oxidative metabolism). In the subsequent work, the authors demonstrated the uptake of the same antibiotics in AMs from smokers with pulmonary disease (Hand et al., 1985). Although the extent of accumulation of most of the antibiotics was comparable between healthy non-smokers and smokers with pulmonary disease, AMs of smokers accumulated a number of drugs to a greater extent than those of non-smokers (e.g., rifampicin, clindamycin) (Hand et al., 1984; Hand et al., 1985; Carlier et al., 1987), as summarised in Appendix Tables 7-6 and 7-7. In addition to the comparison of antibiotic uptake in AMs of smokers and non-smokers, the uptake of the same mentioned antibiotics was investigated in rabbit AMs at a single concentration and thereby provided species comparison in drug uptake (Johnson et al., 1980). However, there has been no study to date investigating drug uptake simultaneously in NR8383 and human AMs to provide comparison between these two systems. Similarly, despite the presence of a number of studies investigating lysosomal trapping of drugs in NR8383 (Togami et al., 2009; Togami et al., 2010; Togami et al., 2011; Togami et al., 2013), there has been no attempt to characterise this process in human AMs. It is particularly important to demonstrate that such processes in human AMs can be predicted in advance by studies in either animals or in appropriate *in vitro* systems. Therefore, there is a need to
investigate uptake and lysosomal accumulation of respiratory drugs in both human AMs and NR8383 to evaluate the appropriateness of the latter in vitro system in predicting the behaviour of respiratory drugs in humans.

4.2 Aims

One of the aims of this current chapter is to characterise the uptake of 10 drugs in human AMs which were previously assessed in the NR8383 cell line. Drug uptake was investigated at 37°C and 4°C to delineate active uptake and passive diffusion processes at a single substrate concentration (5 µM). Further aims include assessment of lysosomal trapping of clarithromycin in human AMs at 37°C at a single substrate concentration in the presence of chemical agents, namely NH₄Cl, monensin and nigericin. Imipramine, prototypical lysosomotropic drug, was used in those studies as a positive control. The accumulation of drugs in human AMs was assessed by determining their $K_{p,h\text{AM}}$. Furthermore, $CL_{\text{uptake}}$ of drugs was determined in addition to the contribution of passive diffusion and active uptake processes. $K_{p,AM}$ and $CL_{\text{uptake}}$ were further determined in the absence and presence of the agents as an indication for lysosomal trapping of the two compounds. Lysosomes of human AMs were localised and the lysosomal trapping of the two compounds were assessed qualitatively using LTR. The comparison of the findings from the drug uptake and lysosomal trapping studies in human AM and NR8383 was performed.
4.3 Methods

4.3.1 Chemicals and Reagents
In addition to the chemicals and reagents used in Chapter 2 and 3, the reagents used were as follows; Roswell Park Memorial Institute (RPMI)-1640 medium from Sigma-Aldrich, Dorset, UK, 200 mM L-glutamine from Life Technologies, Paisley, UK and Ficoll-Paque from GE Healthcare, Buckinghamshire, UK.

4.3.2 Source and preparation of human alveolar macrophages
The human alveolar macrophages were kindly provided from the Respiratory and Allergy Clinical Research Facility in the University Hospital of South Manchester NHS Foundation Trust. The cells were obtained from patients undergoing lung surgery at the hospital. The isolation of the cells was performed by the research technical staff in the mentioned Research Centre, as described below.

4.3.2.1 Isolation of alveolar macrophages from lung surgery sections
Areas of lung distant from the tumour were perfused with 0.1M sodium chloride. The resulting cell suspension was centrifuged (400g, for 10 minutes, at room temperature) and the cell pellet was re-suspended in RPMI-1640 medium. The cells were layered over a Ficoll-Paque gradient. The mononuclear cells at the Ficoll interface were extracted, washed and re-suspended in complete media: RPMI-1640 medium containing 10% v/v FBS, 1% v/v 200 mM L-glutamine, 1% v/v 100 units/ml penicillin and 100 µg/ml streptomycin. Following isolation, the cells were maintained in this complete media in the fridge before the start of the experiments. All experiments were completed within 36 hours of surgery.

4.3.3 Patient demographics
Nine patients undergoing surgical resection for suspected or confirmed lung cancer were recruited for human alveolar macrophage assays (Table 4-1). Patients were categorised as either smokers (≥1 pack year history) or non-smokers (<1 pack year history). All patients with the exception of one had normal lung function as predicted forced expiratory volume in 1 second (FEV1) was greater than 80%, and FEV1/Forced vital capacity (FVC) ratio was greater than 70%. One patient had predicted FEV1 of 69% and FEV1/FVC of 68%. This patient was asymptomatic, not diagnosed as having
chronic obstructive pulmonary disease and was thought to have airway obstruction due to tumour’s anatomical position. All patients were known to be free from any medications except one patient who had salbutamol in her medication history. The information regarding whether the patient was having the medication prior to surgery is unknown. The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents. All subjects gave written informed consent. The study was approved by the local research ethics committee (South Manchester Research Ethics Committee).

Table 4-1: The demographics of the patients recruited for human AM experiments. Data are presented as mean with standard deviation in brackets.

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>NS</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F/M)</td>
<td>1/0</td>
<td>5/3</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63</td>
<td>66.0 (6.10)</td>
</tr>
<tr>
<td>FEV1% predicted</td>
<td>109</td>
<td>91.8 (11.9)</td>
</tr>
<tr>
<td>FEV1/FVC ratio (%)</td>
<td>78</td>
<td>77.6 (6.60)</td>
</tr>
<tr>
<td>Smoking history</td>
<td>n/a</td>
<td>49.3 (25.6)</td>
</tr>
<tr>
<td>Medications</td>
<td>Nil</td>
<td>1/nil</td>
</tr>
</tbody>
</table>

NS, Non-smoker; S, Smoker; FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity; Smoking history is pack years; n/a, not applicable.

4.3.4 Optimisation of in vitro uptake conditions

Optimisation of in vitro conditions for human AMs was based on the experimental conditions set up for NR8383 and the observation of the cell’s attachment behaviour in the first experiment. While some of the conditions including the coating type of 24-well plates and drug incubation time points were adapted from NR8383, two conditions were optimised for human AMs specifically due to different attachment characteristics of these two cells and limited number of human AMs provided on each occasion. These conditions were cell seeding density to collagen-coated 24-well plates and culturing time before uptake experiments.

In the initial experiments cells were observed under the light microscope (Olympus CK2, Olympus Optical Co., Japan) for size, shape and brief inspection of cytoplasmic appearance. Due to the limited number of human cells provided per subject, cell density
of 0.3 million cells/well relative to 0.5 million cells/well used for NR8383 was also seeded in order to compare the cell confluency and distribution between different conditions. Following the seeding of the cells in 24-well plates, cells were examined under the light microscope every hour for attachment. When the cells were found to have >95% attachment to the collagen support of the 24-well plates, the culture time was deemed sufficient.

Cell seeding density into collagen-coated 24-well plates was reduced and found to be optimal when they were plated between 0.3-0.4 million cells/well. At this reduced seeding density, the cell confluency was still at least >70% due to human AMs being larger than NR8383 cells. Furthermore, the cells were found to be >90% attached after 2 hours and were almost completely attached after 4 hours, therefore the latter was chosen as an optimal culture time of human AMs before starting the uptake experiments. These conditions were comparable to those used for NR8383 cells.

4.3.5  

**In vitro drug uptake and lysosomal trapping of drugs in human AMs**

4.3.5.1  

**Investigation of drug accumulation at single concentration at 37 and 4°C**

In the current study, the uptake of 9 drugs was assessed in human AMs using the method adapted from NR8383 cells. The uptake of ciprofloxacin could not be assessed in human AMs due to limited provision of the cells and problems associated with the analytical quantification of this drug. Following the provision of the cells in RPMI 1640 medium, the cells were centrifuged at 400 g for 10 minutes (Eppendorf Centrifuge 5804, Cambridge, UK). The supernatant medium was gently discarded and the cell pellet was re-suspended in pre-warmed 1 mL RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% L-glutamine and 1% 100 units/mL penicillin-100 µg/mL streptomycin (CGM). Cell count and viability were assessed with trypan-blue exclusion method using a haemocytometer under the light microscope (Leica Microsystems ATC2000, Milton Keynes, UK). The initial cell suspension was then further supplemented with complete growth medium and 0.4 mL of the cell suspension was dispensed into collagen coated 24-well plates (BD Biosciences, Oxford, UK), seeding the wells at a density of 0.4 x 10^6 cells (0.3 x 10^6 in cases of insufficient number of cells provided). On occasions where limited cells were available, the seeding density was reduced to 0.2 x 10^6 cells/well in order to perform the experiments. The plates were placed into 37°C 5% CO₂ incubator (CO₂ incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK) to allow the cells to adhere to collagen. After two hours of plating,
the cells were observed under the light microscope for attachment and the existing medium was replaced with pre-warmed fresh medium to remove any unattached remnant cells (e.g., red blood cells) other than AMs which remained after their isolation. The plates were returned to 37°C incubator and cultured for another 2 hours before the start of the uptake experiment. Following the observation of the cells under the light microscope, the CGM was removed, the cells were washed twice with 800 µL of either pre-warmed or ice-cold DPBS and pre-incubated with 500 µL of pre-warmed or ice-cold ABT/DPBS for 20 minutes. Where ABT pre-treatment was not necessary, cell monolayers were treated with 500 µL of DPBS only. After the removal of this pre-incubation medium, the incubation was started by the addition of 400 µL of substrate solution (with maximum DMSO content of 1% and pre-warmed or ice-cold for 37 and 4°C incubations, respectively) in each well. The incubations were performed either on a dry heater blocked maintained at 37°C (Tecam Dri-block DB-3, Tecam, Princeton, NJ) or on ice for 4°C incubations. The incubation of the substrate solution with the cells was performed for 1, 2, 5 and 10 minutes, with each time point being carried out in duplicate at 37°C. At 4°C, the incubation was performed at 2 and 10 minutes, as a single measurement. In the case of clarithromycin which was used as a control throughout the experiments, the incubation was performed once for 1, 2, 5 and 10 minutes at 37°C and for 2 and 10 minutes at 4°C. Incubation with substrate was stopped by removal of the substrate solution and rinsing the monolayers three times with 800 µL ice-cold DPBS. The cells were lysed by adding 200 µL ice-cold deionised water to the wells. Cell lysates were kept at -20°C overnight, until they were further processed next day for LC-MS/MS analysis. Experiments in human AMs were performed on a single occasion for each drug investigated. The amount of protein in each well was measured to determine the cell number using BCA protein assay, as described in Section 2.3.3.5. A mean cell calibration was performed with NR8383, assuming it would be representative of human AMs, and used to determine the cell number from measured protein concentration in human AMs. Due to the limited provision of human AMs, a calibration standard could not be prepared with human AMs and comparison with NR8383 calibration standard was not possible.

4.3.5.2 Assessment of lysosomal trapping in human AMs using Lysotracker® Red
Localisation of lysosomes and assessment of lysosomal trapping in human AMs were performed as described in Section 3.3.3.1. Briefly, a clear flat bottom Ibidi 8-well
chamber slide (Thistle Scientific, Glasgow, UK) was coated with 50 µg/mL collagen-I solution (4.05 mg/mL Collagen type I rat tail, BD Biosciences, Oxford, UK) in advance of the experiment. Human AMs were centrifuged, after which cell count and viability were assessed as described in the previous section. The cells were resuspended in complete growth medium to give a cell suspension of 0.66 x 10^6 cells/ml. 0.3 mL of this cell suspension was then dispensed into the wells of the 8-well chamber slide (seeding density of 200,000 cells/well) and the cells were cultured for 4 hours at 37°C 5% CO_2 (CO_2 incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK). This cell density was chosen as it would give cell confluency of at least 70% based on previous experiences with NR8383 cells. Solutions of NH_4Cl, monensin and nigericin were prepared by diluting their stock solutions in serum-free RPMI 1640 medium resulting in final incubation concentrations of 20 mM, 5 µM and 10 µM, respectively. LTR was added into the solutions of the chemical agents to achieve a final concentration of 200 nM as previously used in studies with NR8383. In addition, a control solution of LTR at the same concentration was prepared by diluting the original DMSO stock in serum-free RPMI 1640 medium. Following 4 hours culturing of human AMs, the complete growth medium was removed and the cells were treated with prepared solutions of LTR for 1 hour at 37°C in the presence of 5% CO_2 and protected from light (CO_2 incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK). The incubation was stopped by washing the cells three times with 300 µL ice-cold DPBS after which the cells were fixed immediately with 4% (v/v) formaldehyde in DPBS for 10 minutes at room temperature, protected from light. Fifty millimolar NH_4Cl in DPBS was then added into the wells for 5 minutes at room temperature to quench the residual formaldehyde fluorescence. The cells were subsequently washed with ice-cold DPBS and were examined with a confocal laser scanning microscope (Zeiss LSM 510, Jena, Germany) for the detection of LTR. The quantification of LTR fluorescence intensity was performed as described in Section 3.2.3.1.

4.3.5.3 Investigation of lysosomal trapping of selected drugs in human AMs using chemical agents

The investigation of lysosomal trapping of basic drugs in human AMs using chemical agents was performed as described for NR8383 in Section 3.3.3.2. Among the compound dataset, clarithromycin was selected for the assessment of lysosomal trapping in human AMs, following positive findings of lysosomal sequestration in
NR8383 cells. In addition, imipramine was included as a control, consistent with experiments in NR8383. Uptake of the two drugs into human AMs was performed in the absence and presence of 20 mM NH₄Cl, 5 µM monensin and 10 µM nigericin, as previously used in NR8383 cells. The concentration of imipramine and clarithromycin in the incubation was 5 µM. Following culturing of the human AMs for 4 hours at 37°C 5% CO₂ (CO₂ incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK), incubation was started by the removal of complete growth medium and the addition of 400 µL of the pre-warmed drug solution with or without each of the chemical agents. In experiments with NH₄Cl, the incubation time points were 1, 2, 5 and 10 minutes, performed in duplicate. However, upon provision of a limited number of cells, the number of time points were reduced to 2, 5 and 10 minutes, which were studied either in duplicate or single. In the case of monensin and nigericin, the incubations were performed only up to 5 minutes and time points were studied at least in duplicate. The incubations were stopped by rinsing the cell monolayers three times with 800 µL ice-cold DPBS, followed by cell lysis in 200 µL deionised water. The removed incubation media was retained for the measurement of drug concentrations and subsequent Kᵢd determination. The pH of the incubation media containing NH₄Cl was monitored for any changes in pH due to NH₄Cl. All cell lysates and media samples were kept at -20°C overnight before analysis by LC-MS/MS. The amount of protein per well was measured using the BCA protein assay to determine the cell number in each well as described in Section 4.3.5.1.

### 4.3.5.4 Assessment of cytotoxicity of NH₄Cl in human AMs

The cytotoxicity of NH₄Cl in human AMs was assessed as described in Section 3.3.3.4. At the time of the assessment, media samples from a single experiment in human AMs were available, hence incubation media consisting of clarithromycin and imipramine in the absence and presence of NH₄Cl were tested for cytotoxicity. Extensive assessment of cytotoxicity including testing the viability of the untreated cells (i.e., DPBS treated - negative control) and the effect of NH₄Cl alone on the cells could not be performed as done in NR8383 cells due to limited number of human AMs provided. The LDH assay kit was used to measure the cytotoxicity of clarithromycin and imipramine alone or in the presence of NH₄Cl in media samples. The assay was performed and the activity of LDH in experimental samples was measured as described in Section 2.3.4.3.
4.3.5.5 Sample preparation and mass spectrometry analysis
Preparation and analysis of the cell lysate and media samples were performed as described in Section 2.3.4.4. Briefly, both the cell lysates and media samples were thawed and quenched with methanol containing internal standard. The concentration of the internal standard was 1 µM for all drugs except for budesonide and rifampicin for which 0.1 µM was used. Equal volume of cell lysates or media samples were mixed with equal volume of methanol/internal standard mixture (200 µL). Samples were then kept at -20°C freezer for at least an hour before centrifugation at 2500 rpm for 10 minutes (Eppendorf Centrifuge 5804, Cambridge, UK). Ten or twenty microliters of supernatant was analysed by a Waters Alliance 2795 HPLC system coupled to a Micromass Quattro Ultima mass spectrometer (Waters, Watford, UK). The details of the LC-MS/MS system and the conditions for each drug investigated are presented in Section 2.3.4.4 and Table 2-2, respectively. A calibration standard, containing the compound of investigation at a concentration range covering that of the experimental samples with an additional zero blank, was prepared in the same matrix of the experimental samples when sufficient number of human AMs was provided. When this was not the case, NR8383 cell lysates were used; a matrix made of NR8383 cells was considered to be the closest to human AMs due to the use of the same cell type. The calibration standards were analysed twice, one before and one after the experimental samples, during each run in order to confirm compound stability, consistent peak area for internal standard and any potential compound carry over. Samples were quantified as described in Section 2.3.4.4.

4.3.5.6 Confocal microscopy
Images from human AMs treated with LTR were taken using a Zeiss LSM (Zeiss LSM 510, Jena, Germany) equipped with a C-Apochromat 40x/1.2 NA (Numerical Aperture) water-immersion objective. The equipment settings used for the visualisation of LTR in human AMs and the image processing were the same as described in Section 3.3.3.7.

4.3.5.7 Data analysis
4.3.5.7.1 Determination of total uptake and passive diffusion clearances
$CL_{\text{uptake}}$ and $CL_{\text{diff}}$ were estimated at 5 µM substrate concentration for each compound from the 37 and 4°C data, respectively. Uptake rate was determined from the slope of the linear regression of the cell concentration versus time plot and was divided by the
substrate concentration to calculate uptake clearance. Both CL_{uptake} and CL_{diff} were determined for the investigated compounds on a single occasion except for clarithromycin and imipramine for which CL_{uptake} was determined on at least three separate occasions. The inter-individual variability in clarithromycin and imipramine CL_{uptake} was therefore assessed. CL_{active} was determined from the difference between total CL_{uptake} and estimated CL_{diff} from 4°C data for each drug. Maximal % contribution of passive diffusion and active process to the total uptake were calculated for each drug. In addition, the relative importance of active uptake was expressed as the ratio of CL_{active} and CL_{diff}.

4.3.5.7.2 Determination of cell-to-medium partition coefficient (K_p) of drugs in human AMs
Accumulation of drugs in human AMs was determined by calculating K_{p,hAM} at 5 µM drug concentration at final incubation time point (10 minutes). K_{p,hAM} was calculated from cell-to-medium concentration ratio, as described in Section 2.3.4.5.2. For a number of compounds where measured media concentrations were not available, nominal media concentration (i.e., 5 µM) was used. In the case of clarithromycin, K_{p,hAM} for 3 out of 9 patients was obtained using mean media concentration from the remaining experiments where they were measured (n=6). The cell volume used to calculate the total cell concentration of the drugs in human AMs was 3 µL/10^6 cells in contrast to the value used for NR8383 (1 µL/10^6 cells). This value represents the mean of a number of morphometric studies reporting AM cell volume in healthy non-smoker subjects (Crapo et al., 1982; Stone et al., 1992; Krombach et al., 1997). Even though the cells used in the studies presented here were from smokers, it was assumed that cell size would not differ greatly between smokers and non-smokers based on the majority of previously published studies (Harris et al., 1970; Reynolds and Newball, 1974; Territo and Golde, 1979) (Discussed in Section 4.4.1) and this assumption was applied for the data analysis. Similar to CL_{uptake}, the inter-individual variability in clarithromycin and imipramine K_{p,hAM} was assessed.

4.3.5.7.3 Determination of lysosomal trapping of selected drugs in human AMs
Determination of lysosomal trapping of drugs in human AMs was performed in a similar manner as performed previously for NR8383 cells. Lysosomal trapping of imipramine and clarithromycin was evaluated indirectly as a % change in CL_{uptake} and
$K_{p,hAM}$ investigated in the presence of either $NH_4Cl$, monensin or nigericin relative to the control. These agents were assumed to reduce lysosomal distribution of basic drugs in human AMs based on currently available literature (Appendix Table 7-3) and NR8383 data (Chapter 3). $CL_{uptake}$ and $K_{p,hAM}$ were determined as described in Sections 2.1.4.5.1 and 3.2.2.7.1, respectively. The cell volume of human AMs used to calculate cellular drug concentrations was $3 \mu L/10^6$ cells as described in the previous section. $CL_{uptake}$ and $K_{p,hAM}$ in the presence and absence of $NH_4Cl$ was determined in at least 3 separate experiments for both compounds. In the case of monensin and nigericin, $K_{p,hAM}$ was determined in single experiment performed to provide comparison with NR8383 data and confirm the findings in this cell line. Lysosomal trapping of the two drugs was assessed by the comparison of both $CL_{uptake}$ and $K_{p,hAM}$ determined under control and chemical agent treatment conditions. A value of 50% reduction in both $CL_{uptake}$ and $K_{p,hAM}$ was set as cut-off for lysosomal trapping as applied for NR8383 before.

**4.3.5.8 Statistical analysis**

The arithmetic mean, standard error and coefficient of variation (CV) were calculated for uptake clearances ($CL_{uptake}$ and $CL_{diff}$) and $K_{p,hAM}$ where the data were determined on more than one occasion. Geometric mean fold error (gmfe) was calculated (Equation 4-1) in order to assess the discrepancy in NR8383 total uptake clearance data (predicted) relative to human AMs (observed). When assessing lysosomal trapping, the control and chemical agent treated cells were compared using the two-tailed, paired Student’s t-test in order to determine the existence of a statistically significant difference between the two conditions. The data was considered to be statistically significant when $p < 0.05$.

Equation 4-1

$$gmfe = 10^{\frac{1}{N} \Sigma |log{\log{\frac{predicted}{observed}}}|}$$

where $N$ is the number of observations.
4.4 Results

4.4.1 Determination of uptake clearances of selected drugs in human AMs

Uptake of 9 drugs was assessed in human AMs at 5 µM at 37 and 4°C in order to determine active uptake and passive diffusion clearances and compare the data to those obtained in NR8383 cells. For the majority of compounds, assessment of CL\text{uptake} and CL\text{diff} was performed once due to limited availability of human cells. The CL\text{uptake} and CL\text{diff} of investigated drugs in human AMs are presented in Table 4-2. The contribution of CL\text{active} to total uptake in human AMs in comparison to NR8383 cells is shown in Figure 4-1. Over 3000-fold range in CL\text{uptake} of investigated drugs was observed in human AMs. For the majority of compounds, the uptake in human AMs was mainly driven by an active process, in agreement with NR8383 cells (Section 2.2). A further agreement between the two systems was that imipramine showed the highest CL\text{uptake} in human AMs (66.2 µL/min/10^6 cells). Its CL\text{uptake} showed 81% variation between patients. The CL\text{uptake} of imipramine in NR8383 was 4-fold lower than this value. Imipramine also showed the highest CL\text{diff} in human AMs (11.02 µL/min/10^6 cells) which was 2-fold of its value in NR8383. However, the contribution of passive process to total uptake was higher in NR8383 (30.2%) than in human AMs (16.8%). Consequently, the contribution of CL\text{active} to total uptake was almost 5-fold higher in human AMs than in NR8383 (Figure 4-1). The second highest CL\text{uptake} in human AMs was observed for clarithromycin with a value of 15.8 µL/min/10^6 cells. Although CL\text{uptake} of clarithromycin was 2.6-fold lower in NR8383 than in human AMs, its CL\text{diff} was low in both systems (0.03 µL/min/10^6 cells). Contribution of passive process to total uptake was the lowest in the case of clarithromycin in both systems (0.5% or less). Consequently, the importance of an active process in accumulation in both cells was highest (CL\text{active}/CL\text{diff} >500 and >180 in human AMs and NR8383, respectively) (Table 4-2).
Table 4-2: Total uptake (CL\textsubscript{uptake}) and passive diffusion (CL\textsubscript{diff}) clearances and % contribution of CL\textsubscript{diff} estimated at 5 µM substrate concentration in human alveolar macrophages (AMs). CL\textsubscript{uptake} data for imipramine and clarithromycin in human AMs represent mean ± SD of at least 3 experiments. All other data in human AMs are from a single experiment.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CL\textsubscript{uptake}</th>
<th>CL\textsubscript{diff}</th>
<th>CL\textsubscript{diff}/CL\textsubscript{uptake} (%)</th>
<th>CL\textsubscript{active}/CL\textsubscript{diff}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µL/min/10\textsuperscript{6} cells)</td>
<td>(µL/min/10\textsuperscript{6} cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td>66.2 ± 53.8</td>
<td>11.09</td>
<td>16.8</td>
<td>5.00</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>15.8 ± 13.9</td>
<td>0.03 ± 0.02</td>
<td>0.19</td>
<td>524</td>
</tr>
<tr>
<td>Formoterol</td>
<td>2.15</td>
<td>0.16</td>
<td>7.44</td>
<td>12.4</td>
</tr>
<tr>
<td>Budesonide</td>
<td>2.00</td>
<td>2.72</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>3.16</td>
<td>0.05</td>
<td>1.58</td>
<td>62.2</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>0.10</td>
<td>0.02</td>
<td>19.8</td>
<td>4.05</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>0.03</td>
<td>0.01</td>
<td>16.7</td>
<td>5.00</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>0.12</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.02</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a, not available
CL<sub>active</sub> could not be determined in human AMs for budesonide, terbutaline and ipratropium bromide. The second largest contribution of active process to uptake in human AMs was observed for rifampicin (CL<sub>active</sub>/CL<sub>diff</sub> of 62.2-fold) and the value in NR8383 (Table 2-3) was comparable to that in human AMs (52.4-fold). Human AMs showed further agreement with NR8383 in CL<sub>uptake</sub> of rifampicin, formoterol and budesonide which were also found to be similar in human AMs. The contribution of passive diffusion to total uptake was greater for formoterol than rifampicin in both cells. However, in the case of budesonide, passive diffusion was the predominant mechanism for its accumulation in both systems, showing more than 95% contribution to total uptake. The remaining drugs including tiotropium and ipratropium bromide, fenoterol and terbutaline, showed very low accumulation in human AMs, with CL<sub>uptake</sub> < 0.5 µL/min/10<sup>6</sup> cells. The same trend was also observed in NR8383 cells. The rank order of compounds in CL<sub>uptake</sub> and CL<sub>diff</sub> was generally in good agreement between the two systems. The correlation between CL<sub>uptake</sub> of human AMs and NR8383 cells is illustrated in Figure 4-2. The discrepancy of the uptake data in NR8383 was 2.6-fold relative to the data in human AMs. CL<sub>uptake</sub> for budesonide and formoterol showed a particular good agreement (<2-fold), whereas the most pronounced outliers were imipramine, tiotropium bromide and fenoterol (>3.5-fold).

**Figure 4-1:** Active uptake clearance (µL/min/10<sup>6</sup> cells) of drugs in human alveolar macrophages (AMs) (■) and NR8383 cells (□). CL<sub>active</sub> could not be determined in human AMs for budesonide, terbutaline and ipratropium bromide.
Figure 4.2: Correlation of total uptake clearance ($CL_{uptake}$) between human alveolar macrophages (AMs) and NR8383 cells. Solid line represents the line of unity and dashed lines represent 3-fold deviation from the line of unity. Gmfe represents the geometric mean fold error and represents the discrepancy between human AM and NR8383 data. (■) imipramine; (●) clarithromycin; (☆) budesonide; (♦) rifampicin; (▲) formoterol; (++) ipratropium bromide; (▼) tiotropium bromide; (★) fenoterol; (●) terbutaline.
The active uptake and passive diffusion ratio of 7 drugs in NR8383 cells and human AMs was also evaluated across the range of lipophilicity of investigated drugs (Figure 4-3). Five out of seven drugs with logP > 2 had a CL_{diff} ratio within 3-fold from the line of unity. The two outliers were fenoterol and tiotropium bromide (CL_{diff} ratio 5.2 and 3.1, respectively) which had logP < 1.5. In the case of CL_{active} ratio, 3 drugs (clarithromycin, formoterol and rifampicin) were within 3-fold, indicating better agreement in CL_{diff} data between the two systems than CL_{active} data.

Figure 4-3: Uptake clearance ratio of 7 drugs in NR8383 and human alveolar macrophages (AMs) across their lipophilicity range. Solid line represents the line of unity and dashed line represents 3-fold from the line of unity. Blue and orange symbols represent active uptake and passive diffusion clearance ratio, respectively. (■) imipramine; (●) clarithromycin; (●) rifampicin; (★) budesonide; (▲) formoterol; (★) fenoterol; (▼) tiotropium bromide. For budesonide, CL_{active} in human AMs could not be determined as CL_{diff} was the predominant process (~100% of CL_{uptake}).
4.4.2 Variability in clarithromycin uptake in human AMs

The total uptake clearance values of clarithromycin were obtained in human AMs from 9 different donors, as shown in Figure 4-4. The overall mean clearance was 15.8 µL/min/10^6 cells with associated coefficient of variation of 88% across all patients. In male subjects (n=3), the mean clearance value was 25.6 µL/min/10^6 cells which was 2.4-fold higher than the mean CL_{uptake} in female subjects (10.8 µL/min/10^6 cells). There was a larger variability in CL_{uptake} of the compound obtained from males compared to females (87 and 44%, respectively). Pronounced variability in clarithromycin CL_{uptake} was driven by the value obtained in a single donor (50 µL/min/10^6 cells); which was determined using only two time points in single due to limited number of cells for that patient. The K_{p,hAM} of clarithromycin was also determined across all patients which is shown in Appendix Figure 7-4. The trends were similar to those observed for CL_{uptake}. The overall mean K_{p,hAM} was 84 and showed 74% variation between patients. In male and female patients, mean K_{p,hAM} was 135 (CV=67%) and 59 (CV=42%), respectively.

![Figure 4-4](image_url)

**Figure 4-4:** Variation in clarithromycin uptake clearance (CL_{uptake}) in human alveolar macrophages from 9 patients. (■) Male, (■) Female. Numbers below the bars indicate patient number.
4.4.3 Determination of cell-to-medium partition coefficients in human AMs

The assessment of the accumulation of drugs in human AMs was performed by determining their $K_{p,hAM}$ at 5 µM at 10 minutes of incubation (Table 4-3). The most extensive accumulation in human AMs was observed for imipramine with $K_{p,hAM}$ of 626 which showed 63% variation between patients. Clarithromycin showed the second highest accumulation in human AMs with $K_{p,hAM}$ of 84.4 (mean of all investigated patients) which was 74% variable. Budesonide accumulated in human AMs to a greater extent ($K_{p,hAM}$ 22.6) than formoterol and rifampicin ($K_{p,hAM}$ 8.43 and 12, respectively), although data in human AMs were based on a single experiment.

Table 4-3: Cell-to-medium concentration ratio ($K_{p,hAM}$) of 9 drugs in human alveolar macrophages (AMs). Data are from a single experiment for all drugs except for clarithromycin and imipramine for which data represent mean ± SD of at least three separate experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{p,hAM}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>626 ± 395</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>84.4 ± 62.5</td>
</tr>
<tr>
<td>Formoterol</td>
<td>8.43*</td>
</tr>
<tr>
<td>Budesonide</td>
<td>22.6</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>12.0*</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>0.74</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>0.51</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>1.94</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*Nominal media concentration were used in the estimation of $K_{p,hAM}$

Poor accumulation was observed in human AMs for fenoterol, tiotropium bromide and terbutaline. Their cellular concentrations remained lower than extracellular media concentrations for the entire incubation period ($K_{p,hAM} < 1.0$). Ipratropium bromide
achieved a slightly higher cellular accumulation at the end of the incubation period ($K_{p,hAM}$ 1.94).

Comparison of the data between human AMs and NR8383 showed that the ratio of $K_{p,NR8383}$ and $K_{p,hAM}$ for 6 out of 9 drugs was within 3-fold from the line of unity (Figure 4-5). The outliers were rifampicin (3.2-fold), fenoterol and tiotropium bromide (~11.5-fold for both drugs). The overall bias between the two systems was 2.9, in agreement with the bias observed for CL_{uptake} between the two systems.

Figure 4-5: Correlation of cell-to-medium concentration ratio ($K_p$) between human alveolar macrophages (AMs) and NR8383 cells. Solid line represents the line of unity and dashed lines represent 3-fold deviation from the line of unity. Gmfe represents the geometric mean fold error and represents the discrepancy between human AM and NR8383 data. (■) imipramine; (●) clarithromycin; (▲) budesonide; (▲) rifampicin; (▲) formoterol; (⊕) ipratropium bromide; (▼) tiotropium bromide; (※) fenoterol; (●) terbutaline.

Comparison of the $K_p$ data for all drugs between human AMs and NR8383 cells is further illustrated in Figure 4-6. Imipramine showed the most extensive accumulation in both systems. Its $K_p$ was approximately 1.5-fold lower in NR8383 than in human AMs ($K_{p,NR8383}$ 391, Table 2-4) however, the data in human AMs were variable. The $K_p$ of clarithromycin in NR8383 was comparable to its value in human AMs ($K_{p,NR8383}$ 81.6). The accumulation of budesonide, formoterol and rifampicin was comparable in NR8383 cells ($K_{p,NR8383}$ ranged between 24.5 and 38.6), as opposed to human AMs where
budesonide showed higher accumulation than formoterol and rifampicin. However, $K_{p,hAM}$ of formoterol and rifampicin was determined using the nominal media concentration which may lead to the discrepancy in the $K_p$ estimates for the three compounds. Terbutaline and ipratropium bromide showed comparable accumulation in human AMs and NR8383 (Table 2-4), whereas fenoterol showed higher accumulation in NR8383. In the case of tiotropium bromide, higher accumulation in NR8383 was observed compared to human AMs, although data in NR8383 showed large variation (CV=83%).

![Figure 4-6](image)

**Figure 4-6:** Comparison of $K_p$ of all investigated drugs in human AMs (■) and NR8383 cells (□). Data in human AMs are from a single experiment except for clarithromycin and imipramine for which data represent mean ± SD of at least 3 experiments as the case for NR8383 cells.

### 4.4.4 Assessment of lysosomal trapping in human AMs using LysoTracker® Red

Localisation of lysosomes and qualitative assessment of lysosomal trapping in human AMs were performed using LTR as previously done in NR8383 cells. Human AMs (Patient 6) were treated with LTR either in the absence or presence of the chemical agents and the visualisation of the stained cells was performed with a confocal microscope (Figure 4-7). The presence of large number of lysosomes was evident in human AMs by the detection of LTR in these organelles (Figure 4-7B).
Treatment of human AMs with LTR in the presence of 20 mM NH₄Cl showed a maximal reduction in the fluorescent intensity of LTR by 67%, indicating reduced accumulation of LTR in lysosomes of human AMs (Figure 4-7C). This reduction was less than that observed by NH₄Cl in NR8383 cells (85%, Section 3.4.2), however it was in agreement with the effect of NH₄Cl on CL-uptake or Kₚ,hAM of imipramine in the same patient (Patient 6, Figure 4-9). In the case of monensin and nigericin, the maximal reduction in LTR accumulation in the presence of the agents in the same donor was 84% and 85%, respectively (Figure 4-7D and 7E) and was in agreement with the reduction observed by these agents in NR8383 (85%, Section 3.4.2). The effect of these agents on imipramine or clarithromycin uptake in the same donor is unknown. The observed inhibitory effects of the chemical agents towards LTR accumulation in the lysosomes demonstrated the lysosomal targeting of this basic dye in human AMs and its accumulation by a pH gradient dependent mechanism. These results are in good agreement with those obtained in NR8383 cells (Section 3.4.2).
Figure 4-7: Confocal microscopic images of human alveolar macrophages (AMs) treated with LysoTracker® Red (LTR) in the absence and presence of chemical agents. (A) A contrast image of human AMs treated with 200 nM LTR; (B) the same cells being excited to detect LTR localised in lysosomes under control conditions; (C) the localisation of LTR in the lysosomes of a human AM cell was reduced in the presence of 20 mM NH₄Cl, (D) 5 µM monensin and (E) 10 µM nigericin.
4.4.5 Assessment of lysosomal trapping of selected drugs in human AMs by chemical agents

Lysosomal trapping of imipramine and clarithromycin in human AMs was assessed using NH$_4$Cl (20 mM), monensin (5 µM) and nigericin (10 µM) at 37°C. The concentrations of the chemical agents and the incubation time points used were the same as in NR8383 to allow comparison between the two systems. The incubation was performed up to 10 minutes with NH$_4$Cl and for 5 minutes with monensin and nigericin. When the pH of incubation media containing NH$_4$Cl was measured, a minor reduction (≤ 0.05 unit) in the pH was observed relative to control media solutions. The effect of NH$_4$Cl on CL$_{uptake}$ of 5 µM clarithromycin in a subset of patients and the comparison to NR8383 are shown in Figure 4-8A. The reduction in CL$_{uptake}$ of clarithromycin ranged from 65% to 75% as measured in human AMs from 4 patients. There was a similar level of reduction in CL$_{uptake}$ of the compound regardless of the different uptake clearances measured under control conditions. There was no clear trend between the extent of reduction in CL$_{uptake}$ of clarithromycin and the initial CL$_{uptake}$ under control conditions. The mean CL$_{uptake}$ under control conditions was 10.9 µL/min/10$^6$ cells which was reduced to 3.5 µL/min/10$^6$ cells in the presence of NH$_4$Cl, leading to an average 68% reduction in CL$_{uptake}$ (Appendix Table 7-5).
Figure 4-8: Clarithromycin total uptake clearance (CL$_{uptake}$) (A) and cell-to-medium concentration ratio (K$_p$) (B) in human alveolar macrophages (AMs) and NR8383 in the absence (■) and presence (▲) of 20 mM NH$_4$Cl. Data in human AMs represent single measurements in individual patients (numbered below bars) whereas in NR8383, data represent mean ± SD of 3 separate experiments. Numbers above bars indicate % reduction in either CL$_{uptake}$ or K$_p$ due to NH$_4$Cl treatment (*, p < 0.05, **, p < 0.01 by Student’s t-test).
Alveolar macrophages from the patients used to assess lysosomal trapping of clarithromycin (Patients 5, 7, 8, 9), in addition to another patient (Patient 6), were also used to assess lysosomal trapping of imipramine. A wider range in the reduction in imipramine CL$_{uptake}$ was observed compared to clarithromycin (Figure 4-9A). The reduction in CL$_{uptake}$ of imipramine ranged from 34% to 88% when measured in 5 patients and the mean value was 71%.

The change in K$_{p,hAM}$ of imipramine and clarithromycin as a result of chemical agent treatment was also evaluated in the assessment of lysosomal trapping of the two compounds. The trends in K$_{p,hAM}$ data were in line with CL$_{uptake}$ data for both compounds. The mean K$_{p,hAM}$ of clarithromycin in four patients under control conditions was 59.6 (Appendix Table 7-5) and showed a large variation between patients (CV=51%, Figure 4-8B). In the presence of NH$_4$Cl, the K$_{p,hAM}$ was reduced by 61% on average (ranged between 57-67%). The mean K$_{p,hAM+NH4Cl}$ of clarithromycin was 23.3 in human AMs with a CV of 62%. Despite the variability in K$_{p,hAM}$, control and K$_{p,hAM+NH4Cl}$, the ratio between the two was 2.7 on average and was consistent between patients (CV=13%). Imipramine showed much higher partitioning in human AMs compared to clarithromycin, consistent with NR8383 data. The mean K$_{p,hAM}$ of imipramine under control conditions was 707 in human AMs (Appendix Table 7-5) and showed 54% variation between patients. The individual % reduction in K$_{p,hAM}$ values are shown in Figure 4-9B. K$_{p,hAM,control}$ was reduced on average by 67% in the presence of NH$_4$Cl (ranged between 47-71%). The mean imipramine K$_{p,hAM+NH4Cl}$ was 231 (CV=37.6%) and showed less variation than clarithromycin. Similar to clarithromycin, the imipramine K$_{p,hAM}$ ratio between control and NH$_4$Cl conditions was consistent between patients (on average 2.9); however, the variability was more pronounced (CV=28.8%).
Figure 4-9: Imipramine total uptake clearance (CL\textsubscript{uptake}) (A) and cell-to-medium concentration ratio (K\textsubscript{p}) (B) in human alveolar macrophages (AMs) and NR8383 in the absence (■) and presence (□) of 20 mM NH\textsubscript{4}Cl. Data in human AMs represent single measurements in individual patients (numbered below bars) whereas in NR8383, data represent mean ± SD of 3 separate experiments. Numbers above bars indicate % reduction in CL\textsubscript{uptake} or K\textsubscript{p} due to NH\textsubscript{4}Cl treatment (**, p < 0.01 by Student’s t-test).
Comparison of human AMs and NR8383 with regards to the effect of NH₄Cl showed more pronounced reduction in clarithromycin uptake in NR8383 cells (85% in CL uptake and 84% in Kₚ,NR8383) in comparison to human AMs (68% in CL uptake and 61% in Kₚ,hAM). The mean Kₚ+NH₄Cl of clarithromycin in NR8383 (13.3) was 1.8-fold lower than Kₚ+NH₄Cl in human AMs. In the case of imipramine, the reduction in CL uptake by NH₄Cl in NR8383 (68%) was comparable to that observed in human AMs (71%). Kₚ,control was 2-fold lower in NR8383 than the value in human AMs. However, the reduction in Kₚ,control in the presence of NH₄Cl was also comparable between the two systems (67% and 71% in human AMs and NR8383, respectively). The mean Kₚ+NH₄Cl of imipramine in NR8383 was 2.2-fold lower than Kₚ in human AMs under the same conditions.

Assessment of lysosomal trapping of clarithromycin in human AMs in the presence of all three agents (monensin and nigericin and NH₄Cl) was performed in one patient (Patient 9). In addition, the effects of these agents were compared to those observed in NR8383 cells. In the presence of monensin, the CL uptake of clarithromycin was reduced by 64% (Figure 4-10A), whereas nigericin caused 56% reduction in clarithromycin CL uptake (Figure 4-10B). When compared to NH₄Cl, both agents caused a smaller reduction in CL uptake of clarithromycin in this patient (75% by NH₄Cl). Furthermore, the % reduction values in CL uptake of the drug caused by the two agents in this particular patient were lower than those observed in NR8383 (78% by monensin and 84% by nigericin).
Figure 4-10: Clarithromycin total uptake clearance (CL_{uptake}) in human alveolar macrophages (AMs) and NR8383 in the absence and presence of 5 μM monensin (A) or 10 μM nigericin (B). (■) control; (▲) treated with monensin; (■) treated with nigericin. Data in human AMs represent single measurements in an individual patient (Patient 9) whereas in NR8383, data represent mean ± SD of at least 3 separate experiments. Numbers above bars indicate % reduction in CL_{uptake} caused by treatment with chemical agents (**, p < 0.01 by Student’s t-test).
Monensin and nigericin were also used in the assessment of lysosomal trapping of imipramine in AMs from the same patient in which lysosomal trapping of clarithromycin was investigated. The reduction in CL\textsubscript{uptake} of imipramine by both agents was higher compared to that of clarithromycin (Figure 4-11). Monensin caused 73\% reduction in CL\textsubscript{uptake} of imipramine and this reduction was 66\% by nigericin. Both values were lower than the observed reduction in CL\textsubscript{uptake} observed by NH\textsubscript{4}Cl in this patient (88\%). However, these values were comparable to those obtained by using the two agents in NR8383 cells (72\% by monensin and 62\% by nigericin) (Appendix Table 7-4).

The K\textsubscript{p} values of both compounds was also determined in the absence and presence of monensin and nigericin in human AMs. The reduction in K\textsubscript{p,hAM} of clarithromycin by monensin and nigericin was 62 and 58\%, respectively (Appendix Figure 7-5). These values are in good agreement with the % reduction of clarithromycin CL\textsubscript{uptake} by the two agents (Figure 4-10). In addition, they were comparable with the effect of NH\textsubscript{4}Cl on K\textsubscript{p,hAM} of clarithromycin (reduction by 67\%) (Figure 4-8B). In the case of imipramine, similar levels of reduction in K\textsubscript{p,hAM} were observed by monensin (78\%) and nigericin (81\%) (Appendix Figure 7-6). These values were in agreement with the reduction of imipramine K\textsubscript{p,hAM} caused by NH\textsubscript{4}Cl (71\%). Comparison of the data with those in NR8383 showed that both agents caused a larger reduction in clarithromycin K\textsubscript{p,NR8383} (76 and 82\% by monensin and nigericin, respectively) than in K\textsubscript{p,hAM}. For imipramine, the effect of monensin on K\textsubscript{p} in human AMs and NR8383 was comparable whereas nigericin caused a greater reduction in K\textsubscript{p,hAM} than in K\textsubscript{p,NR8383} (65\%).
Figure 4-11: Imipramine total uptake clearance (CL_\text{uptake}) in human alveolar macrophages (AMs) and NR8383 in the absence and presence of 5 µM monensin (A) or 10 µM nigericin (B). (■) control; (▲) treated with monensin; (■) treated with nigericin. Data in human AMs represent single measurements in individual patients (numbered in italic) whereas in NR8383, data represent mean ± SD of at least 3 separate experiments. Numbers above bars indicate % reduction in CL_\text{uptake} caused by treatment with chemical agents (*, p < 0.05 by Student’s t-test).
4.4.6 Assessment of cytotoxicity of NH₄Cl in human AMs
The assessment of the cytotoxicity of NH₄Cl presented in the incubation medium containing clarithromycin or imipramine showed that the LDH activity of the samples was less than 1% compared to 100% of the positive control. Therefore, no cytotoxicity was associated with NH₄Cl in human AMs which is in agreement with the findings in NR8383 cells (Section 3.4.5). The viability of the cells without drug or NH₄Cl treatment (i.e., negative control) or the effect of NH₄Cl on the cells on its own have not been assessed due to limited availability of the cells. However, the cells were 98% viable before plating as assessed by the trypan-blue exclusion method. Furthermore, the cytotoxicity of NH₄Cl or the drugs on their own on NR8383 cells were previously found to be minimal (< 2%). Therefore, as the combined presence of NH₄Cl and the drugs on human AMs were not cytotoxic, it was assumed that the agent had minimal effect on the cell viability. In addition, although the effect of monensin and nigericin on human AM viability could not be assessed, they were assumed to have no cytotoxic effect on human AMs as no issues were previously associated with these agents in NR8383 cells (Section 3.4.5).
4.5 Discussion

4.5.1 Assessment of drug uptake in human AMs

In the present chapter, drug uptake was assessed in human alveolar macrophages obtained from patients who were suspected or confirmed for having lung cancer and therefore underwent lung surgery. With the exception of one non-smoker patient, all other patients were smokers at the time of cell isolation. Provision of human AMs from healthy subjects is generally limited, whereas procurement of cells from patients undergoing either lung surgery for cancer or bronchoscopy for diagnostic purposes is more realistic. Previously, several studies have investigated the impact of smoking and cancer on AM morphology and function including particle recognition by surface receptors, cytokine secretion and phagocytosis. In general, no significant size difference in AMs between smokers and non-smokers was reported across a number of studies (Harris et al., 1970; Reynolds and Newball, 1974; Territo and Golde, 1979). Some studies used size classification and reported that the majority of the cells were either 25 µm in size (Cohen and Cline, 1971) or between 14-19 µm in range (Reynolds and Newball, 1974). The findings of Cohen and Cline (1971) were in agreement with others reporting an AM size between 23-26 µm for both group of subjects (Harris et al., 1970; Territo and Golde, 1979). In contrast, one study reported a significant size difference in AMs between smokers (21.6 µm) and non-smokers (16.2 µm) (Davis et al., 1980).

Previously, cigarette smoking was shown to cause changes to AM phenotype; differences in the expression of cell membrane antigens between smokers and non-smokers were reported (Sköld et al., 1996). In another study, the phagocytic ability of AMs and their expression of several membrane-associated proteins from healthy smokers were shown to be reduced compared to AMs from healthy non-smokers (Hodge et al., 2007). In contrast, no apparent difference in the capacity of AMs from healthy smokers and non-smokers to phagocytise bacteria was found (Taylor et al., 2010). The findings of Taylor et al. (2010) are supported by many earlier studies which showed no difference in bacterial phagocytosis between AMs of smokers and non-smokers (Harris et al., 1970; Cohen and Cline, 1971; Mann et al., 1971). However, the pinocytic activity of AMs from smokers was shown to be less than that of AMs from non-smokers (Yeager et al., 1974). Despite the changes in AM phenotype observed with smoking, no differences in the expression of membrane antigens between healthy subjects and lung cancer patients have been reported (McDonald et al., 1993). In one
study, the changes in human AM function in different types of lung cancer were investigated and reduced production of cytokines or chemotactic activity of AMs were noted in certain types of cancer (Pouniotis et al., 2006). However these observations were based on AMs isolated from the site of the lung lesion rather than away from the tumour site.

While the mentioned changes in AM function and phenotype by cancer and smoking were at times controversial and related to the endocytotic and immunoregulatory roles of AMs, it is not known whether other mechanisms of drug uptake (e.g., transporter-mediated uptake and passive diffusion) are affected. The AMs used in the current work were isolated from areas of lungs distant from the tumour sites and therefore, only smoking was considered to potentially cause variability in cell phenotype and function.

4.5.2 Drug accumulation in human AMs

The accumulation of 9 mainly respiratory drugs was assessed in human AMs at 5 µM at 37°C. The accumulation of investigated drugs was evaluated by their cell-to-medium partition coefficients determined at 10 minutes of incubation. The most extensive accumulation in human AMs was observed for imipramine. This accumulation was approximately <2-fold higher than that in NR8383 cells but $K_p$ in human AMs showed high inter-patient variability (CV=63%). As $K_p$ reflects active uptake, passive diffusion, lysosomal sequestration and intracellular binding, this may suggest higher contribution or involvement of one or more of these processes in imipramine accumulation in human AM than in NR8383. Clarithromycin showed the second most extensive accumulation in human AMs which was also associated with large inter-patient variability (CV=74%). No clear trend was observed between patients’ age, gender, smoking history, the time to complete the experiments and extent of accumulation observed for these two drugs. Although the $K_p$ data seemed to be more variable in males than in females, any conclusions are limited by a small dataset (particularly in males). Therefore, further work is required to confirm the initial trends in the data.

High accumulation of clarithromycin in human AMs was previously reported in in vivo studies which demonstrated AMs to attain several hundred fold higher concentrations of clarithromycin compared to plasma concentrations (AM-to-plasma concentration ratios between 400-1300 at 24 hour following the last dose) (Honeybourne et al., 1994; Conte et al., 1995; Patel et al., 1996; Rodvold et al., 1997). This high intracellular
accumulation of clarithromycin in human AMs has been reported to be important for effective killing of intracellular bacteria that are resistant to biocidal mechanisms of AMs (Togami et al., 2013).

Fenoterol, terbutaline and tiotropium bromide showed poor accumulation in human AMs as their $K_p$ remained below 1 throughout the incubation period. This finding further suggested that equilibrium between intracellular and extracellular drug concentration was not achieved for these hydrophilic drugs. There are examples in the literature showing the $K_p$ of a number of antibiotics (mostly hydrophilic and largely ionised at physiological pH) remaining <1 for as long as 60 minutes in incubation in human AMs (Hand et al., 1984).

Studies in NR8383 cells correctly assigned relatively lower accumulation ($K_p < 5$) of the four respiratory drugs (terbutaline, fenoterol, tiotropium and ipratropium bromide) in human AMs. Low accumulation of these inhaled drugs in human AMs is considered favourable, as it may imply they are less likely to be removed by AMs before they interact with their targets and exert their effects in the airways. In the case of formoterol and particularly budesonide, their relatively higher accumulation in AMs in vitro may suggest potentially higher removal of these two drugs by AMs from the airways in vivo. $K_{p,hAM}$ of formoterol and rifampicin was lower than $K_{p,NR8383}$. For these two drugs, the nominal media concentration of 5 µM was used in estimating $K_{p,hAM}$, as the actual media concentrations were not measured. Therefore, this may have caused the discrepancy in the values between the two systems. Whilst an attempt was made to compare the accumulation of drugs between NR8383 and human AMs, the results presented here by no means are definite as the data in human AMs were from a single subject for most of the compounds investigated. However, they provide important preliminary information regarding the differences in drug uptake between the two systems which is of importance in consideration of NR8383 as a surrogate in vitro tool for human AMs. The overall discrepancy of the $K_p$ data obtained in NR8383 relative to human AMs was 2.9-fold. Such a bias may be considered reasonable, given the large variability in both NR8383 and human AM data (where available). However, further studies are required to confirm the trends in data between the two systems.

The paucity of drug uptake studies and $K_p$ data in human AMs does not permit extensive comparison of the data presented in this chapter with the literature. To our knowledge, there are few published studies reporting $K_p$ of drugs in human AMs (summary in Appendix Table 7-6 and 7-7). In one of these studies (Hand et al., 1984),
the accumulation of rifampicin (25 µM) was assessed in AMs of healthy non-smokers. Its $K_p$ was 4.67 at 15 minutes which is a similar incubation time to that used in the current study. The authors suggested that good lipophilicity of the compound was possibly responsible for its accumulation in human AMs. The $K_{p,hAM}$ of rifampicin at 10 minutes was at least 2.5-fold higher than this value which could be a result of the cells being exposed to cigarette smoke. Previous transmission and scanning electron micrographs of AMs from smokers showed large amounts of “cytoplasmic inclusions” in the cells (Pratt et al., 1969; Cohen and Cline, 1971; Pratt et al., 1971; Hocking and Golde, 1979; Finch et al., 1982) compared to AMs from non-smokers which contained few inclusions. The cytoplasmic inclusions were proposed to be lysosomes and other acidic derivatives due to presence of acid phosphatase (Pratt et al., 1971). In addition, they showed that these inclusions contained lipid-like structures and suggested that they may represent cellular lipids which were left undigested by lysosomal enzymes. The abundance of cytoplasmic inclusions was greater in smokers and similar observations were obtained in smoke exposed rats (Davies et al., 1978). Consequently these lipid-like structures may contribute to the binding of lipophilic compounds and increase their partitioning in AMs of smokers compared to non-smokers. Some supporting evidence shows that rifampicin (Appendix Table 7-6) and a number of macrolide antibiotics (Appendix Table 7-7) accumulated to a larger extent in AMs from smokers. These studies suggested that increased lipid content or occurrence of enlarged lysosomes led to enhanced accumulation of these drugs in AMs. Hand et al. (1985) measured rifampicin $K_p$ in smokers as 6.7 which is lower than its $K_{p,hAM}$ in this study (12). The difference could be due to higher concentration of rifampicin (25 µM) being used in that study compared to 5 µM of the drug used in the current study.

4.5.3 Determination of uptake clearances in human AMs

The uptake of 9 drugs was assessed in human AMs at 5 µM at 37°C and 4°C in an attempt to determine the contribution of active uptake and passive diffusion processes, respectively. The uptake of drugs in human AMs was performed only once except for clarithromycin and imipramine due to the limited provision of human AMs. Clarithromycin was used as a control drug in each experiment in order to assess the inter-patient variability in drug uptake as opposed to studies in NR8383 in which it was used to assess inter-assay variability. Its uptake was assessed further when investigating
its lysosomal trapping along with lysosomotropic drug imipramine which was used as a positive control.

The highest uptake in human AMs was observed for imipramine, analogous to in NR8383. Its accumulation in AMs was mainly dependent on an active process although some contribution of passive diffusion (by 16.8%) to total uptake was evident. However, the latter was assessed only once by 4°C experiments in which CL_{diff} data were variable; therefore, further studies are needed to confirm the contribution of passive diffusion. The uptake of imipramine was assessed in a total of 6 patients and large inter-individual variation (CV=81%) was observed. The second highest uptake clearance in human AMs was observed for clarithromycin. Its uptake was driven predominantly by an active process as in NR8383. Similar to imipramine, large inter-individual variability in uptake was also observed for clarithromycin (CV=88%). The uptake clearances of the remaining drugs investigated were relatively lower (CL_{uptake} <4 \mu L/min/10^6 cells) as in NR8383 and mainly dependent on an active process. Contribution of passive diffusion clearance could not be determined for a number of drugs such as terbutaline and ipratropium bromide due to the limited data points available from 4°C experiments not yielding a positive slope in the cell concentration-time profiles. The discrepancy observed in NR8383 CL_{uptake} data relative to human AMs was reasonably small (gmfe of 2.6-fold) and was in line with the discrepancy observed for the K_{p} data.

Possible mechanisms by which the investigated drugs are taken up by NR8383 including passive permeation, transporter-mediated uptake and endocytosis have been discussed in Chapter 2 and also apply for human AMs. Although there is relatively more information regarding transporter expression in human AMs compared to NR8383 cells, such information is currently limited to efflux transporters. Previously, expression of P-gp and MRP1 was demonstrated in human AMs (van der Valk et al., 1990; Scheffer et al., 2002). Even though the expression of OCTN1 was indicated in human AMs (Bosquillon, 2010), this transporter has not been shown to be expressed directly in AMs in the original study (Horvath et al., 2007b). The most recent data (Moreau et al., 2011) report the expression of SLC and ABC transporters in primary human macrophages generated from blood monocytes and demonstrated low expression of OCTN1/2, OCT1, but high expression of OATP2B1 (mRNA) and efflux transporter MRP1 (both mRNA and activity), whereas expression of BCRP (mRNA) was intermediate. However, the expression of P-gp (mRNA and activity) was found to be
very low or not detectable. P-gp expression in human AMs was found to be contradictory in earlier studies relying on immunohistochemical staining of lung tissue; positive and variable (or partial) staining of AMs have been reported depending on the antibodies used (van der Valk et al., 1990; Scheffer et al., 2002). In this regard, the more recently available mRNA and activity data provide valuable information with regards to the expression of mentioned transporters in human AMs (Moreau et al., 2011).

4.5.4 Lysosomal trapping of basic drugs in human AMs
In this chapter, the contribution of lysosomal trapping to drug accumulation was investigated in human AMs from a number of patients for the first time by both LysoTracker® Red staining and chemical agents in vitro. The abundance of lysosomes in human AMs was revealed by localisation of basic LTR in the organelles (Figure 4-7). When the basic dye was co-incubated with the cells in the presence of the chemical agents NH₄Cl, monensin and nigericin, its measured fluorescent intensity was reduced, indicating its targeting of the lysosomes and the effect of the agents on disturbing the lysosome-cytosol pH-gradient. While this work provided a qualitative confirmation to the results obtained with the chemical agents, data regarding the potency (IC₅₀ values) of the investigated drugs in reducing LTR accumulation in lysosomes (as a measure of their lysosomotropic properties) could not be generated due to the paucity of the human AMs. Therefore, it was not possible to compare this method with that involving the chemical agents regarding the rank order of imipramine and clarithromycin for lysosomal trapping.

Quantitative analysis of lysosomal trapping of imipramine and clarithromycin in human AMs was performed indirectly by assessing their CLuptake and Kp,hAM in the presence and absence of NH₄Cl, monensin and nigericin. For both drugs, a reduction in CLuptake and Kp,hAM was observed in the presence of the chemical agents, confirming their lysosomal sequestration. As the trends in Kp,hAM and CLuptake were comparable to each other, the discussion in this section will focus on the Kp,hAM data as representative. For imipramine, the reduction in Kp,hAM in the presence of NH₄Cl was > 50% in most of the patients (mean 67%), suggesting important contribution of lysosomal sequestration to its accumulation in human AMs. Considering its high lipophilicity, the remaining accumulation unaffected by NH₄Cl (Kp+NH₄Cl) can largely be attributed to partitioning of imipramine into membranes. However, some additional unaccounted contribution of pH partitioning is feasible if lysosomal pH did not equilibrate with cytosolic pH by NH₄Cl.
Nonetheless, the contribution of the latter is expected to be relatively small compared to membrane partitioning. Variability was observed in $K_{p,hAM}$ of imipramine in control (CV=54%) and NH$_4$Cl conditions (CV=38%) between different patients. One source of variability in data may be attributed to potential differences in the extent of lysosomal sequestration (assuming no transporter mediated active uptake) and membrane binding processes between subjects. Morphometric studies comparing human AMs from smokers and non-smokers have previously shown increased abundance of lysosomes (enlarged in some cases) and lipid content in AMs from smokers compared to non-smokers as mentioned in the previous section. Therefore, such physiological changes may contribute to the differences in data observed between NR8383 and human AMs, as well as between different patients. Another possible source of variability could be differences in AM cell preparations which may have been contaminated with red blood cells. Although the growth medium covering the cell monolayers was replaced with fresh medium before the start of uptake experiments in order to remove any unattached red blood cells, some may have remained attached with AMs and could have contributed to drug partitioning. Finally, the determination of human AM cell numbers from NR8383 cell calibration standards (different absorbance values leading to different protein content) may cause variation. For instance measurement of low cell number (0.031 x 10$^6$ cells) and protein concentration (25 µg/ml) was associated with the highest uncertainty (CV=40-50%). Among all patients, the cell number used to determine clarithromycin CL$_{uptake}$ and $K_{p,hAM}$ in Patient 2 was 0.07 x 10$^6$ cells which is at the lower end of the cell calibration standard. Moreover, the variability associated with the measurement of this number (across n=4 wells) was 28%. Regardless of the variability in $K_{p,hAM,control}$ and $K_{p,hAM+NH4Cl}$, a similar reduction in $K_{p,hAM}$ was observed between patients, with low CV (20%). The $K_{p,hAM,control}/K_{p,hAM,+NH4Cl}$ ratio between patients ranged between 1.9-3.5 suggesting a similar magnitude of saturable processes. Although some variability in lysosomal trapping is expected due to possible differences in number and/or volume of lysosomes as suggested earlier, these differences may not result in significant differences in the extent of lysosomal trapping between patients. Consequently, the variability in $K_{p,hAM,control}$ may be a reflection of all other processes, as discussed above.

In the case of clarithromycin, the reduction in $K_{p,hAM}$ in the presence of NH$_4$Cl ranged from 57 to 67% between patients (CV=7.7%), indicating a similar contribution of lysosomal sequestration to its accumulation in human AMs. This was in agreement with
similar $K_{p,hAM,control}/K_{p,hAM,treated}$ ratio observed between the patients (ranged between 2.3-3). High lysosomal sequestration of clarithromycin in human AMs can explain its high accumulation in these cells demonstrated by previous clinical studies (Honeybourne et al., 1994; Conte et al., 1995; Patel et al., 1996; Rodvold et al., 1997). It also indicates that certain intracellular pathogens that are preferentially localised in subcellular organelles like lysosomes (Van Bambeke et al., 2006) will be targeted by clarithromycin due to high concentration of the drug in lysosomes relative to cytosol. Similar to imipramine, the $K_{p,hAM}$ of clarithromycin was variable in control and treated cells; however, the $K_{p,hAM,control}/K_{p,hAM,+NH4Cl}$ ratio was consistent between patients (ranged between 2.3-2.9).

The effect of monensin and nigericin on imipramine and clarithromycin accumulation (reduction in $K_p$ ranged between 58-81%) in human AMs was in good agreement with the effect of NH$_4$Cl (reduction by 67-71%). Although these findings are based on an individual patient, it is encouraging that the trends seen in NR8383 cells are in line with these preliminary data in human AMs.

### 4.5.5 Conclusion

To conclude, the present chapter presents the first most extensive characterisation of the accumulation of a range of drugs in human alveolar macrophages. A wide range in drug accumulation ($K_{p,NR8383}$ between 0.4-626) was observed in human AMs, consistent with data previously generated in NR8383 under comparable experimental conditions. An active mechanism seems to be the main contributor for their accumulation and may involve endocytosis and/or uptake transporters. These results and the rank order of the CL$_{uptake}$ and $K_p$ data in NR8383 were in reasonable agreement (within 3-fold) with those in human AM.

Imipramine and clarithromycin accumulated in human AMs extensively compared to the remaining drugs. These two drugs allowed the assessment of inter-individual variability in drug accumulation in human AMs. The lysosomal trapping potential of both drugs was investigated using a number of different methods and agents as this process was shown to be an important contributor to their accumulation in NR8383 cells. Similar trends were observed in human AMs and the inter-patient variability in their lysosomal sequestration was assessed. While the similarities observed in in vitro data between the two systems may potentially propose NR8383 as a surrogate system to be utilised in pre-clinical studies, the presented work in human AMs are preliminary

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and further investigations are needed to confirm the observed similarities and
differences between the two systems. Morphometric studies in AMs from rats and
humans can provide useful information regarding the size, volume and the structures of
the cells and organelles which can potentially be used to parameterise physiological and
mechanistic in silico models to predict total and lysosomal accumulation of drugs in
AMs. To that end, the presented in vitro data can be used to evaluate the performance of
such predictive models (Chapter 5).
**Chapter 5  Application of an *in silico* mechanistic cell model for the prediction of lysosomal trapping of drugs in alveolar macrophages**

I would like to acknowledge CAPKR’s former research associate Dr. Michael Gertz for his contributions in implementing the model presented here in Matlab v7.14® (2012) (Mathworks, Natick, MA) and performing model validation and sensitivity analysis.

5.1 Introduction

Lysosomes can accumulate CADs with $pK_a$ between 6.5 and 11 up to 100-fold or more relative to cytosol (de Duve et al., 1974; Duvvuri et al., 2005; Nadanaciva et al., 2011) and therefore contribute significantly to their intracellular concentrations. This is in contrast to the fact that lysosomes represent <1% and up to 7.8% of the total cell volume for hepatocytes (Alberts et al., 1994) and alveolar macrophages (References in Table 5-1), respectively. The contribution of lysosomal trapping to total tissue uptake was reported to be 20-70% for a number of antipsychotic drugs, depending on the organ investigated (Appendix Table 7-3). Extensive accumulation of investigated drugs was often observed in tissues with the abundance of lysosomes including the lungs, liver and the kidneys. In addition to the influence of lysosomal trapping on the whole body distribution of lipophilic amine drugs, its role in drug efficacy and induction of phospholipidosis, and potential involvement in drug resistance and DDIs have been highlighted in the previous chapters. Although it is straightforward to measure total intracellular drug concentrations and relate that to external media drug concentrations, determination of unbound cytosolic and lysosomal concentrations is experimentally challenging. Consequently, use of *in silico* mechanistic compartmental models consisting of system and drug properties is useful in providing dynamic assessment of intracellular concentrations and several processes occurring in cells such as transporter uptake/efflux, passive diffusion, metabolism, intracellular binding and lysosomal trapping (Chu et al., 2013). While a number of these mechanistic modelling approaches have been described in Section 1.3.4.3, a particular focus will be given here to the work by Trapp and colleagues, as it provides the basis for the cell model presented in this chapter (Trapp and Horobin, 2005; Trapp et al., 2008). This four-compartment mechanistic model uses passive diffusion as the transport mechanism of drug species and allows the prediction of cytosolic, lysosomal, mitochondrial and total cell
concentrations based on cell specific and drug physiochemical properties (LogP and pKₐ). To the best of our knowledge, currently there is no in silico cell-based model specifically developed for AMs. Therefore, exploring the application of this mechanistic model for AMs can provide a step in explaining the accumulation behaviour of respiratory drugs in these cells and complement the in vitro accumulation and lysosomal trapping data in AMs presented in this Thesis.

5.2 Aims

The aim of this chapter is to assess the utility of an in silico mechanistic cell model reported by Trapp et al (2008) in predicting lysosomal trapping of basic drugs in AMs (Trapp and Horobin, 2005; Trapp et al., 2008). The model was implemented in Matlab v7.14® (2012) and modified for alveolar macrophages by using AM cell parameters collated from the literature where available. Drug concentrations in cytosol, lysosomes, mitochondria and total cell were predicted for all investigated compounds. The predictive performance of the model was assessed by comparing the predicted Kᵰ with experimentally determined Kᵰ for each compound. In addition, the pH and volume of lysosomes, as well as the total cell volume were modified to simulate the alkalinisation of lysosomes by NH₄Cl, monensin and nigericin (or basic drugs) and the effect of smoking in human AMs. The changes in predicted Kᵰ were compared to those observed in vitro. The advantages and the limitations of the cell model were discussed and recommendations for future improvements were addressed.
5.3 Methods

5.3.1 Cell model structure

The cell model consists of 4 compartments, namely extracellular medium, cytosol, mitochondria and lysosomes. The cell compartments are associated with an aqueous and lipid fraction and surrounded by a membrane (Trapp et al., 2008). In the model, drug accumulation in the cell and sub-cellular compartments occurs via passive diffusion which is driven by i) pH differences between medium, cytosol, lysosomes and mitochondria, ii) partitioning of neutral and ionised molecules into lipids within the cytosol and the organelles in conjunction with the assumption that ionised molecules show a reduced lipophilicity (and consequently permeability) relative to neutral molecules, and iii) electrostatic attraction/repulsion of the ionised molecules with electrically charged membranes. The uptake of a weak basic drug involving these processes are further illustrated in Figure 5-1.

**Figure 5-1:** The cell model scheme demonstrates the processes involved in uptake of a weak base into the cell and sub-cellular compartments. The figure was adapted from Trapp et al. (2008). (↔) indicate diffusion of neutral species (B) across membranes, (⇒) indicate limited diffusion of ionised species (BH⁺) across membranes, (⇌) indicate ionisation according to Henderson-Hasselbalch and (→) indicate partitioning into lipids.
5.3.2 Cell model equations

The equations used to define lipophilicity, permeability, lipid partitioning, electrostatic interactions, diffusive flux and free molecule fractions (neutral and ionised), are described individually below. Consequently, they were incorporated in the mass-balance equations used to estimate concentrations in each compartment.

**Permeability, lipophilicity and lipid partitioning:**

The permeability of molecules across a membrane is related to their partition coefficient by an equation defined as;

$$P = \frac{D_C K}{dx},$$

where $D_C$ refers to the diffusion coefficient and was assumed to be $10^{-14} \text{m}^2\text{s}^{-1}$ for all drugs (Trapp and Horobin, 2005). $K$ is the partition coefficient and $dx$ is the membrane thickness. The partition coefficient $K$ was replaced by $K_{ow}$ (octanol-water partition coefficient) assuming the same partitioning of drug molecules into membranes and octanol. Here, $K_{ow}$ refers to neutral species and is equal to $10^{\log P}$ ($\log K_{ow} = \log P$). $K_{ow}$ for monovalent ions was considered to be $3.5$-log unit lower than that for neutral species (Trapp et al., 2008).

Drug partition into lipids was described based on the assumption that this process was adequately defined by drug partitioning into octanol. Therefore, the lipid sorption parameter $K_L$ is defined as;

$$K_L = L \cdot K_{ow}$$

where $L$ is the fractional lipid content ($\text{L} \cdot \text{L}^{-1}$). Therefore, from the above equations, it is shown that both neutral and ionised molecules permeate through membranes and partition into lipids. However, ionised species do so to a lower extent than the neutral species.

**Flux of neutral and ionised molecules across membranes:**

The permeability of neutral and ionised molecules described above was used to define the diffusive flux of both species across membranes. The flux of neutral species is driven by the chemical potential as described by Fick’s 1st Law of Diffusion:

$$J_N = P_N (a_{N,\text{out}} - a_{N,\text{in}})$$

where $J_N$ is the flux of the neutral molecules from the outside (out) to the inside (in) of the membrane ($\text{kg.m}^{-2}\cdot\text{s}^{-1}$), $P_N$ is the permeability of the membrane to neutral molecules
(m.s\(^{-1}\)) and \(a\) is the activity of the molecule (kg.m\(^{-3}\)). Activity is the measure of the thermodynamic strength of ionic species in solution and relates the chemical potential of an ion in solution to its concentration (Olander, 2008). It has been stated as the driving force for the diffusion of freely dissolved molecules (neutral or ions) (Trapp, 2004). The activity of molecules is related to their concentration by activity coefficient by the equation:

**Equation 5-4**

\[ a = AC \cdot C \]

where \(AC\) is the activity coefficient and \(C\) is concentration. The activity coefficients were used as in Trapp et al. (2008). The authors calculated it as 1.23 for neutral molecules, 0.74 for monovalent ions and 0.3 for bivalent ions at ionic strength of 0.3 mol.L\(^{-1}\) inside the cell using a number of thermodynamic equations.

The flux of ionised species across membranes is governed by the chemical and electrical potential and is described by the Nernst-Planck equation as follows;

**Equation 5-5**

\[ J_D = P_D \cdot \frac{N}{e^N - 1} (a_{D, out} - a_{D, in} \cdot e^N) \]

where \(J_D\) is the flux of dissociated (ionised) molecules (kg.m\(^{-2}\).s\(^{-1}\)), \(P_D\) is the permeability of the membrane to dissociated molecules (m.s\(^{-1}\)), \(N = zE_mF/(RT)\); \(z\) is the electric charge of ionic species (e.g., +1 for a monoprotic base and -1 for monoprotic acid), \(E_m\) is the membrane potential (V), \(F\) is the Faraday constant (96,484.56 C.mol\(^{-1}\)), \(R\) is the universal gas constant (8.314 J.mol\(^{-1}\).K\(^{-1}\)) and \(T\) is the absolute temperature (310.16 K). The total net flux will be the sum of \(J_N\) and \(J_D\):

**Equation 5-6**

\[ J = P_N (a_{N, out} - a_{N, in}) + P_D \cdot \frac{N}{e^N - 1} (a_{D, out} - a_{D, in} \cdot e^N) \]

**Equations defining freely dissociated molecule fractions:**

Under physiological conditions, acids, bases and zwitterionic compounds may exist in ionised or neutral form in a solution (Trapp and Horobin, 2005). The ionisation of drug molecules was calculated by Henderson-Hasselbalch equation. The fraction of neutral and ionised species was calculated from the activity and concentration. The activity of free molecules in solution is related to the total concentration by the equation:

**Equation 5-7**

\[ a = f \times C_T \]

where \(f\) represents the fraction which can freely diffuse across membranes. The units of activity and \(C_T\) are kg.m\(^{-3}\) or g.L\(^{-1}\). The total measurable concentration \(C_T\) of the drug is comprised of neutral and dissociated molecular species both of which can be in solution.
or adsorbed. Only the free, non-adsorbed molecules, neutral or dissociated, will move across membranes by diffusion. Therefore, total concentration $C_T$ is described by the following equation:

$$ C_T = W \cdot C_{N,f} + K_L \cdot C_{N,s} + W \cdot C_{D_1,f} + K_L \cdot C_{D_1,s} + W \cdot C_{D_2,f} + K_L \cdot C_{D_2,s} $$

Equation 5-8

where $W$ refers to the fractional water content (L.L$^{-1}$). $C$ refers to concentration and indices $T$, $N$, $D$, $f$ and $s$ refer to total, neutral, dissociated (ionised), free and sorbed. Replacing concentration $C$ and sorption parameter $K_L$ in the equation will give the following equation:

$$ C_T = \frac{W \cdot a_{N,f}}{A C_N} + \frac{L \cdot K_{ow,N} \cdot a_{N,f}}{A C_N} + \frac{W \cdot a_{D1,f}}{A C_{D1}} + \frac{L \cdot K_{ow,D1} \cdot a_{D1,f}}{A C_{D1}} + \frac{W \cdot a_{D2,f}}{A C_{D2}} $$

Equation 5-9

Substitution of $a_D$ by $a_N$ in accordance with Henderson-Hasselbalch equation (i.e., $a_{D1} = a_{N} \cdot 10^{(pK_{a1}-pH)}$) gives the fraction of the neutral species ($f_N$) which can freely permeate membranes in relation to the total measureable concentration:

$$ f_N = \left( \frac{W + L \cdot K_{ow,N}}{A C_N} + \frac{(W + L \cdot K_{ow,D1}) \cdot 10^{(pK_{a1}-pH)}}{A C_{D1}} \right)^{-1} + \left( \frac{(W + L \cdot K_{ow,D2}) \cdot 10^{(pK_{a1}-pH) + i(pK_{a2}-pH)}}{A C_{D2}} \right)^{-1} $$

Equation 5-10

For ions, the fractions are as follows:

$$ f_{D1} = 10^{(pK_{a1}-pH)} \cdot f_N $$

Equation 5-11

$$ f_{D2} = 10^{(pK_{a1}-pH) + i(pK_{a2}-pH)} \cdot f_N $$

Equation 5-12

The computation of ionisation, lipophilicity, membrane permeation, electrostatic interactions and molecular fractions inside the cell and associated compartments was performed in Matlab v7.14® (2012) (Appendix 7.1.1.1). The numerical solution of the implemented equations was performed using ordinary differential equation 15 (ODE15i) solver.
Mass-balance equations and model output predictions

A set of differential equations describing the mass exchange between five compartments were implemented in Matlab v7.14® (2012) (Appendix 7.1.1.2). These compartments were medium, cytosol, lysosome, mitochondria and total cell defined in Matlab in this order (e.g., compartment 1: medium, compartment 2: cytosol). An example of the mass exchange of neutral and ionised species between cytosol and lysosome is shown with the following equation:

\[ \frac{dC_l}{dt} = \left( (C_c \cdot f_N \cdot P_N \cdot SA_l) + \left( C_c \cdot f_D \cdot P_D \cdot \frac{N}{e^N - 1} \cdot SA_l \right) \right) - \left( C_l \cdot f_N \cdot P_N \cdot SA_l \right) / V_l \]

Equation 5-13

where \( C_l \) and \( C_c \) are drug concentrations in lysosome and cytosol, respectively. \( f_N \) and \( f_D \) are free fractions of neutral and ionised species as described in above equations. \( SA_l \) is surface area of the lysosome and \( V_l \) is volume of lysosome. As multiplying concentration with free fraction gives activity (Equation 5-7) and \( aP = J \) (Equation 5-3 and 5-5 for flux of neutral and ionised species, respectively), then \( J \cdot SA = m \) and \( C = m/V \). The respective equations for all other compartments are provided in the Matlab script in Appendix 7.1.1.2. The final output of the model was drug concentrations in each compartment which were simulated to steady-state condition \( (t = 1 \text{ h}) \). The estimation of \( K_p \) for cell and sub-cellular compartments was then performed both relative to medium, and relative to cytosol in the case of lysosomes and mitochondria.

5.3.3 Collation of alveolar macrophage parameters for parameterisation of the cell model

The original model was parameterised using both cell and drug specific parameters. A database was generated from the collation cell parameters from the literature. The drug specific parameters were the octanol-water partition coefficient, \( \text{LogP} \), and acid-base dissociation constant(s), \( \text{pK}_a \), which are shown in Table 1-4 of the first chapter. The cell specific parameters are listed in Table 5-1 and include pH, volume, surface area and water and lipid content of the subcellular compartments. The literature was searched for morphometric studies in primary human and/or rat AMs for the collation of mentioned cell parameters. Where neither of rat or human AM cell parameters were available, data were taken from primary macrophages or macrophage cell lines of other species, or rat hepatocytes. In the case of human AMs from non-smokers, lysosome and mitochondria volumes were determined from \( V_{\text{lys}}/V_{\text{cell}} \) ratio of 7.8% and \( V_{\text{mid}}/V_{\text{cell}} \) ratio of 2.8%,
respectively, as determined in primary rat AMs, under the assumption that the ratios are the same in both systems. The organelle surface areas were determined from the volumes, assuming they were spheres. The pH and membrane potential of the cell, lysosome and mitochondria were the same as used for rat AMs. In the case of fractional water and lipid content of the cell and associated organelles (lysosome, mitochondria), generic human cell values were taken from Trapp and colleagues (Trapp and Horobin, 2005; Trapp et al., 2008). These values were 0.95 and 0.05 L.L⁻¹ for water and lipid, respectively. Furthermore, the information regarding plasma and organelle membrane thickness of AMs was also not readily available, therefore a value generic to plasma membranes (9 nm) was used (Korn, 1966).

As drug uptake studies were performed using human AMs from smokers, the literature was also searched for information regarding size differences in AMs from smokers and non-smokers in order to explore the impact of modifying the cell and organelle size on predicted drug accumulation by the model. As the cell volume was not reported in smokers, the parameter was calculated either from the surface area or the cell diameter, assuming that AMs are spherical (Table 5-2). This assumption was first tested in primary rat AMs for which both measured cell volume and surface area were reported (references provided in Table 5-1). Calculated and measured values were compared to determine the deviation from sphericity. This allowed the justification for the use of this assumption in human AMs. The calculated cell volume was then used to determine the organelle volumes as performed in non-smokers (from the organelle/cell volume ratio), assuming it is the same in smokers. The organelle surface areas were also determined in the same manner as in non-smokers.

5.3.4 Model assumptions

Drug related assumptions regarding membrane partitioning (same as in octanol) and reduced permeability and lipid partitioning of ionised drugs relative to neutral drugs were covered in Section 5.3.2. Model assumptions regarding cell parameters including organelle volumes and surface areas, water and lipid contents and membrane thickness were covered in Section 5.3.3.
5.3.5 Applications of the model

1. Using the AM cell model, the $K_p$ in each compartment was first predicted under control conditions when lysosomal pH was acidic (pH 4.75). These initial predictions were performed assuming 3.5-log unit reduced lipophilicity of the ionised species for all drugs. The performance of the cell model was assessed by the comparison of the predicted and observed total $K_{p,cell}$ for each compound.

2. Following the initial predictions, the impact of using the lipophilicity penalty on the prediction of the accumulation of imipramine, clarithromycin, ciprofloxacin and terbutaline was assessed by modifying the penalty value with the LogP difference reported for neutral and ionised forms of these drugs in the literature (Appendix Table 7-8). Following model predictions for these 4 drugs were performed using the modified penalty values. The accuracy of the revised model predictions was assessed by calculating the gmfe (Equation 4-1) to determine the model predictive bias.

3. The in silico AM model predictions mentioned above were performed using the experimental (for 5 drugs) and ADMET Predictor™ v7.0 predicted (for 5 drugs) LogP and pK$_a$ as drug specific input (Table 1-4). The accumulation of 5 drugs which was initially predicted using the experimental LogP and pK$_a$ was further predicted when these input parameters were calculated by ADMET™ Predictor (Appendix Table 7-9). The impact of the change in input drug physicochemical data on the model predictions was then evaluated.

4. Next, the performance of the model in mimicking the effect of the chemical agents (e.g., NH$_4$Cl) used in the assessment of lysosomal trapping of drugs was evaluated. Therefore, $K_p$ values were predicted when lysosomal pH was increased in the model to either cytosolic pH (7.2) or 6.3. The latter pH value was based on the experimental observation where lysosomal pH of mouse peritoneal macrophages was measured in the presence of 10 mM NH$_4$Cl (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981). Following the increase in lysosomal pH in the model, the predicted lysosomal $K_p$ (relative to medium) values were compared to the predictions performed under control conditions. Furthermore, the predicted reduction in total and lysosomal $K_p$ were compared to the observed reduction in $K_{p,NR8383}$ for all drugs.

5. The final application of the model was to predict cell and organelle $K_p$ values in human AMs and compare the predictions with those obtained using rat AM
parameters. Furthermore, the human AM cell parameters (cell and organelle volume and surface area) were changed in the model to mimic AMs from smokers and the impact of the changes on the predictions was evaluated.
Table 5-1: Alveolar macrophage parameters used as input for the *in silico* mechanistic cell model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>CV (%)</th>
<th>N</th>
<th>Range</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (pL)#</td>
<td>1.13</td>
<td>31</td>
<td>11</td>
<td>0.623 - 1.949</td>
<td>Rat alveolar macrophages</td>
<td>1-2, 4-5, 10-16</td>
</tr>
<tr>
<td>Surface area (m²)</td>
<td>4.6x10^{10}</td>
<td>54</td>
<td>4</td>
<td>1.2x10^{-10} - 6.8x10^{10}</td>
<td>Rat alveolar macrophages</td>
<td>1-2, 12, 14</td>
</tr>
<tr>
<td>pH</td>
<td>7.17</td>
<td></td>
<td>1</td>
<td></td>
<td>Rabbit alveolar macrophages</td>
<td>7</td>
</tr>
<tr>
<td>Eₘ (V)</td>
<td>-0.032</td>
<td>31</td>
<td>2</td>
<td>-0.021 - 0.04</td>
<td></td>
<td>4, 9</td>
</tr>
<tr>
<td><strong>Lysosome</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (pL)#</td>
<td>0.09</td>
<td>54</td>
<td>5</td>
<td>0.042 - 0.157</td>
<td>Rat alveolar macrophages</td>
<td>1-2, 10, 12, 14</td>
</tr>
<tr>
<td>Surface area (m²)</td>
<td>9.62x10^{11}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>4.75</td>
<td>1.3</td>
<td>1</td>
<td></td>
<td>Mouse peritoneal macrophages</td>
<td>3</td>
</tr>
<tr>
<td>Eₘ (V)</td>
<td>0.019</td>
<td></td>
<td>1</td>
<td></td>
<td>RAW264.7 mouse macrophages</td>
<td>18</td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (pL)#</td>
<td>0.03</td>
<td>39</td>
<td>5</td>
<td>0.022 - 0.053</td>
<td>Rat alveolar macrophages</td>
<td>1-2, 10, 12, 14</td>
</tr>
<tr>
<td>Surface area (m²)</td>
<td>4.9x10^{11}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.50</td>
<td></td>
<td>1</td>
<td></td>
<td>Rat hepatocytes</td>
<td>8</td>
</tr>
<tr>
<td>Eₘ (V)</td>
<td>-0.158</td>
<td>2.6</td>
<td>2</td>
<td>-0.155 - 0.161</td>
<td>Rat hepatocytes</td>
<td>6, 17</td>
</tr>
</tbody>
</table>

\# Volume represents per 10^6 cells; Eₘ is membrane potential; CV, coefficient of variation; n, number of studies.

**References:** 1 Davies et al., 1977; 2 Davies et al., 1978; 3 Ohkuma and Poole, 1978; 4 Castranova et al., 1979; 5 Reasor et al., 1979; 6 Hoek et al., 1980; 7 Laman et al., 1981; 8 Roos and Boron, 1981; 9 Cameron et al., 1983; 10 Lum et al., 1983; 11 Chang et al., 1986; 12 Kradin et al., 1986; 13 Gladue et al., 1989; 14 Sebring and Lehnert, 1992; 15 Stone et al., 1992; 16 Krombach et al., 1997; 17 Cortese, 1999; 17 Koivusalo et al., 2011
Table 5-2: Human alveolar macrophage parameters for non-smokers (NS) and smokers (S) used as input for the *in silico* mechanistic cell model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cell</th>
<th>Lysosome</th>
<th>Mitochondria</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (pL) - NS</td>
<td>3.0 (61%)</td>
<td>0.23</td>
<td>0.08</td>
<td>Human alveolar macrophages</td>
<td>5, 7-8</td>
</tr>
<tr>
<td>Volume (pL) - S</td>
<td>7.50⁻ – 7.90⁻</td>
<td>0.58</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface area (m²) - NS</td>
<td>1.87 x 10⁻⁹</td>
<td>1.83 x 10⁻¹⁰</td>
<td>9.24 x 10⁻¹¹</td>
<td>Human alveolar macrophages</td>
<td>6</td>
</tr>
<tr>
<td>Surface area (m²) - S</td>
<td>1.85 x 10⁻⁹</td>
<td>3.37 x 10⁻¹⁰</td>
<td>1.7 x 10⁻¹⁰</td>
<td>Human alveolar macrophages</td>
<td>6</td>
</tr>
<tr>
<td>pH – NS/S</td>
<td>7.17</td>
<td>4.75</td>
<td>7.50</td>
<td>Rabbit alveolar macrophages, Mouse peritoneal macrophages, rat hepatocytes</td>
<td>1, 3-4</td>
</tr>
<tr>
<td>Eₘ(V) – NS/S</td>
<td>-0.032</td>
<td>0.019</td>
<td>-0.158</td>
<td>RAW264.7 mouse macrophages, rat hepatocytes</td>
<td>2, 9-10</td>
</tr>
</tbody>
</table>

Number in bracket indicates the coefficient of variation. Cell volume was calculated from surface area and diameter.

References: ¹ Ohkuma and Poole, 1978; ² Hoek et al., 1980; ³ Laman et al., 1981; ⁴ Roos and Boron, 1981; ⁵ Crapo et al., 1982; ⁶ Nakstad et al., 1989; ⁷ Stone et al., 1992; ⁸ Krombach et al., 1997; ⁹ Cortese, 1999; ¹⁰ Koivusalo et al., 2011
5.4 Results

5.4.1 Model parameterisation

The cell parameters were collated from the literature for primary rat and human AMs. Cell and organelle volumes and cell surface area data were taken from rat AMs. The remaining parameters including pH and $E_m$ were taken from macrophages from other species or rat hepatocytes. The input cell parameters for AMs are shown in Table 5-1 (includes references). The rat AM cell volume was 1.13 pL (1.13 µL/10$^6$ cells) and was comparable to NR8383 cell volume (1.04 µL/10$^6$ cells). This value was 31% variable. A higher uncertainty was associated with the cell surface area (CV=54%). Lysosomal volume was 0.09 pL and 7.8% of the total cell volume. Similar to cell surface area, a large variation was observed for lysosomal volume (CV=54%). Mitochondrial volume was 0.03 pL and 2.8% of the total cell volume. Its value was 39% variable. In the case of human AMs, the cell volume was reported to be 3 pL (CV=61%) for non-smokers (Table 5-2). Lysosome and mitochondrial volumes were 0.23 (7.8% of $V_{cell}$) and 0.085 pL (2.8% of $V_{cell}$), respectively.

In order to simulate the effect of smoking in human AMs, the literature was first searched for an indication of the impact of smoking on cell and organelle volume and surface area. While a number of studies reported cell diameter in both cells, there was only one study reporting cell surface area. There was no significant difference in the surface area of AMs from smokers and non-smokers (1.85 x 10$^{-9}$ and 1.87 x 10$^{-9}$, respectively) (Nakstad et al., 1989). As the cell volume in smokers was unknown, it was determined from this reported surface area using the sphere assumption (7.5 pL). Furthermore, 3 out of 4 studies reporting cell diameter showed no significant difference in cell size from smokers and non-smokers (Harris et al., 1970; Reynolds and Newball, 1974; Territo and Golde, 1979; Davis et al., 1980). The mean cell diameter was 24.7 ± 2.7 µm (± SD) in smokers and 21.2 ± 4.3 µm (± SD) in non-smokers. Using these diameter values led to a cell volume of 7.9 pL in smokers (Table 5-2) and 4.97 pL in non-smokers. This allowed the comparison of these values with that calculated from the surface area in smokers and the measured value in non-smokers (3 pL).

Finally, although there was no study reporting the differences in cell and organelle volume between smokers and non-smokers in human AMs, one study reported cell, lysosomal and mitochondrial volume of AMs in control and smoke-exposed rats (Davies et al., 1977). While the study showed a small decrease in lysosomal volume and
increase in cell and mitochondrial volume in 30-day smoke exposed rat AMs, when the rats were exposed to cigarette smoke for 60 days, cell and lysosomal volumes increased by 2-fold and mitochondrial volume was increased by 2.5-fold. These results were found to be in good agreement with the changes in parameters due to smoking in human AMs (Table 5-2).

5.4.2 Model predictions at acidic lysosomal pH and comparison with in vitro data

Using the AM cell parameters (Table 5-1) and experimentally determined drug physicochemical data (where available), simulations were performed at lysosomal pH 4.75 and the K_p for each compartment was predicted for all drugs. These predictions were initially made assuming 3.5-log unit reduced lipophilicity of ionised drugs for all drugs. The predictions relative to medium and the comparison of predicted K_p,cell with observed K_p,NR8383 are shown in Table 5-3. The predicted K_p,lysosome and K_p,mitochondria relative to cytosol are also presented in the table. The cell model predicted high lysosomal accumulation for all basic drugs and for the zwitterionic ciprofloxacin. This accumulation was selective in the lysosomes as lysosomal K_p was predicted relatively higher than cytosolic and mitochondrial K_p. Among basic drugs, the highest and the lowest lysosomal accumulation were predicted for formoterol and imipramine, respectively; lysosome-to-medium concentration ratios were 234 and 51, respectively. The lysosome-to-cytosol concentration ratio was highest for fenoterol (102) and lowest for imipramine (2). In the case of imipramine, its distribution in cytosol and mitochondria was relatively higher than the remaining drugs. In the case of zwitterions, rifampicin was predicted to accumulate almost equally in all compartments, opposite to ciprofloxacin. The same was observed for neutral budesonide, whereas for permanently positively charged ipratropium and tiotropium bromide, the model predicted high accumulation in mitochondria (mitochondria-to-medium concentration ratio >2000), although their overall predicted cellular accumulation was low (K_p,cell of 71-74). Similar trends were observed for ciprofloxacin and all basic drugs where predicted lysosomal accumulation exceeded total K_p,cell.
Table 5-3: *In silico* cell model predicted K$_p$ values (expressed relative to medium) at acidic lysosomal pH and the comparison of the predicted K$_{p,cell}$ with observed K$_{p,NR8383}$ for all drugs. Observed K$_{p,NR8383}$ are from in vitro lysosomal trapping experiments and represent mean ± SD of at least 3 experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Predicted K$_p$</th>
<th>K$_{p,NR8383}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Lysosome</td>
</tr>
<tr>
<td>Imipramine</td>
<td>26.0</td>
<td>51.0 (2.0)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>3.71</td>
<td>67.8 (18)</td>
</tr>
<tr>
<td>Formoterol</td>
<td>2.58</td>
<td>234 (91)</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>2.14</td>
<td>118 (55)</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>2.07</td>
<td>210 (102)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.91</td>
<td>29.0 (32)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>3.10</td>
<td>2.48 (0.8)</td>
</tr>
<tr>
<td>Budesonide</td>
<td>12.8</td>
<td>12.8 (1.0)</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>1.87</td>
<td>0.92 (0.5)</td>
</tr>
<tr>
<td>Tiotropium bromide</td>
<td>1.83</td>
<td>0.90 (0.5)</td>
</tr>
</tbody>
</table>

* Predictions were performed assuming 3.5-log unit reduced lipophilicity for all drugs. Numbers in brackets indicate K$_p$ relative to cytosol.

Following the initial prediction of accumulation of all drugs under the assumption that ionised drugs show 3.5-log unit reduced lipophilicity than the neutral drugs, the lipophilicity penalty was modified in the model for 4 drugs including imipramine, clarithromycin, terbutaline and ciprofloxacin for which the measured octanol-water partition coefficients of neutral and ionised forms were reported (Appendix Table 7-8). While the lipophilicity difference between the neutral and positively charged forms of imipramine and clarithromycin was close to 3.5 (3.9 and 3.61 for imipramine and clarithromycin, respectively), this difference was 1.89 and 0.61 for terbutaline and ciprofloxacin, respectively. The predicted K$_p$ data for these 4 drugs are shown in Table 5-4. The *in silico* model predicted higher lysosomal accumulation for clarithromycin and imipramine; K$_{p,lysosome}$ increased < 2-fold for both drugs as a result of increased lipophilicity difference between the neutral and ionised forms of the drug (reduced permeability of ionised drug). This finding was in agreement with the simulations which
showed increased lysosomal trapping of imipramine with increasing lipophilicity (permeability) penalty applied (Appendix Figure 7-7). In contrast, a significantly reduced lysosomal accumulation was predicted for terbutaline and ciprofloxacin due to reduced permeability penalty; \( K_{p,\, \text{lysosome}} \) was predicted as 5.4 and 3.2 for terbutaline and ciprofloxacin, respectively. These values are 22 and 9-fold less compared to those predicted earlier. The magnitude of the increase in predicted \( K_{p,\, \text{cell}} \) was < 1.5-fold for imipramine and clarithromycin. In the case of terbutaline and ciprofloxacin, \( K_{p,\, \text{cell}} \) was predicted 4 and 2.8-fold less, respectively. The predicted \( K_p \) of all drugs by the revised model (for 4 drugs) in comparison to the observed \( K_{p,\, \text{NR8383}} \) are illustrated in Appendix Figure 7-8.

**Table 5-4:** *In silico* cell model predicted \( K_p \) (relative to medium) of 4 drugs and comparison of \( K_{p,\, \text{cell}} \) with observed \( K_{p,\, \text{NR8383}} \). Predictions were performed at acidic lysosomal pH and using the literature reported LogP difference between neutral and ionised forms of the drugs. Observed \( K_p \) data in NR8383 are from in vitro lysosomal trapping experiments and represent mean ± SD of at least 3 experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Predicted ( K_p )</th>
<th>Observed ( K_{p,, \text{NR8383}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Lysosome</td>
</tr>
<tr>
<td>Imipramine</td>
<td>24.1</td>
<td>80.0 (3.3)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>3.69</td>
<td>83.6 (23)</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>2.49</td>
<td>5.41 (2.2)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.87</td>
<td>3.22 (3.6)</td>
</tr>
</tbody>
</table>

Numbers in brackets represent \( K_p \) relative to cytosol. LogP difference between neutral and ionised forms (measured in octanol-water) was 3.9, 3.61, 1.89 and 0.61 for imipramine, clarithromycin, terbutaline and ciprofloxacin, respectively.

The correlation of predicted \( K_{p,\, \text{cell}} \) and experimental \( K_{p,\, \text{NR8383}} \) illustrates the overall large predictive bias of the model (gmfe of 7) (Figure 5-2). The accumulation of permanently positively charged ipratropium and tiotropium bromide, and hydrophilic bases fenoterol and terbutaline was over-predicted by the model (only terbutaline was within 3-fold). In the case of moderately lipophilic base formoterol, the model correctly predicted its accumulation in NR8383 cells. For the remaining drugs, including lipophilic
imipramine, clarithromycin, rifampicin and budesonide, and hydrophilic ciprofloxacin, \( K_{p,NR8383} \) was under-predicted by the model. Out of all drugs under-predicted, only budesonide \( K_p \) ratio was within 3-fold from the unity.

![Figure 5-2](image.png)

**Figure 5-2:** In silico cell model predicted \( K_{p,cell} \) versus observed \( K_{p,NR8383} \) for all investigated drugs. The solid line represents the line of unity and the dashed lines represent the prediction bias of 3-fold deviation from the unity. Geometric mean fold error (gmfe) was 7. (■) imipramine; (●) clarithromycin; (☆) budesonide; (×) ciprofloxacin; (♦) rifampicin; (▲) formoterol; (●) ipratropium bromide; (▼) tiotropium bromide; (●) fenoterol; (●) terbutaline.

### 5.4.3 Impact of increase in lysosomal pH on model predictions

The accumulation of basic drugs in the lysosomes can lead to an increase in the pH of these organelles, subsequently resulting in reduced accumulation of the same drugs in the lysosomes. The lysosome-cytosol pH gradient can also be disrupted as a result of chemical agents increasing lysosomal pH by buffering (e.g., \( \text{NH}_4 \text{Cl} \)), modifying intracellular ionic gradients (e.g., monensin, nigericin) and directly inhibiting lysosomal V-H\(^+\)-ATPase (e.g., bafilomycin A1) (Kazmi et al., 2013). In order to evaluate the model’s performance in predicting the reduction in basic drug accumulation as a result of an increase in lysosomal pH, this cell parameter in the model was increased. First, the pH was increased to 6.5 in order to account for the possibility that \( \text{NH}_4 \text{Cl} \) or the
ionophores did not completely abolish the pH-gradient in vitro. The predicted $K_{p,\text{lysosome}}$ and $K_{p,\text{cell}}$ at this pH value in comparison to the predictions at acidic pH are shown in Table 5-5. The predicted values are shown only for 6 drugs for which a reduction in $K_{p,\text{lysosome}}$ and $K_{p,\text{cell}}$ was observed. Furthermore, the cytosolic and mitochondrial $K_p$ values were not presented as there was no change in drug accumulation in these compartments. With increase in pH, a reduction in predicted $K_{p,\text{lysosome}}$ was observed for all drugs indicating the model could simulate the alkalinisation of lysosomes by NH$_4$Cl. This reduction was minimum 26% for terbutaline and maximum 96% for formoterol and fenoterol. For the latter two drugs, this change translated to >20-fold change in lysosomal accumulation. For imipramine and clarithromycin, the reduction in $K_{p,\text{lysosome}}$ was 53 and 87%, respectively. In terms of total cell accumulation, the fold change ranged from < 1.5 to 7 (reduction of 12-86%) for all drugs.

Next, the pH of the lysosomes was increased further to the pH value in the cytosol. This resulted in further reduction in the accumulation of the drugs in both compartments, particularly in lysosomes. The predicted $K_{p,\text{lysosome}}$ was minimum < 1 for ciprofloxacin and maximum 23 for imipramine. The reduction in $K_{p,\text{lysosome}}$ was at least 65% for terbutaline and was highest for formoterol and fenoterol (99%). The magnitude of the predicted reduction in $K_{p,\text{cell}}$ was less than that in $K_{p,\text{lysosome}}$. The reduction in $K_{p,\text{cell}}$ ranged between 11 and 89% for terbutaline and fenoterol, respectively. At pH 7.2, the \textit{in silico} model predicted reduction in $K_{p,\text{cell}}$ for clarithromycin (63%), terbutaline (11%) and ciprofloxacin (21%) was in good agreement with the observed reduction in $K_{p,\text{NR8383}}$ for these drugs (84, 22 and 13% for clarithromycin, terbutaline and ciprofloxacin, respectively). For imipramine, the predicted reduction in $K_{p,\text{lysosome}}$ (71%) was in good agreement with the observed reduction in $K_{p,\text{NR8383}}$ (71%). In the case of fenoterol and formoterol, the predicted reduction in both $K_{p,\text{lysosome}}$ and $K_{p,\text{cell}}$ exceeded the observed reduction in $K_{p,\text{NR8383}}$ by at least 2.8 and 3.4-fold, respectively. For the remaining 4 drugs (budesonide, rifampicin, tiotropium and ipratropium bromide), an increase in lysosomal pH did not lead to further reduction in $K_{p,\text{lysosome}}$ or $K_{p,\text{cell}}$. 

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Table 5-5: *In silico* cell model predicted $K_p$ values at different lysosomal pH and comparison of predicted reduction in $K_{p,lys}$ ($K_{p,lys}$) and $K_{p,cell}$ with observed reduction in $K_{p, NR8383}$ for all compounds.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{p,lys}$</th>
<th>$K_{p,cell}$</th>
<th>$K_{p,lys}$</th>
<th>$K_{p,cell}$</th>
<th>$K_{p,lys}$</th>
<th>$K_{p,cell}$</th>
<th>$K_{p,lys}$</th>
<th>$K_{p,cell}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>80.0</td>
<td>28.5</td>
<td>37.9</td>
<td>25.2</td>
<td>53</td>
<td>12</td>
<td>23.5</td>
<td>24.1</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>83.6</td>
<td>9.93</td>
<td>10.7</td>
<td>4.21</td>
<td>87</td>
<td>58</td>
<td>3.51</td>
<td>3.65</td>
</tr>
<tr>
<td>Formoterol</td>
<td>234</td>
<td>20.7</td>
<td>9.22</td>
<td>3.07</td>
<td>96</td>
<td>85</td>
<td>2.45</td>
<td>2.54</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>5.41</td>
<td>2.79</td>
<td>3.98</td>
<td>2.67</td>
<td>26</td>
<td>3.6</td>
<td>1.95</td>
<td>2.51</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>210</td>
<td>18.4</td>
<td>8.90</td>
<td>2.58</td>
<td>96</td>
<td>86</td>
<td>1.94</td>
<td>2.03</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>3.22</td>
<td>1.05</td>
<td>1.11</td>
<td>0.89</td>
<td>66</td>
<td>19</td>
<td>0.85</td>
<td>0.87</td>
</tr>
</tbody>
</table>
5.4.4 Use of experimental versus predicted drug physicochemical data as input

The initial prediction of $K_p$ values was performed using the experimentally determined $\text{LogP}$ and $pK_a$; these data were available for imipramine, clarithromycin, terbutaline, ciprofloxacin and rifampicin. For the remaining drugs, either LogP and/or $pK_a$ were predicted using ADMET Predictor™ v7.0 software. For rifampicin, the predicted $pK_a$ showed 60% difference relative to its experimentally determined value. Therefore, in order to investigate the impact of the predicted physicochemical data as inputs, the simulations were also performed for the above mentioned drugs using the calculated physicochemical data. The predictions of $K_{p,\text{cell}}$ and $K_{p,\text{lysosome}}$ were compared for the 5 drugs and are shown in Figure 5-3.

![Figure 5-3](image-url)

**Figure 5-3:** Predicted lysosomal and cell $K_p$ of 5 drugs (imipramine, clarithromycin, terbutaline, ciprofloxacin and rifampicin) using experimental and predicted (with ADMET Predictor™ v7.0) LogP and p$K_a$ data. $K_{p,\text{lysosome}}$ using experimental (■) and predicted (■) LogP and p$K_a$; $K_{p,\text{cell}}$ using experimental (■) and predicted (■) LogP and p$K_a$.

The most prominent change in predictions was observed for rifampicin. The drug was initially predicted to distribute evenly in all compartments, whereas the use of ADMET Predictor™ $pK_a$ data predicted 5 and 21-fold more pronounced accumulation in the cell and lysosomes, respectively. The difference was due to the higher predicted basic p$K_a$. 
estimate (10.7) compared to experimental basic pK$_a$ (6.7). For imipramine and clarithromycin, the use of predicted physicochemical data resulted in a further increase in the predicted extent of lysosomal accumulation; the fold changes in predicted K$_{p,lysosome}$ were 3 and 1.6 for imipramine and clarithromycin, respectively. In the case of terbutaline and ciprofloxacin, there was a minimal decrease in the predicted K$_{p,lysosome}$ and the K$_{p,cell}$ was almost unchanged.

5.4.5 Prediction of drug accumulation in human alveolar macrophages

Following the prediction of the accumulation of investigated drugs in primary rat AMs, the developed in silico cell model was applied to human AMs by modifying the input cell and organelle volume and surface area in the model. The predictions were performed for clarithromycin and imipramine as representative drugs as they were shown to accumulate in the lysosomes of human AMs in the in vitro experiments. The model predictions for non-smoker human AMs were compared to those predicted for primary rat AMs and to the in vitro K$_{p,hAM}$ of the compounds (Figure 5-4). Both cell and organelle volumes were almost 2.5-fold higher in human AMs relative to primary rat AMs. An increase in these parameters did not translate into an increase in the predicted accumulation of both drugs in human AMs. As in NR8383 cells, the accumulation of both drugs was under-predicted by the cell model (row data shown in Appendix Table 7-10).

![Graph](image)

**Figure 5-4:** Comparison of the predicted K$_{p,lysosome}$, K$_{p,lysosome}$ and K$_{p,cell}$ in primary rat AMs (■) and non-smoker human AMs (□) with observed K$_{p,cell}$ in human AMs (■).
Next, the cell and organelle volume and surface area were further increased by 2.5-fold in the model to simulate the drug accumulation in AMs in smokers. Although no significant changes in cell size were reported between human AMs from smokers and non-smokers, a 2-fold increase in both cell and organelle volumes was reported in rat AMs exposed to smoke. When cell volume in smokers was calculated from the measured surface area, the increase in cell and organelle volume (2.5-fold) was in line with the increase observed in rat AMs. The predicted $K_p$ for cytosol, lysosomes and total cell in AMs from smokers and non-smokers is presented in Figure 5-5. The trends in predictions were in agreement with those observed when the cell parameters were first increased to adapt the model to human AMs. The further increase in the mentioned cell parameters did not change the predicted accumulation of both drugs, indicating the insensitivity of the cell model towards the changes in volume and surface area.

**Figure 5-5:** Comparison of the predicted $K_{p,\text{cytosol}}$, $K_{p,\text{lysosome}}$ and $K_{p,\text{cell}}$ in human alveolar macrophages from non-smokers (■) and smokers (□). The $K_p$ values in smokers were the same when the cell volume was 7.5 and 7.9 pL.
5.5 Discussion

5.5.1 *In silico* cell model parameterisation

The original cell model developed by Trapp and colleagues considered a generic cell in parameterising the model to predict lysosomal sequestration of compounds (Trapp et al., 2008). The current model presented in this chapter has been modified and adapted to AMs by changing the input cell parameters. For this purpose, a thorough literature search was performed to collate AM specific parameters including cell and organelles’ (lysosome and mitochondria) diameter, volume, surface area, pH, membrane potential and water and lipid content. Where available, measured volume and surface area for the cell and organelles were used and deriving these parameters from the diameter (as in Trapp et al. (2008)) was avoided since that would lead to the assumption that the cell, lysosomes and mitochondria are all spheres. While a number of morphometric studies have indicated AMs to be spherical in shape (Finch et al., 1982; Sebring and Lehnert, 1992), Davies et al. (1977) have found up to 37% deviation from the sphericity of AMs from rats, both control and smoke-exposed, by comparing the measured surface area with that calculated from the diameter. Indeed, when the cell volume and surface area were calculated from rat AM diameter assuming the spherical shape of the cell, the deviations from the measured values were 16 and 1.6%, respectively. Likewise, the calculation of cell volume and surface area from each other led to deviations of < 20%.

However, lysosomes have been reported to be more irregular in shape; some were defined as “oval or round”, some as “U-shaped” or “concave” (Pratt et al., 1971; Kradin et al., 1986; Sebring and Lehnert, 1992). In the case of mitochondria, certain authors have suggested their spherical shape (Sebring and Lehnert, 1992), whereas transmission electron micrographs reported both spherical and non-spherical shapes (Nakstad et al., 1989). Therefore, although it is reasonable to assume the AMs to be spherical, in the case of organelles, this assumption may lead to uncertainties in the model parameters. Similar uncertainty for the remaining cell parameters (e.g., $E_m$, pH) also arises as these were taken from macrophages from other species or from hepatocytes. In the current model, the fractional water and lipid contents (95 and 5% of the total cell volume, respectively) were directly taken from Trapp et al. (2008) due no data reported directly for AMs. Castranova et al. (1979) reported the water content of rat AMs to be 72% of the cell volume; however, the lipid content was not measured/specified which did not permit the parameterisation of the cell model (Castranova et al., 1979).
In order to explore the potential impact of the water-lipid content, $K_p$ of imipramine and clarithromycin was predicted assuming the lipid content was 28% (Appendix Figure 7-9). With increasing lipid content of the cell, the model predicted much higher accumulation of imipramine in all compartments. In contrast, in the case of clarithromycin, higher accumulation was predicted in all compartments except in lysosomes. The reason of this discrepancy is unclear as increased lipid content was assumed to be the same in all compartments and an increase in lysosomal accumulation was expected as for imipramine. Running simulations across a range of LogP when the lipid content was set to 28% resulted in an increase in lysosomal $K_p$ only for highly lipophilic compounds (when LogP > 4).

### 5.5.2 Comparison of model predictions with experimental $K_p$ data

The developed *in silico* cell model was used to predict the total cell and sub-cellular accumulation of the 10 drugs investigated in this study. The predicted $K_{p,\text{cell}}$ data were compared to those observed *in vitro* for both NR8383 and human AMs. The model predicted selective lysosomal accumulation for 4 out of 10 drugs of which are all basic; imipramine, clarithromycin, formoterol and fenoterol. As lysosomal drug concentrations were not measured, direct comparison with predicted lysosomal $K_p$ was not possible. Nevertheless, the results from the indirect assessment of lysosomal trapping (reduction in $K_{p,\text{NR8383}}$ *in vitro* by NH$_4$Cl) were in agreement with the model’s predictions for clarithromycin and imipramine. For these two drugs, the reduction in $K_{p,\text{lysosome}}$ was compared to the observed reduction in $K_{p,\text{NR8383}}$ assuming the reduction observed experimentally reflects the contribution of their lysosomal accumulation. In the case of formoterol and fenoterol, over-prediction of the extent of lysosomal accumulation relative to the experimental data was evident. When lysosomal pH was raised to 7.2 in the model, the predicted $K_{p,\text{cell}}$ was reduced by 88 and 89% for formoterol and fenoterol, respectively, whereas observed reduction in $K_{p,\text{NR8383}}$ was 26-28% for these compounds. However, the model could correctly predict non-lysosomal targeting of neutral, zwitterionic and permanently positively charged compounds as the reduction in predicted $K_{p,\text{cell}}$ was in agreement with the observed reduction in $K_{p,\text{NR8383}}$. One striking observation from the model predictions was that, despite the high accumulation of the drugs in the lysosomes or mitochondria, their overall predicted cellular accumulation was relatively much lower, possibly owing to these organelles representing 7.8 and 2.8% of the total cell volume, respectively. The overall predictive performance of the
model was poor and a large bias was observed (gmfe of 7.0). $K_{p,NR8383}$ of 5 drugs, of which two were basic lipophilic, was under-predicted, indicating the presence of other mechanisms contributing to their high accumulation that are currently not considered in the model. These are discussed in the following section.

Different trends were observed for a number of basic drugs; while the over-prediction of lysosomal accumulation of formoterol led to predicted $K_{p,cell}$ that was comparable with its $K_{p,NR8383}$, for fenoterol, this led to an over-prediction of its $K_{p,NR8383}$. The reason for the over-prediction of $K_{p,lysosome}$ for these drugs is unclear. One possible explanation could be the 3.5-log unit lipophilicity penalty for the ionised drugs that was applied. Previously, the difference in the octanol-water partition coefficients of neutral and ionised forms of basic imipramine, propranolol and amiodarone has been reported to range between 2.7 to 3.92 (Avdeef et al., 1998; Avdeef, 2012). Therefore, it seems reasonable to apply this value to monoprotic bases. However, for basic terbutaline which has multiple acidic (phenolic) groups in addition to an amine group, the $\text{LogP}$ difference between the neutral and ionised form was 1.89 (Appendix Table 7-8). Modifying this value in the model for terbutaline led to an accurate prediction of its lysosomal non-targeting in NR8383 (predicted and observed reduction in $K_{p,cell}$ were 11 and 22%, respectively). As fenoterol and formoterol also have multiple ionisation centres similar to terbutaline, the applied high penalty value possibly caused the over-prediction of their lysosomal accumulation. For tiotropium and ipratropium bromide, the implementation of the model to consider them permanently cationic predicted high mitochondrial accumulation, due to their attraction by the strong negative electrical field of mitochondria and has led to over-prediction of the $K_{p,NR8383}$. This discrepancy may be explained by the hydrophilicity of these compounds limiting their permeation into cells in vitro and that short incubation times used did not allow cell concentrations to reach equilibrium. Finally, the assessment of the impact of using calculated $\text{LogP}$ and $pK_a$ data showed that inaccurate determination of these parameters by predictive software (as in over-prediction of rifampicin basic $pK_a$) can lead to inaccurate prediction of cell and sub-cellular drug concentrations. This highlights the importance of using experimentally determined drug physicochemical data where reported.

5.5.3 Advantages and limitations of the model
An important advantage of the current cell model is that it provides information regarding the drug concentrations in the lysosomes relative to cytosol and other
organelles. The model is able to account for the lysosomal trapping behaviour of basic drugs with relatively simple ionisation profiles (clarithromycin, imipramine). However, when the ionisation of drugs becomes more complex, the model cannot correctly predict their actual accumulation behaviour. The input pKₐ and applied lipophilicity penalty are highly influential and may lead to a misprediction of lysosomal trapping of drugs (including hydrophilic ones). Moreover, the model assumes that drugs behave the same when partitioning into membranes and octanol. A number of studies have demonstrated that the lipophilicity difference between neutral and ionised forms of basic drugs is much smaller in liposome-water membrane system compared to octanol-water system (Avdeef et al., 1998; Balon et al., 1999). The small difference measured in liposome-water system is expected due to strong association of ionised species with membranes by electrostatic and hydrophobic interactions, whereas electrostatic interactions are ignored in octanol-water system. Nonetheless, the LogP measurements made in liposome-water systems are sparse compared to those made in octanol-water system which are more likely to be used as input in in silico models.

The inaccuracy of the input physicochemical data, particularly when predicted by different in silico tools, introduces an uncertainty in the model predictions. The same also applies to the system specific input parameters. For instance, the model predictions are highly sensitive to the changes in lysosomal and mitochondrial pH as revealed by the Monte Carlo analysis (data not shown). For this reason, the model could well mimic the reduced accumulation of basic drugs in the lysosomes in the presence of NH₄Cl. Consequently, any uncertainty in these parameters can propagate further into predicted concentrations. Similarly, the cell and organelle volumes used as input parameters are highly variable (CV > 30%) and surface areas were derived from the volumes (for the organelles) which brings additional uncertainty on the predictions, although Kᵃ was shown to be relatively insensitive to these parameters.

Another important advantage of the current cell model is that it can be adapted to any cell system by modifying the system specific input parameters in the model. This is particularly important for identifying a tendency of compounds to accumulate in lysosome rich tissues such as lungs, liver and kidneys in order to predict potential adverse effects (e.g., phospholipidosis). Furthermore, the cell model allows investigation of the impact of the changes in cell morphology and physiology due to environmental factors (e.g., increased cell volume in smoking), disease or the presence of chemicals (e.g., disturbance of pH gradients in some cancer cells or due to
accumulation of basic drugs). The model’s ability to limit membrane permeability of ionised drugs relative to neutral forms brings an important mechanistic feature to the model. Although several studies have shown increased fluidity and permeability of biological membranes associated with the interaction with weakly basic lipophilic compounds (Drori et al., 1995; Mingeot-Leclercq et al., 2001; Maruoka et al., 2007; Murata et al., 2007; Kornhuber et al., 2010a), this has not been considered in the model as yet. Kornhuber et al. (2010) used a similar cell model consisting of cytosol and lysosomes and incorporated a number of feedback mechanisms. These included the impact of drug concentrations on lysosomal membranes on the permeability of these membranes, and the increase in lysosomal pH due to uptake of amine drugs into these organelles, which has also not been considered in the current model. Therefore, these processes can potentially be implemented in the existing model. Further feedback mechanisms which have not been taken into account include accumulation of basic drugs in the lysosomes on lysosomal volume and membrane potential. In addition to these mechanisms, a number of processes have also not been considered including transporter-mediated saturable active transport and membrane internalisation by endocytosis (Trapp et al., 2008). One other important limitation of the model is it does not take into consideration the binding to cytosolic proteins (applicable to neutral drugs in the dataset) and membrane lipids and phospholipids. The current assumption of the model is drug species partition into one type of lipids with no specification of these lipids being made. The interaction of cationic lipophilic bases with acidic phospholipids is an important mechanism contributing to intracellular concentrations. A number of recent publications have emphasized the necessity to incorporate membrane compartments in the in silico cell models for apical efflux transporters in order to improve the prediction of intracellular drug concentrations (Korzekwa et al., 2012; Korzekwa and Nagar, 2014; Nagar et al., 2014). These additional membrane compartments account for partitioning of drug molecules into and out of the cell membranes and provide a more dynamic assessment of cell and sub-cellular drug concentrations (Zamek-Gliszczynski et al., 2013). Therefore, modification of membrane compartments with appropriate binding and dissociation constants as well as AM phospholipid content may potentially resolve the under-prediction of the $K_{p_{cell}}$ values.
5.5.4 Conclusion and future directions

In conclusion, an *in silico* mechanistic cell model was developed for alveolar macrophages and used to predict drug concentrations in cell and sub-cellular compartments including lysosomes. To date, this is the first cell model specifically developed for AMs. Currently, the cell model performs as a qualitative tool and is useful in ranking drugs for their potential for lysosomal sequestration.

The model is able to capture the lysosomal trapping behaviour of a number of basic, neutral, zwitterionic and permanently cationic compounds. The impact of pH partitioning on overall cellular accumulation was greater for basic drugs than for the others. Whilst being a promising *in silico* model, efforts towards making the model more mechanistic may improve its predictive performance. The future work includes modification of membrane compartments (for cell and lysosomal membrane) to account for the interactions of ionised drugs with membrane acidic phospholipids. Furthermore, issues regarding the over-prediction of lysosomal trapping need to be addressed. Direct assessment of lysosomal trapping of drugs by measuring lysosomal concentrations *in vitro* will further enable direct comparison of the data with the cell model predictions and confirmation of lysosomal $K_p$ values.
Chapter 6  Final Discussion

Despite the presence of a number of inhaled drugs used for asthma and COPD treatment, development of new drugs for lung diseases and systemic delivery has been reported to be challenging. The occurrence of increased number of AMs and/or formation of foamy cells have been a significant safety issue in the development of inhaled drugs (Forbes et al., 2014). Formation of foamy AMs is commonly associated with lysosomal trapping of CADs leading to phospholipidosis. Many clinically used drugs are basic and lipophilic, with a high propensity to accumulate in lysosome rich tissues, including the lungs (Rodgers et al., 2005a). The preferential distribution of these drugs in the lysosomes has advantages and disadvantages depending on the location of intracellular drug targets (Duvvuri et al., 2006; Logan et al., 2012).

Most of studies using macrophage cell lines (including those that are not of alveolar origin and are cancer-derived) have so far investigated the mechanisms of macrophage phagocytosis and expression of inflammatory proteins caused by either environmental particles and/or inhaled pathogens (Forbes et al., 2014). In contrast, there is paucity of in vitro literature data on drug accumulation and lysosomal distribution in both primary human AMs and macrophage cell lines and comparison of the data between different systems. In this study, an in vitro uptake method was developed for the rat AM cell line NR8383 and primary human AMs in order to assess the accumulation of a range of respiratory drugs in both systems. Lysosomal trapping of drugs was investigated by a number of in vitro methods in order to understand the potential contribution of this process to drug accumulation in AMs. In addition, a published in silico mechanistic cell model (Trapp et al., 2008) was modified in order to predict cell and lysosomal distribution of investigated drugs in both rat and human AMs and to compare the model predictions with the data obtained in vitro. The current chapter will discuss the major findings of the work presented in this Thesis together with the existing challenges. In addition, recommendations will be provided with respect to the in vitro methods to assess accumulation and lysosomal sequestration of drugs in AMs and data interpretation. Furthermore, the chapter will discuss areas of improvement for the prediction of cell and lysosomal accumulation of drugs in AMs using the in silico mechanistic cell model.
6.1 *In vitro* assessment of drug accumulation in alveolar macrophages

A plated uptake methodology from the work with hepatocytes (Menochet et al., 2012) was adapted and optimised for NR8383 cells and human AMs to assess drug accumulation. This method was comparable to the previously reported methods for NR8383 cells in that, incubation of drugs with the cells was performed in multi-well plated format and ceased with media removal and subsequent washes with DPBS (Togami et al., 2009; Togami et al., 2011; Togami et al., 2013). The main challenge in method optimisation using NR8383 was the detachment of the cells from experimental plates during washing steps due to semi-adherent nature of the cells. Although measures were taken to minimise the cell detachment by using collagen-coated plates, increasing initial culture time and gentle handling of plates, significant detachment of the cells (>50%) could not be avoided. Further refinement of the method and measurement of protein relative to the cell number resulted in a reproducible method to assess drug uptake in AMs. In contrast to NR8383, the cell detachment was not as pronounced in the case of human AMs as the adhesion of these cells to the experimental support was better in comparison to NR8383 cells. One way of addressing this cell detachment and potentially reducing the associated variability in data could be to perform the incubations when cells are in suspension. The oil-spin assay, which has previously been used to assess drug uptake in primary hepatocytes (Hallifax and Houston, 2006; Soars et al., 2009; Yabe et al., 2011), rabbit and human AMs (Johnson et al., 1980; Hand and King-Thompson, 1982; Hand et al., 1984) may be considered as an alternative method. However, issues with this method are potential HPLC contamination associated with oil or the requirement for radiolabelled compounds. In addition to experimental, cell-associated variation can be a potential contributor to the high variability in data observed for all investigated drugs. In the case of NR8383, the difference in passage numbers may be one source of cell-associated variation (Sambuy et al., 2005; Hughes et al., 2007). In addition, it has been indicated that studying AM biology is complicated as their functional activity, protein and gene expression and their regulation can be altered quickly in response to very subtle changes in their microenvironment (Cassetta et al., 2011; Forbes et al., 2014). Reasons discussed above may all contribute to inter-individual variation in data observed between different patients.
6.2 Drug accumulation in NR8383 cells

The present study demonstrated for the first time the characterisation of accumulation of 10 drugs in the NR8383 cell line. The accumulation of selected drugs in NR8383 was assessed systematically by determining both CL_{uptake} and K_p at 5 µM for all drugs. Active uptake and passive diffusion processes were delineated by assessing drug uptake at 37 and 4°C, respectively. Both CL_{uptake} and K_p of investigated drugs showed a wide range (>400-fold) in NR8383. For most drugs (8 out of 10), active uptake was the predominant process contributing to total uptake in NR8383 (CL_{active}/CL_{diff} ranged between 2-189 for imipramine and clarithromycin, respectively). The limitations in determining passive diffusion clearance at 4°C and the challenges in estimating the parameter for most drugs were highlighted in Sections 1.3.3 and 2.5.2, respectively. Where there was indication for the transport characteristics or passive diffusion of investigated drugs in macrophages (regardless of the species or location), the results were compared with the available data. However, for the majority of drugs, particularly inhaled ones, such data were limited or reported in different transfected cell lines. For instance, the presence of transporter affinity data for rifampicin and ipratropium bromide in the literature was supportive when interpreting the uptake kinetic data in AMs for rifampicin and structurally-related tiotropium bromide. In the case of passive diffusion, the permeability characteristics across Caco-2 cells were also utilised to support the importance of this process in the accumulation of drugs in AMs. For a number of selected drugs, the K_{p,NR8383} showed a decrease with increasing drug concentration. This observation was more evident for basic imipramine, clarithromycin and formoterol (Figure 2-6 A-C). For these drugs, both saturable and nonsaturable processes were proposed to be involved in their accumulation. Saturable process may be attributed to transporter-mediated uptake and/or lysosomal trapping, whereas passive diffusion and nonspecific binding to membrane acidic phospholipids represent nonsaturable processes (Ishizaki et al., 2000; Hallifax and Houston, 2006; Hallifax and Houston, 2007). It was also evident from the accumulation profiles in the current work that maximum uptake of these three drugs occurred at concentrations < 5 µM. Although this may suggest the limitation of the concentration chosen to assess drug uptake, the challenge in the analytical quantification of most drugs at lower concentrations did not permit using lower substrate concentrations.
To our knowledge, the current study is the first one to assess cellular accumulation of a larger number of respiratory drugs in both NR8383 and human AMs under optimised experimental conditions. The analysis has shown an overall 3-fold bias when the $CL_{uptake}$ and $K_p$ data between the two systems were compared (Figure 4-2 and 4-5, respectively). For the majority of drugs, $CL_{uptake}$ and $K_p$ data were up to 12-fold higher in NR8383. The $K_p$ and $CL_{uptake}$ data were comparable (within 3-fold) between the two systems for 6 drugs. The two outliers which were outside of the 3-fold limit in both cases were fenoterol and tiotropium bromide. It is noteworthy that the data in human AMs are yet preliminary for most of the investigated drugs, highlighting the necessity to investigate further the findings in this system. Furthermore, establishment of the NR8383 cell line as a surrogate for human AMs in pre-clinical research and drug development studies requires extending the current dataset to a broader range of compounds and further comparison.

6.3 In vitro assessment of lysosomal trapping of drugs in alveolar macrophages

The in vitro assessment of lysosomal trapping of investigated drugs in NR8383 and human AMs was performed using a number of indirect methods. First, the accumulation of the drugs was assessed in the presence of $NH_4Cl$, monensin and nigericin. These agents have been widely used for more than two decades to investigate lysosomal sequestration of drugs in hepatocytes, AMs and in slices of various tissues (summarised in Appendix Table 7-3), as they abolish the intracellular pH gradients (Ishizaki et al., 1996; Daniel and Wojcikowski, 1997; Ishizaki et al., 1998b). Secondly, LysoTracker® Red was used to qualitatively assess the effect of the agents above in NR8383 and human AMs and lysosomal accumulation of selected drugs in NR8383 cells. As LTR is a lysosomotropic dye, its reduced accumulation in lysosomes in the presence of the chemical agents or basic drugs could be confirmed both visually and from the assessment of the reduction in its fluorescent intensity. The same principle was applied here to provide a preliminary assessment of lysosomal accumulation of the drugs in AMs; the reduction in $CL_{uptake}$ and $K_p$ in the presence of the chemical agents was attributed to lysosomal trapping and the remaining accumulation was mainly attributed to nonspecific binding to membrane acidic phospholipids.
Indirect methods have a number of advantages; the experimental procedure is relatively easy and less time-consuming compared to cellular fractionation. In addition, these methods do not have the issues associated with homogenisation and fractionation (e.g., lysosomal damage, contamination, drug diffusion from lysosomes) and allow larger number of compounds to be assessed simultaneously. However, when evaluating lysosomal accumulation of drugs using these agents, a number of factors need to be considered. The contribution of lysosomal trapping relative to phospholipid binding may be under-estimated if the pH gradient between lysosomes and cytosol is not abolished completely by the mentioned chemical agents. This would cause a lower reduction of drug accumulation by the agents and lead to over-estimation of the contribution of phospholipid binding to the cellular accumulation. The concentration of the chemical agents and the incubation conditions have been shown to influence the increase in lysosomal pH or extent of intracellular CAD accumulation (Ohkuma and Poole, 1981; Novelli et al., 1987; Ishizaki et al., 1996). For instance, 10 mM NH₄Cl has been shown to increase lysosomal pH to ~6.3 in mouse peritoneal macrophages (Ohkuma and Poole, 1981), whereas the corresponding pH level caused by 50 mM of the agent was indicated to be ~6.5-7 in lysosomes from rat liver, although the data were not shown (Ishizaki et al., 1996). Based on these literature data, it is possible that use of 20 mM NH₄Cl raised the lysosomal pH to the value below cytosolic, resulting in a potential under-estimation of the extent of lysosomal trapping by experimental methods. In the case of nigericin, it has been suggested that this agent abolishes the intracellular pH gradients in acidic buffer conditions (Rotin et al., 1987). This was based on the finding that the intra- and extracellular pH in Chinese hamster ovary cells became almost equal when pH of the medium was 6.5 and below, whereas under more alkaline buffer conditions, a small pH difference between intra- and extracellular pH was still evident (Rotin et al., 1987). Some of the previous studies have used acidic buffer pH to investigate lysosomal trapping (Funk and Krise, 2012), whereas others have maintained the extracellular buffer in the pH 7.4 region (Ohkuma and Poole, 1978). In the latter study, the intralysosomal pH of macrophages increased to 6.2 at its highest, indicating that a small pH gradient between lysosomes and cytosol still remained in the presence of nigericin. In this study, the pH of the buffer was not adjusted to acidic pH, as this would impact the ionisation and diffusion of the drugs into the cells even in the absence of nigericin. Therefore, it is possible that a small pH difference had remained between lysosomes and cytosol, potentially leading to an under-estimation of the contribution of
lysosomal trapping in a similar manner. Nonetheless, a significant reduction in $K_{p,NR8383}$ of imipramine (71%) and clarithromycin (84%) by NH$_4$Cl at 5 µM was observed and the results were comparable with those observed in the presence of monensin and nigericin (Table 3-5). This is in agreement with the literature studies in which similar effect of NH$_4$Cl and monensin on the accumulation of a number of drugs in rat lung and liver tissue slices has previously been demonstrated (Appendix Table 7-3). Furthermore, this is the first study to date demonstrating the comparable effect of all three agents on a number of drugs. The findings imply that for both clarithromycin and imipramine, >70% of their accumulation in NR8383 cells relies on lysosome-cytosol pH gradient.

Similar findings were observed in human AMs in which >75% of imipramine and 60% of clarithromycin (both represent the mean reduction in $K_{p,hAM}$ caused by the 3 agents) accumulated due to the pH gradient. Therefore, based on above considerations, it could be suggested that the remaining accumulation of both drugs represents mainly nonspecific binding to phospholipids, although a small contribution of additional lysosomal trapping cannot be neglected.

The selection of the appropriate in vitro method to assess lysosomal trapping of drugs depends on the goals of the research. When the purpose is to obtain an indication for the extent of lysosomal trapping occurring in a cell system for a large dataset, indirect methods are practical and appropriate. If the aim is to obtain lysosomal drug concentrations for a smaller number of compounds, then lysosomal isolation should be considered.

### 6.4 Lysosomal trapping of drugs in NR8383 cells

The reduction in cellular accumulation of investigated drugs in NR8383 showed a wide range in the presence of the chemical agents. No or minimal reduction in $K_{p,NR8383}$ (< 15%) was observed for drugs including zwitterions rifampicin and ciprofloxacin, neutral budesonide and permanently cationic tiotropium bromide. Among basic drugs, the reduction in $K_{p,NR8383}$ was in the range of 20-40% for formoterol, fenoterol and terbutaline. Out of all investigated drugs, significant lysosomal accumulation in NR8383 was observed only for basic imipramine and clarithromycin (reduction in $K_{p,NR8383}$ > 60%). In the case of imipramine, the results were in agreement with the use of this drug as a prototypical CAD for the assessment of lysosomal trapping, as demonstrated in numerous different in vitro systems either in the presence of the
chemical agents (Appendix Table 7-3) or LTR (Nadanaciva et al., 2011; Kazmi et al., 2013). Although clarithromycin is cationic, it is not an amphiphilic drug as it lacks the typical hydrophobic moiety (an aromatic and/or aliphatic ring structure) that is present in many CADs (Fischer et al., 2012). However, macrolides have been indicated to be one of the few drug classes which are exceptional by lacking this typical CAD structure, yet causing phospholipidosis as a result of lysosomal sequestration (Reasor et al., 2006).

Lysosomal sequestration of clarithromycin in NR8383 has also been reported in the recent study (Togami et al., 2013) where 48% reduction in its accumulation in the granule fraction of NR8383 cells was observed in the presence of NH$_4$Cl (Togami et al., 2013). This reported extent of reduction was lower compared to the findings observed in NR8383 cells in the current work (>75%); the discrepancy can be rationalised by the differences in experimental methodologies used. Firstly, Togami and co-workers incubated clarithromycin with the granule fraction isolated from NR8383, whereas in this study, intact cells were used, which should provide a closer representation of drug accumulation in AMs in vivo. Furthermore, a lower concentration of NH$_4$Cl (10 mM) was used with the granule fraction which may have failed to sufficiently alkalinise the lysosomes. It is also possible that the energy source necessary to provide ATP to the V-H$^+$-ATPase was absent and this may have raised the pH during fractionation (Ishizaki et al., 1998a) and incubation with clarithromycin, potentially leading to the underestimation of its lysosomal sequestration. In fact, when the authors performed the incubations in intact cells prior to fractionation of NR8383, the amount of clarithromycin in the granule fraction was reduced by 88%, in agreement with our findings.

The basic drugs in this project were initially selected considering their basic pK$_a$ (range from 8.14 to 9.9), which together with the lysosome-cytosol pH gradient suggested potential accumulation in the lysosomes (Table 3-1). However, 3 out of 5 basic drugs with LogP < 3 failed to show substantial lysosomal accumulation in the follow up experimental studies, suggesting both sufficient lipophilicity and ionisation are required for lysosomal accumulation. This finding was in agreement with a number of studies which showed that among drugs with a wide range of physicochemical properties investigated, lysosomal trapping was observed for the majority of drugs that met criteria of LogP > 2 and pK$_a$ between 6.5-11 (Nadanaciva et al., 2011; Kazmi et al., 2013). However, it was also evident from those studies that the mentioned physicochemical space was not clear-cut and that a number of drugs with these properties failed to
accumulate in the lysosomes. The discrepancy may be explained by the earlier findings of Duvvuri et al (2004) who showed that compounds with similar octanol-water partition coefficients in neutral and ionised forms did not accumulate in the lysosomes as much as those whose partition coefficients were much lower in ionised relative to neutral form (all have the above physicochemical properties).

Another finding of the current lysosomal trapping studies was the altered accumulation kinetics of clarithromycin in NR8383 cells when its lysosomal sequestration was significantly reduced in the presence of NH₄Cl. While it was not possible to determine the uptake kinetic parameter estimates for clarithromycin under control incubation conditions, in the presence of NH₄Cl, the initial uptake rates were greatly reduced and the kinetic parameter estimates could be obtained. Currently, whether these estimates truly reflect the existence of a membrane transporter in NR8383 is uncertain without the knowledge of transporters expressed in this cell line. At present, there is no study against which these findings can be compared. Although such comparison would strengthen the validity of the presented findings, the lack of literature data highlights the novelty of the work presented here. The results further suggest that for CADs, macrolides and other basic lipophilic drugs which accumulate in the lysosomes, the uptake kinetic parameter estimates obtained without complete elimination of this process in vitro may not represent the true transporter affinities and uptake rates. This is particularly important if in vitro generated uptake kinetic parameters will be used in physiologically-based pharmacokinetic models to predict in vivo PK of transporter substrates (Poirier et al., 2009; Watanabe et al., 2009; Jones et al., 2012; Zamek-Gliszczynski et al., 2013). Considering many lysosomotropic drugs are cationic and amphiphilic, these results warrant the need for studies to investigate the impact of lysosomal sequestration on uptake kinetics of known OCT and OCTN substrates.

6.5 Accumulation and lysosomal trapping of drugs in human alveolar macrophages and their clinical importance

At present, existing in vitro data regarding drug accumulation in human AMs are limited (Hand et al., 1984; Hand et al., 1985; Carlier et al., 1987). While the previous studies have focused on the accumulation of antibiotics, the accumulation of a range of drug classes in human AMs was investigated here, including a number of inhaled drugs used in the treatment of asthma and COPD. A significant challenge in investigating
drug accumulation in human AMs is the limited availability of cells from healthy volunteers isolated by bronchioalveolar lavage procedure. In addition, *in vitro* uptake assays generally require large number of cells which is difficult to obtain from a single non-smoker subject. In this study, the accumulation of drugs in human AMs from lung cancer patients who were mainly smokers was assessed in the same manner as in NR8383 cells. A wide range of the extent of drug accumulation was evident in human AMs, with imipramine showing the most extensive accumulation (K<sub>p,hAM</sub> 626), whereas terbutaline accumulated the least (K<sub>p,hAM</sub> 0.39). The contribution of an active process to total uptake, relative to passive diffusion was greater for the majority of investigated drugs (n=6) as the CL<sub>active</sub>/CL<sub>diff</sub> ratio was > 4-fold. However, this ratio ranged a great deal between almost 0 to 524 for budesonide and clarithromycin, respectively.

In addition, the inter-individual variability in the accumulation and lysosomal trapping of clarithromycin and imipramine was evaluated. Large variability in K<sub>p,hAM</sub> between patients was observed for both drugs (63 and 73% variation for imipramine and clarithromycin, respectively). Both drugs showed lysosomal sequestration in human AMs, in line with the findings in NR8383. The lack of variation in the % reduction of CL<sub>uptake</sub> and K<sub>p,hAM</sub> of clarithromycin and imipramine caused by the agents in the same patient may imply that the agents caused a similar increase in lysosomal pH at the *in vitro* conditions they were used at (i.e., concentration and incubation time).

Furthermore, although the % reduction in both parameters caused by NH<sub>4</sub>Cl was consistent between the patients, suggesting little variation in lysosomal-cytosol pH gradient between individuals, the relatively larger variation in control CL<sub>uptake</sub> and K<sub>p,hAM</sub> may indicate potential differences in the lysosomal volume and/or the extent of membrane acidic phospholipid binding of both drugs between the patients. The extent of lysosomal accumulation of both drugs in human AMs was comparable; the reduction in K<sub>p,hAM</sub> by NH<sub>4</sub>Cl was 67 and 61% for imipramine and clarithromycin, respectively. It was evident that in all the patients in which lysosomal trapping of both drugs was assessed, the baseline accumulation of clarithromycin (in the presence of NH<sub>4</sub>Cl) was much lower than that of imipramine. In addition, the variability associated with clarithromycin K<sub>p,hAM+NH4Cl</sub> (62%) was larger than in the case of imipramine (38%).

These results may be explained by the differences in lipophilicity and amphiphilicity of these drugs and their interactions with membrane acidic phospholipids. A study investigating the interactions of macrolides including clarithromycin with dodecylphosphocholine as a membrane mimetic has shown that macrolides bind close
to the micelle surface, close to polar lipid head groups where electrostatic interactions occur with positively charged amino groups (Kosol et al., 2012). In the case of imipramine, partitioning into membranes (defined by $K_{p,\text{AM}+\text{NH}_4\text{Cl}}$) is expected to be much greater, as a result of both electrostatic interactions with phosphate head groups and hydrophobic interactions with the fatty acid chains in the core of the membrane bilayer (Joshi et al., 1988; Fisar et al., 2004).

An important implication of drug accumulation in AMs is the formation of foamy macrophages which can occur due to a number of different processes, namely, phospholipidosis, intracellular lipid accumulation due to uptake of excess surfactant and phagocytosis of poorly soluble particles (reported for β2-agonists, anti-muscarinics and corticosteroids) (Forbes et al., 2011; Lewis et al., 2014). It has been indicated that foamy AMs are often observed in pre-clinical studies in rats due to uptake of insoluble particles and consequences can be both adverse and non-adverse (Jones and Neef, 2012; Owen, 2013; Lewis et al., 2014). The potential of foamy AM formation to exacerbate COPD in humans and the difficulty in monitoring it in clinical trials have been indicated to be the major concerns (Reasor et al., 2006; Owen, 2013; Lewis et al., 2014). A Phospholipidosis Working Group has been formed by the FDA to address the concerns associated with phospholipidosis (Chatman et al., 2009; FDA, 2010); however, specific regulatory guidance in interpretation of AM responses (e.g., increase in number, foamy cell formation) are currently lacking (Forbes et al., 2014).

6.6 Prediction of the accumulation and lysosomal trapping of drugs using the in silico AM cell model

In addition to the in vitro work presented in the current study, an in silico modelling approach was also used to assess lysosomal trapping of drugs investigated. This in silico mechanistic cell model (Trapp et al., 2008) was adapted to AMs using cell parameters from rat and human AMs where available in the literature. The cell model was used to predict steady-state accumulation of investigated drugs in lysosomes and total cell compartments of AMs. In general, lysosomal trapping behaviour of most of the basic drugs as well as neutral, zwitterionic and positively permanently charged drugs was predicted well (Tables 5-3 and 5-5). Comparison of the predicted $K_{p,\text{cell}}$ and experimentally determined $K_{p,\text{NR8383}}$ data revealed a 7-fold bias; an under-prediction by 2.4- to 40-fold for 5 drugs and an over-prediction by 2.8- to 16-fold for 4 drugs.
observed. The limitations of the current model were discussed and a number of recommendations regarding model parameterisation were made in Chapter 5. Two main issues encountered were the over-prediction of lysosomal accumulation observed for a number of basic and zwitterionic drugs and the under-prediction of total cellular accumulation of half of the investigated drugs. The *in silico* model recognises the permeability difference of neutral and ionised species across membranes and the default 3.5-log unit lipophilicity penalty was initially set for ionised drugs. This set up was supported by the findings that for a large number of charged species, partitioning into octanol in the presence of aqueous solutions with physiologically relevant salt concentrations leads to ~3-4 unit LogP difference between neutral and ionised forms of weak bases and acids (Avdeef, 2012). The penalty may have an important impact on the predicted lysosomal drug concentrations, and was therefore scrutinised in an attempt to address the over-prediction issue. A number of conditions have been indicated where the mentioned LogP difference may not apply: 1) smaller LogP difference is observed when a molecule has several polar groups (e.g., hydroxyl) or large polar surface over which charge can be delocalised, 2) when hydroxyl groups are located next to amines or carboxylic groups which can lead to ion pair formation, 3) larger LogP difference is observed in cases where the association between the atoms of the solvent and the drug molecule is hindered by the spatial arrangement of the atoms of the drug molecule (i.e., steric hindrance to solvation as seen in tertiary amines compared to primary ones) (Avdeef, 2012). The reported logP difference was lower for terbutaline and ciprofloxacin. The refinement of the values for these two drugs led to accurate prediction of their lysosomal accumulation. As both formoterol and fenoterol have multiple hydroxyl groups and show resemblance in structure to terbutaline, the default lipophilicity penalty value may also be smaller for these drugs. In addition to above conditions, a smaller LogP difference has also been reported in biological membranes as discussed in Section 5.5.3 (Avdeef et al., 1998; Balon et al., 1999). Therefore, the presented model has been revised for a number of drugs with regards to the lipophilicity penalty value, and the over-prediction of lysosomal trapping of basic drugs has been addressed. Therefore, it is recommended that for future applications of the model, the above mentioned conditions are considered and if available, measured LogP difference between the neutral and ionised species is used to parameterise the model. Otherwise, if that data were not available, then 3.5-log unit penalty should be applied.
The electrostatic and hydrophobic interactions of basic, lipophilic drugs with acidic phospholipids have been reported by numerous studies (Lüllmann and Wehling, 1979; Joshi et al., 1988; Fisar et al., 2004; Fisar, 2005). These interactions have been identified as the primary driving force for the tissue distribution of moderate-to-strong bases (Rodgers et al., 2005b). In the initial set up of the *in silico* model presented here this process has not been accounted for; the absence of this process in the cell model is likely to be the main reason for the under-prediction of cellular accumulation of basic drugs such as imipramine and clarithromycin.

The *in vitro* data from the current study and the literature suggest that uptake transporters have a minimal influence on the accumulation of both drugs in AMs. Therefore, it is expected that for both drugs, the cause of underprediction is the lack of partitioning into acidic phospholipids in the model. Imipramine is more lipophilic than clarithromycin and this reflected to the magnitude of the underprediction of $K_{p, NR8383}$ (8 and 12-fold underprediction for clarithromycin and imipramine, respectively) and $K_{p,hAM}$ (6 and 24-fold underprediction for clarithromycin and imipramine, respectively) in the model. It has been reported that binding of CADs to membranes is affected by membrane phospholipid composition due to the different types of interactions with phospholipids involved (Fisar et al., 2004). For instance, monoprotic imipramine has been reported to interact with both phosphatidyl choline and phosphatidyl serine mainly by hydrophobic and electrostatic interactions, respectively (Joshi et al., 1988; Fisar et al., 2004), whereas diprotic chloroquine has been indicated to interact mainly with phosphatidyl serine via electrostatic interactions (Lüllmann and Wehling, 1979; Joshi et al., 1988). Both the total acidic phospholipid content and the fractions of individual phospholipids have been reported for rat AMs (Nishiura et al., 1988). In addition, the binding affinities of a number of CADs including imipramine to each of these phospholipids have been measured (Murakami and Yumoto, 2011). Therefore, incorporation of membrane phospholipid content of AMs, binding affinities of drugs to phospholipids and the fraction unbound in the cell will lead to a more mechanistic model and improve the prediction of cell and lysosomal drug concentrations.

Over the recent years, there has been an increasing interest in understanding intracellular drug concentrations using *in silico* modelling and simulation approaches (Zhang et al., 2006; Trapp et al., 2008; Korzekwa et al., 2012; Menochet et al., 2012). Some of these mechanistic models currently address a number of processes involved in determining intracellular drug concentrations (e.g., active uptake and efflux, passive
diffusion, intracellular binding) in certain cell systems such as hepatocytes and those expressing P-gp (e.g., MDR1-MDCKII, Caco-2). However, these models do not consider lysosomal accumulation which is of relevance for basic drugs. In the current project, an existing in silico mechanistic cell model which considers intracellular pH gradients and membrane electrostatic interactions was applied specifically for AMs for the first time to predict not only cellular accumulation, but also lysosomal distribution of drugs. A clear advantage of this model is that it is applicable to any cell type, provided that appropriate system specific parameters are used as input. In addition, the model considers the differences in the permeability of neutral and ionised drugs across cell and organelle membranes. However, the model currently does not account for the binding of basic drugs to membrane phospholipids, which is an important limitation. It is clear that there is a need for in silico tools which can be applied to more than one particular cell type and have the ability to account for both system and drug properties. Such models will be a valuable tool to facilitate compound selection in drug discovery and provide a support to in vitro experimental data. Likewise, the in vitro generated data can be used to evaluate the performance of these models for their optimisation and improved prediction of cell and sub-cellular drug concentrations.

6.7 Conclusions

The current study investigated the in vitro accumulation of 10 drugs in AMs. While more extensive characterisation of drug accumulation was carried out in the NR8383 cell line, preliminary assessment was also performed in primary human AMs. Active uptake, passive diffusion and lysosomal trapping were assessed as potential contributors to the accumulation of drugs in both systems. In addition, the inter-individual variability in clarithromycin accumulation in human AMs was investigated by obtaining the $K_p$ data from 9 patients. The selected drugs showed a broad range of uptake clearance (including total and active uptake and passive diffusion) in AMs. The same was observed regarding their cellular partitioning in both NR8383 and human AMs. Large inter-experimental and inter-individual variability of drug accumulation were evident for NR8383 and human AMs, respectively. For the majority of drugs, accumulation in AMs occurred by an active process. The most extensive accumulation and uptake clearance in NR8383 and human AMs were observed for drugs with LogP > 3 and basic $\text{pK}_a$ 9-9.5, which showed significant lysosomal accumulation. The importance of
lysosomal trapping in accumulation of basic lipophilic drugs in AMs and its potential impact on drug accumulation kinetics were emphasized. The comparison of total uptake clearance and cell-to-medium concentration ratio data between NR8383 and human AMs showed an overall 3-fold bias between the two systems, with the majority of drugs showing higher extent of accumulation in NR8383. Based on the current work, NR8383 is a useful in vitro model to assess drug accumulation in AMs. However, the data presented herein should be viewed as initial comparison of the two systems and further investigations are required to confirm the use of NR8383 as a surrogate to human AMs for the assessment of cellular accumulation. Furthermore, lysosomal trapping of a larger number of drugs which are known to accumulate in these organelles can be investigated to confirm the utility of NR8383 in the assessment of this process in AMs. Finally, a mechanistic in silico cell model was successfully adapted to AMs to predict cell and lysosomal accumulation of investigated drugs. The model was sensitive to the changes in lysosomal pH and could correctly predict the lysosomal trapping behaviour of the majority of drugs which were physicochemically diverse. A 7-fold bias was observed between the predicted $K_{p,cell}$ and experimentally determined $K_{p,NR8383}$. In addition to model parameters and a number of processes which are currently not considered in the model, the choice of the in vitro methods and/or the incubation conditions may have also led to the general discrepancy between the observed and predicted values. Currently, the model is recommended to be used as a ranking tool. The strengths and the limitations of the in silico model were highlighted, the areas for improvements were identified and recommendations were made for future applications of this approach to assess subcellular accumulation.
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Chapter 7 Appendix

Appendix Figure 7-1: BSA concentration versus absorbance plot. The absorbance values measured for each cell concentration were read from this plot to determine the protein concentration in different number of cells. Data represent mean ± SD of n=57 measurements.

Appendix Figure 7-2: NR8383 cell number versus absorbance plot used to determine protein concentration in cells. The absorbance values were read from absorbance-BSA protein concentration plot to determine the protein concentration in different number of cells. The protein concentration of experimental samples was then converted to cell number to determine the number of cells. Data represent mean ± SD of n=66 measurements.
Appendix Figure 7-3: NR8383 cell number versus BSA concentration plot used to determine cell number from measured protein concentration. Data represent mean ± SD of n=71 measurements.
Appendix Table 7-1: Literature reported apparent permeability ($P_{\text{app}}$) data for drugs investigated in NR8383.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Physicochemical properties</th>
<th>Apparent permeability</th>
<th>References</th>
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<tbody>
<tr>
<td></td>
<td>LogP</td>
<td>PSA (Å$^2$)</td>
<td>MW (g/mol)</td>
</tr>
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<td>Clarithromycin</td>
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<td>183</td>
<td>747.9</td>
</tr>
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<td>Imipramine</td>
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<td>6.50</td>
<td>280.4</td>
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<tr>
<td>Formoterol</td>
<td>1.99</td>
<td>90.8</td>
<td>344.4</td>
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<td>1.09</td>
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<td>Terbutaline</td>
<td>0.90</td>
<td>72.7</td>
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<td>Budesonide</td>
<td>2.47</td>
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<td>Rifampicin</td>
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<td>Ciprofloxacin</td>
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<td>Mannitol ³</td>
<td>-2.38</td>
<td>121</td>
<td>182.2</td>
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References for experimental LogP data are shown in Table 1-4; ¹ Predicted data by ADMET Predictor™ v7.0 (SimulationsPlus, Lancaster, US); ² Average of literature values for $P_{\text{app}}$ measured in the apical to basolateral direction; ³ mannitol was included as a marker for paracellular transport.
**Appendix Table 7-2:** Literature collated transporter affinity constants and respective expressing systems for rifampicin and ipratropium bromide.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_m$</th>
<th>Transporter</th>
<th>Expressing system</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Rifampicin</td>
<td>13 µM</td>
<td>OATP1B1</td>
<td>Xenopus Laevis</td>
<td>Vavricka et al., 2002</td>
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<tr>
<td></td>
<td>2.3 µM</td>
<td>OATP1B3</td>
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<tr>
<td>Ipratropium</td>
<td>78 µM</td>
<td>OCTN1/2</td>
<td>BEAS-2B</td>
<td>Nakamura et al., 2010;</td>
</tr>
<tr>
<td>bromide</td>
<td>53 µM</td>
<td>OCTN2</td>
<td>HEK293</td>
<td>Nakanishi et al., 2011</td>
</tr>
<tr>
<td></td>
<td>444 µM</td>
<td>OCTN1</td>
<td>HEK293</td>
<td></td>
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<td></td>
<td>0.14 µM</td>
<td>Rat OCT2</td>
<td>HEK293</td>
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</tr>
<tr>
<td></td>
<td>37 µM</td>
<td>Rat OCT1</td>
<td>HEK293</td>
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BEAS-2B; human bronchial epithelial cells
Appendix Table 7-3: Collation of literature studies investigating subcellular distribution of drugs in different in vitro systems using ammonium chloride (NH₄Cl), monensin and nigericin.

<table>
<thead>
<tr>
<th>References</th>
<th>[NH₄Cl] (mM)</th>
<th>In vitro system</th>
<th>Drugs</th>
<th>Concentration (µM)</th>
<th>pKₐ</th>
<th>LogP</th>
<th>Reduction in total uptake (%)</th>
<th>Time (min)</th>
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<tr>
<td>Ohkuma and Poole, 1981</td>
<td>10</td>
<td>Mouse peritoneal macrophages</td>
<td>Methylamine</td>
<td>50</td>
<td>10.6</td>
<td>-0.57</td>
<td>54</td>
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<tr>
<td>MacIntyre and Cutler, 1988a</td>
<td>10</td>
<td>Hepatocytes isolated from male Wistar rats</td>
<td>Chloroquine</td>
<td>10</td>
<td>8.4</td>
<td>5.82</td>
<td>93</td>
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<td></td>
<td>100</td>
<td>10.8</td>
<td></td>
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<td>Daniel et al., 1995</td>
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<td>Tissue slices from male Sprague-Dawley rats</td>
<td>Desipramine</td>
<td>5</td>
<td>10.2</td>
<td>4.53</td>
<td>Lung: 23; Liver: 15</td>
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<td>Chloroquine</td>
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<td>8.1</td>
<td>5.82</td>
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<td>10</td>
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<td>NR</td>
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<td>Ishizaki et al., 1996</td>
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<td>Lysosomes isolated from male Wistar rat liver</td>
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<td>Promazine</td>
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<td>Values</td>
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<td>Fluoxetine</td>
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<td>4.49</td>
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<td>Sertraline</td>
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<td>5.45</td>
<td>4.25</td>
<td>Lung: 42; Liver: 20*</td>
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<td>2.3</td>
<td>2.3</td>
<td>Lung: 5.4; Liver: 16.1</td>
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<tr>
<td>Ishizaki et al., 1998b</td>
<td>10</td>
<td>Lung granule fraction containing lysosomes from male Wistar rats</td>
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<td>54</td>
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<td>8.4, 10.8</td>
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<td>87</td>
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<td>Ishizaki et al., 1998a</td>
<td>3 mg/min/kg (i.v. infusion)</td>
<td>Tissue slices from male Wistar rats</td>
<td>Biperiden</td>
<td>3.2 mg/kg</td>
<td>8.8</td>
<td>4.25</td>
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<td>4.49</td>
<td>Lung: 68; Liver: 11</td>
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* Predicted by MarvinSketch v6.2.1 (ChemAxon Ltd.); * Reported statistically significant difference between control and treatment conditions; NR: no reduction
Appendix Table 7-3 continued:

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<tr>
<th>References</th>
<th>[NH₄Cl] (mM)</th>
<th>In vitro system</th>
<th>Drugs</th>
<th>Concentration (µM)</th>
<th>pKₐ</th>
<th>LogP</th>
<th>Reduction in total uptake (%)</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td>Daniel and Wojcikowski, 1999b</td>
<td>20</td>
<td>Tissue slices from male Wistar rats</td>
<td>Thioridazine, Imipramine, Amitriptyline, Fluoxetine, Sertraline</td>
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<td>Values ranging from 9.4-9.5</td>
<td>Values ranging from 4.6-5.45</td>
<td>Lung: 41°; Liver: 75°</td>
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<td>Lung: 35°; Liver: 57°</td>
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<td>5</td>
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<td></td>
<td>Lung: 56°; Liver: 68°</td>
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<td>5</td>
<td></td>
<td></td>
<td>Lung: 41°; Liver: 52°</td>
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<td>Tissue slices from male Wistar rats</td>
<td>Perazine, Imipramine, Amitriptyline, Fluoxetine, Sertraline</td>
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<td>3.21, 8.14</td>
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<td>Lung: 58°; Liver: 61°</td>
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<td>Daniel et al., 2001</td>
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<td>Brain slices from male Wistar rats</td>
<td>Promazine, Thioridazine, Perazine, Imipramine, Amitriptyline, Fluoxetine, Sertraline</td>
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<td>Values ranging from 9.4-9.5</td>
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<td>Lung: 49°; Liver: 62°</td>
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<td>Lung: 47°; Liver: 67°</td>
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<td>5</td>
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<td></td>
<td>Lung: 39°; Liver: 50°</td>
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<tr>
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<td>NR8383 cell line</td>
<td>Telithromycin</td>
<td>50</td>
<td>2.4, 5.0, 8.7</td>
<td>5.3°</td>
<td>Brain: 20°</td>
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<td>Togami et al., 2013</td>
<td>10</td>
<td>NR8383 cell line and granule fraction (containing lysosomes)</td>
<td>Azithromycin, Clarithromycin</td>
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<td>8.1, 8.8</td>
<td>4.02</td>
<td>Granule: 97°; Granule: 72°</td>
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<td>Kazmi et al., 2013</td>
<td>50</td>
<td>Cell line of immortalized hepatocytes (Fa2N-4)</td>
<td>Propranolol, Imipramine</td>
<td>1</td>
<td>9.67</td>
<td>2.58</td>
<td>Granule: 88°; Granule: 48°</td>
<td>60</td>
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</table>

°Reported statistically significant difference between control and treatment conditions; ‡ before and § after subcellular fractionation.
Appendix Table 7-3 continued;

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<th>[Monensin] (µM)</th>
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<th>Drugs</th>
<th>Concentration (µM)</th>
<th>pKₐ</th>
<th>LogP</th>
<th>Reduction of total uptake (%)</th>
<th>Time (min)</th>
</tr>
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<td>Daniel et al., 1995</td>
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<td>Tissue slices from male Sprague-Dawley rats</td>
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<td>10.2</td>
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<td>Lung: 12%; Liver: NR</td>
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<td>8.1, 10.1</td>
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<td>Lung: 73%; Liver: 44</td>
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<td>3.5</td>
<td>2.9</td>
<td>Lung: 1.8</td>
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<tr>
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<td></td>
<td></td>
<td>Thiopental</td>
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<td>7.5</td>
<td>2.78%</td>
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<td>Promazine</td>
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<td>Values ranging from 9.4-9.5</td>
<td>4.0</td>
<td>Lung: 71%; Liver: 86</td>
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<td>Imipramine</td>
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<td>Values ranging from 9.4-9.5</td>
<td>4.6</td>
<td>Lung: 64%; Liver: 48</td>
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<td>Amitriptyline</td>
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<td>Lung: 70%; Liver: 48</td>
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<td>Lung: 50%; Liver: 19</td>
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<td>Carbamazepine</td>
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<td>Lung: 5.4; Liver: 0</td>
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<td>9.5</td>
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<td>Lung: 35%; Liver: 82</td>
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<td>Daniel and Wojcikowski, 1999a</td>
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<td>Tissue slices from male Wistar rats</td>
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<td>3.21, 8.14</td>
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<td>Lung: 71.6%; Liver: 65</td>
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<td>Daniel et al., 2001</td>
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<td>Brain slices from male Wistar rats</td>
<td>Promazine</td>
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<td>Values ranging from 2.95-5.45</td>
<td>Values ranging from 2.95-5.45</td>
<td>Brain: 35</td>
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NR; no reduction
Appendix Table 7-3 continued;

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<th>LogP</th>
<th>Reduction of total uptake (%)</th>
<th>Time (min)</th>
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<td>4.8</td>
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<td>Ohkuma and Poole, 1981</td>
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<td>Mouse peritoneal macrophages</td>
<td>Methylamine</td>
<td>50</td>
<td>10.6</td>
<td>-0.57</td>
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<th>Drugs</th>
<th>Concentration (µM)</th>
<th>pK\textsubscript{a}</th>
<th>LogP</th>
<th>Reduction of total uptake (%)</th>
<th>Time (min)</th>
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</thead>
<tbody>
<tr>
<td>Kazmi et al., 2013</td>
<td>10 µM + 20 µM</td>
<td>Cell line of immortalized hepatocytes (Fa2N-4)</td>
<td>Propranolol</td>
<td>1</td>
<td>9.67</td>
<td>2.58</td>
<td>67</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Imipramine</td>
<td>1</td>
<td>9.20</td>
<td>4.28</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>

References for experimental drug physicochemical data: Desipramine (Zhao et al., 2002); Diazepam, biperiden and trihexyphenidyl (Ishizaki et al., 1997); Azithromycin (McFarland et al., 1997); Methylamine (Dunn and Nagy, 1992).
Appendix Figure 7-4: Variation in clarithromycin cell-to-media concentration ratio ($K_p$) in human alveolar macrophages (AMs) from 9 patients. (■) Male; (□) Female. Numbers below bars indicate patient number. The third value was obtained from pooled AMs from 2 males. The $K_p$ was determined using mean clarithromycin media concentration for patients 1, 2 and 6.

Appendix Table 7-4: Reduction of uptake clearance ($CL_{uptake}$) of imipramine and clarithromycin (5 µM) by ammonium chloride (NH$_4$Cl) (20 mM), monensin (5 µM) and nigericin (10 µM) in human alveolar macrophages (AMs). Data in human AMs represent single values from one patient for monensin and nigericin, and at least 4 patients for NH$_4$Cl. Data in NR8383 represent mean values from at least 3 separate experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Reduction in $CL_{uptake}$ by $NH_4$Cl</th>
<th>% Reduction in $CL_{uptake}$ by Monensin</th>
<th>% Reduction in $CL_{uptake}$ by Nigericin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human AMs</td>
<td>NR8383</td>
<td>Human AMs</td>
</tr>
<tr>
<td>Imipramine</td>
<td>71$^*$</td>
<td>68$^*$</td>
<td>73</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>68$^*$</td>
<td>85$^*$</td>
<td>64</td>
</tr>
</tbody>
</table>

$^*$, $p < 0.05$; $^{**}$, $p < 0.01$ by Student’s t-test
**Appendix Table 7-5:** Comparison of $K_p$ of imipramine and clarithromycin (5 µM) estimated in human AMs and NR8383 under control and NH₄Cl (20 mM) treatment conditions and the % reduction in $K_p$ as a result of NH₄Cl treatment. Data represent mean ± SD of at least 3 experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Human AMs</th>
<th></th>
<th>Reduction in $K_p$</th>
<th>NR8383</th>
<th></th>
<th>Reduction in $K_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_p$ Control</td>
<td>$K_p$ + NH₄Cl</td>
<td>$K_p$ reduction</td>
<td>$K_p$ Control</td>
<td>$K_p$ + NH₄Cl</td>
<td>$K_p$ reduction</td>
</tr>
<tr>
<td>Imipramine</td>
<td>707 ± 381.9</td>
<td>231.4 ± 86.9</td>
<td>67</td>
<td>347 ± 59.8</td>
<td>102 ± 17.1</td>
<td>71</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>59.6 ± 30.4</td>
<td>23.3 ± 14.5</td>
<td>61</td>
<td>82.7 ± 27.3</td>
<td>13.3 ± 6.5</td>
<td>84</td>
</tr>
</tbody>
</table>
Appendix Figure 7-5: Clarithromycin cell-to-medium concentration ratio ($K_p$) in human alveolar macrophages (AMs) and NR8383 in the absence and presence of 5 µM monensin (A) or 10 µM nigericin (B). (■) control; (□) treated with monensin; (■) treated with nigericin. Data in human AMs represent a single measurement in Patient 9 whereas in NR8383, data represent mean ± SD of at least 3 separate experiments. Numbers above bars indicate % reduction in $K_p$ caused by treatment with chemical agents (**, p < 0.01 by Student’s t-test).
Appendix Figure 7-6: Imipramine cell-to-medium concentration ratio ($K_p$) in human alveolar macrophages (AMs) and NR8383 in the absence and presence of 5 µM monensin (A) or 10 µM nigericin (B). (■) control; (■) treated with monensin; (■) treated with nigericin. Data in human AMs represent a single measurement in Patient 9 whereas in NR8383, data represent mean ± SD of at least 3 separate experiments. Numbers above bars indicate % reduction in $K_p$ caused by treatment with chemical agents (**, p < 0.01 by Student’s t-test).
**Appendix Table 7-6**: Literature collated cell-to-medium concentration ratio ($K_p$) data in AMs for drugs investigated.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Human AMs (Smokers)</th>
<th>Human AMs (Non-smokers)</th>
<th>NR8383 cell line</th>
<th>Primary rat AMs</th>
<th>Primary rabbit AMs</th>
<th>Time of incubation (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rifampicin</strong></td>
<td>ND</td>
<td>3.53 ± 0.71 (3)</td>
<td>n/a</td>
<td>n/a</td>
<td>0.73 ± 0.11 (6)</td>
<td>1</td>
<td>Johnson et al., 1980; Hand et al., 1984; Hand et al., 1985</td>
</tr>
<tr>
<td>(25 µM)</td>
<td>4.16 ± 0.74 (5)</td>
<td>4.09 ± 0.92 (4)</td>
<td>n/a</td>
<td>n/a</td>
<td>ND</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.72 ± 1.18 (4)</td>
<td>4.67 ± 0.67 (4)</td>
<td>2.05 ± 0.3 (5)</td>
<td>15</td>
<td></td>
<td>15</td>
<td>Togami et al., 2011; Togami et al., 2013</td>
</tr>
<tr>
<td></td>
<td>7.88 ± 0.86 (6)</td>
<td>5.20 ± 0.71 (5)</td>
<td>1.84 ± 0.3 (4)</td>
<td>30</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.78 ± 0.51 (5)</td>
<td>4.86 ± 1.17 (3)</td>
<td>1.94 ± 0.2 (6)</td>
<td>60</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.0 ± 0.62 (5)</td>
<td>ND</td>
<td>1.78 ± 0.25 (5)</td>
<td>120</td>
<td></td>
<td>120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>20.0</td>
<td>n/a</td>
<td>n/a</td>
<td>15</td>
<td>Togami et al., 2011; Togami et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>29.0</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td><strong>Clarithromycin</strong></td>
<td>n/a</td>
<td>n/a</td>
<td>15.7</td>
<td>n/a</td>
<td>n/a</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>(50 µM)</td>
<td></td>
<td></td>
<td>29.0</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

* Data reported as mean ± standard error of the mean (number of experiments); ND: not determined; n/a: not available
Appendix Table 7-7: Literature collated cell-to-medium concentration ratio ($K_p$) of drugs in primary alveolar macrophages (AMs) from humans and animals and NR8383 cell line.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Human AMs (Smokers)</th>
<th>Human AMs (Non-smokers)</th>
<th>NR8383 cell line</th>
<th>Primary rat AMs</th>
<th>Primary rabbit AMs</th>
<th>Incubation time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorphentermine (20 µM)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>9.5</td>
<td>n/a</td>
<td>10</td>
<td>Heyneman and Reasor, 1986</td>
</tr>
<tr>
<td>Erythromycin* (25 µM)</td>
<td>6.3 ± 1.8 (4)</td>
<td>15.7 ± 9.3 (2)</td>
<td>n/a</td>
<td>2.17 ± 0.68 (3)</td>
<td>n/a</td>
<td>15</td>
<td>Johnson et al., 1980;</td>
</tr>
<tr>
<td></td>
<td>13.2 ± 3.6 (4)</td>
<td>17.7 ± 6.4 (2)</td>
<td>n/a</td>
<td>8.3 ± 2.64 (5)</td>
<td>n/a</td>
<td>30</td>
<td>Hand et al., 1984;</td>
</tr>
<tr>
<td></td>
<td>22.1 ± 3.8 (3)</td>
<td>ND</td>
<td>n/a</td>
<td>20.6 ± 3.12 (7)</td>
<td>n/a</td>
<td>120</td>
<td>Hand et al., 1985;</td>
</tr>
<tr>
<td></td>
<td>46 ± 4 (4)</td>
<td>38 ± 10 (3)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td>Carlier et al., 1987</td>
</tr>
<tr>
<td>Clindamycin* (25 µM)</td>
<td>50.9 ± 2.1 (3)</td>
<td>20 ± 1.7 (3)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>15</td>
<td>Hand et al., 1984;</td>
</tr>
<tr>
<td></td>
<td>51.2 ± 4.2 (5)</td>
<td>20.2 ± 2.1 (4)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>30</td>
<td>Hand et al., 1985</td>
</tr>
<tr>
<td></td>
<td>47.9 ± 6.0 (4)</td>
<td>23.5 ± 2.9 (3)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Roxithromycin* (12 µM)</td>
<td>190 ± 21 (4)</td>
<td>61 ± 7 (3)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>30</td>
<td>Carlier et al., 1987</td>
</tr>
<tr>
<td>Telithromycin (50 µM)</td>
<td>n/a</td>
<td>n/a</td>
<td>24</td>
<td>n/a</td>
<td>n/a</td>
<td>15</td>
<td>Togami et al., 2009</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>40</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Azithromycin (50 µM)</td>
<td>n/a</td>
<td>n/a</td>
<td>57</td>
<td>n/a</td>
<td>n/a</td>
<td>15</td>
<td>Togami et al., 2011</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>678</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>39</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>10</td>
<td>Togami et al., 2013</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>361</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>731</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>480</td>
<td></td>
</tr>
</tbody>
</table>

Data reported as mean ± standard error of the mean (*) or SD (⁷) (number of experiments); ND: not determined; n/a: not available
7.1. Methods

7.1.1. *In silico* mechanistic cell model implemented in Matlab

7.1.1.1. Equations describing ionisation, permeability, lipophilicity, electrostatic interactions and molecule fractions

```matlab
%% Parameters fixed (physical constants)
F = 96485.3415; % Faraday constant [C/mol]
RT = 8.314;   % gas constant [J/mol/K; VC = J]

%% Physiological values
T = 310.16;   % absolute temperature [K]
DC = 1e-14;  % m2/s
deltax = 9e-9; % m
pH = [7.4;7.17;4.75;7.5]; % [media cytosol lysosomes mitochondria]
W = 0.95;     % water content (L/L)
L = 0.05;     % lipid content (L/L)
ACN = 1.23;   % activity coefficient of neutral form
ACD1 = 0.74;  % activity coefficient of any single charged molecule species
ACD2 = 0.30;  % activity coefficient of any multiple charged molecule species

%% Drug specific parameters
logP = 3.16;  % log value of the octanol to water partitioning coefficient
pKa_a1 = [0]; % acidic pKa (order is irrelevant)
pKa_a2 = [0]; % acidic pKa (order is irrelevant)
pKa_a3 = [0]; % acidic pKa (order is irrelevant)
pKa_a = [pKa_a1 pKa_a2 pKa_a3];
pKa_b1 = [8.99]; % basic pKa (order is irrelevant)
pKa_b2 = [0];  % basic pKa (order is irrelevant)
pKa_b3 = [0];  % basic pKa (order is irrelevant)
pKa_b = [pKa_b1 pKa_b2 pKa_b3];

%% Automated computation;
pKa_a_data = [];
for i = 1:length(pKa_a)
    if pKa_a(i) > 0
        pKa_a_data=[pKa_a_data,pKa_a(i)];
    end
end
pKa_a_data = sort(pKa_a_data,'ascend');
noA = length(pKa_a_data);  % number of acidic fcts

pKa_b_data = [];
for i = 1:length(pKa_b)
    if pKa_b(i) > 0
        pKa_b_data=[pKa_b_data,pKa_b(i)];
    end
end
pKa_b_data = sort(pKa_b_data,'ascend');
noB = length(pKa_b_data);  % number of basic fcts
NSp = noB + 1;   % position of neutral form

pKa = [pKa_a_data,pKa_b_data];
pKa_data = sort(pKa,'ascend');
NSp = length(pKa_data)+1;  % total number of molecule species
```

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% defining the terms required for ionisation
aND_all = [];
for i = 1:length(pH)
    if noB == 3;
        aN_left = (10^(pKa_data(1)+pKa_data(2)+pKa_data(3)-
            3*pH(i))+10^(pKa_data(2)+pKa_data(3)-2*pH(i))+10^(pKa_data(3)-
            1*pH(i)));
    elseif noB == 2;
        aN_left = (10^(pKa_data(1)+pKa_data(2)-
            2*pH(i))+10^(pKa_data(2)-1*pH(i)));
    elseif noB == 1;
        aN_left = (10^(pKa_data(1)-1*pH(i)));
    elseif noB == 0;
        aN_left = 0;
    end

    if noA == 3;
        aN_right = (10^-
            (pKa_data(NSp+2)+pKa_data(NSp+1)+pKa_data(NSp)-3*pH(i))+10^-
            (pKa_data(NSp+1)+pKa_data(NSp)-2*pH(i))+10^-pKa_data(NSp)-1*pH(i)));
    elseif noA == 2;
        aN_right = (10^-(pKa_data(NSp+1)+pKa_data(NSp)-2*pH(i))+10^-
            (pKa_data(NSp)-1*pH(i)));
    elseif noA == 1;
        aN_right = (10^-(pKa_data(NSp)-1*pH(i)));
    elseif noA == 0;
        aN_right = 0;
    end

    aN = (1+aN_left+aN_right)^-1;

    if noB == 3;
        aD_1 = aN*(10^-
            (pKa_data(NSp+2)+pKa_data(NSp+1)+pKa_data(NSp)-3*pH(i)));
        aD_2 = aN*(10^-
            (pKa_data(NSp+1)+pKa_data(NSp)-2*pH(i)));
        aD_3 = aN*(10^-pKa_data(NSp)-1*pH(i)));
    elseif noB == 2;
        aD_2 = aN*(10^-
            (pKa_data(NSp+1)+pKa_data(NSp)-2*pH(i)));
        aD_3 = aN*(10^-pKa_data(NSp)-1*pH(i)));
        aD_1 = 0;
    elseif noB == 1;
        aD_3 = aN*(10^-pKa_data(NSp)-1*pH(i)));
        aD_2 = 0;
        aD_1 = 0;
    elseif noB == 0;
        aD_1 = 0;
        aD_2 = 0;
        aD_3 = 0;
    end

    if noA == 3;
        aD_6 = aN*(10^-pKa_data(NSp)-pKa_data(NSp+1)-
            pKa_data(NSp+2)+3*pH(i)));
        aD_5 = aN*(10^-pKa_data(NSp)-pKa_data(NSp+1)+2*pH(i)));
        aD_4 = aN*(10^-pKa_data(NSp)+1*pH(i)));
    elseif noA == 2;
        aD_5 = aN*(10^-pKa_data(NSp)-pKa_data(NSp+1)+2*pH(i)));
        aD_4 = aN*(10^-pKa_data(NSp)+1*pH(i)));
        aD_6 = 0;
    elseif noA == 1;
\[
a_{D_4} = a_N \times 10^{(-pK_a_{data}(NSp) + 1 \times pH(i))}; \\
a_{D_5} = 0; \\
a_{D_6} = 0;
\]

\[
\text{elseif } \text{noA} == 0; \\
a_{D_4} = 0; \\
a_{D_5} = 0; \\
a_{D_6} = 0;
\]

\[
a_{ND} = [pH(i), a_N, a_{D_1}, a_{D_2}, a_{D_3}, a_{D_4}, a_{D_5}, a_{D_6}]; \\
a_{ND_all} = [a_{ND_all}, a_{ND}];
\]

**defining lipophilicity and permeability (default - affects both partitioning and permeability)**

\[
z = [nan, 0, 3, 2, 1, -1, -2, -3]; \quad \% \text{charge of molecule species}
\]

\[
\text{logP\_all} = \text{logP}\_\text{abs}(z) \times 3.5; \quad \% \text{lipophilicity of molecule species}
\]

\[
\text{KOW\_all} = 10.^
\text{logP\_all}; \quad \% \text{partitioning coefficient of molecule species}
\]

\[
\text{Perm\_all} = DC. \times KOW\_all. / \text{deltax}; \quad \% \text{permeability of molecule species}
\]

% defining electrostatic interactions

\[
\text{E} = [-0.032, 0.019, -0.2]; \quad \% \text{membrane potential of cell compartments}
\]

\[
\text{N\_all} = []; \\
\text{for } i = 1: \text{length}(z) \\
\quad \text{NC} = z(i) \times \text{E}(1) \times F / (RT\_T); \\
\quad \text{NL} = z(i) \times \text{E}(2) \times F / (RT\_T); \\
\quad \text{NM} = z(i) \times \text{E}(3) \times F / (RT\_T); \\
\quad y = [\text{NC}; \text{NL}; \text{NM}]; \\
\quad \text{N\_all} = [\text{N\_all}, y];
\]

\[
a_{ND\_elec} = [a_{ND\_all}, z; \text{KOW\_all}; \text{Perm\_all}; \text{N\_all}]; \quad \% \text{input for simulations}
\]

% calculations of drug fractions inside the cell and cell compartments

\[
\text{FMED\_all} = a_{ND\_elec}(1, 2); \\
\text{FD1MED\_all} = a_{ND\_elec}(1, 3); \\
\text{FD2MED\_all} = a_{ND\_elec}(1, 4); \\
\text{FD3MED\_all} = a_{ND\_elec}(1, 5); \\
\text{FD4MED\_all} = a_{ND\_elec}(1, 6); \\
\text{FD5MED\_all} = a_{ND\_elec}(1, 7); \\
\text{FD6MED\_all} = a_{ND\_elec}(1, 8);
\]

\[
\text{FNC} = (W/ACN) + L \times \text{KOW\_all}(2) / ACN ...
\]

\[
+ \text{aND\_elec}(2, 3) / \text{aND\_elec}(2, 2) \times W/ACD2 + \text{aND\_elec}(2, 3) / \text{aND\_elec}(2, 2) \times L/KOW\_all(3) / ACD2 ...
\]

\[
+ \text{aND\_elec}(2, 4) / \text{aND\_elec}(2, 2) \times W/ACD2 + \text{aND\_elec}(2, 4) / \text{aND\_elec}(2, 2) \times L/KOW\_all(4) / ACD2 ...
\]

\[
+ \text{aND\_elec}(2, 5) / \text{aND\_elec}(2, 2) \times W/ACD1 + \text{aND\_elec}(2, 5) / \text{aND\_elec}(2, 2) \times L/KOW\_all(5) / ACD1 ...
\]

\[
+ \text{aND\_elec}(2, 6) / \text{aND\_elec}(2, 2) \times W/ACD1 + \text{aND\_elec}(2, 6) / \text{aND\_elec}(2, 2) \times L/KOW\_all(6) / ACD1 ...
\]

\[
+ \text{aND\_elec}(2, 7) / \text{aND\_elec}(2, 2) \times W/ACD2 + \text{aND\_elec}(2, 7) / \text{aND\_elec}(2, 2) \times L/KOW\_all(7) / ACD2 ...
\]

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+\text{AND}_\text{elec}(2, 8) / \text{AND}_\text{elec}(2, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(2, 8) / \text{AND}_\text{elec}(2, 2) \cdot L \cdot \text{KOW}_\text{all}(8) / ACD2 \ldots
\}

FD1C = \text{AND}_\text{elec}(2, 3) / \text{AND}_\text{elec}(2, 2) \cdot \text{FNC};
FD2C = \text{AND}_\text{elec}(2, 4) / \text{AND}_\text{elec}(2, 2) \cdot \text{FNC};
FD3C = \text{AND}_\text{elec}(2, 5) / \text{AND}_\text{elec}(2, 2) \cdot \text{FNC};
FD4C = \text{AND}_\text{elec}(2, 6) / \text{AND}_\text{elec}(2, 2) \cdot \text{FNC};
FD5C = \text{AND}_\text{elec}(2, 7) / \text{AND}_\text{elec}(2, 2) \cdot \text{FNC};
FD6C = \text{AND}_\text{elec}(2, 8) / \text{AND}_\text{elec}(2, 2) \cdot \text{FNC};

\text{FNL} = (W / ACN + L \cdot \text{KOW}_\text{all}(2) / ACN \ldots
+\text{AND}_\text{elec}(3, 3) / \text{AND}_\text{elec}(3, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(3, 3) / \text{AND}_\text{elec}(3, 2) \cdot L \cdot \text{KOW}_\text{all}(3) / ACD2 \ldots
+\text{AND}_\text{elec}(3, 4) / \text{AND}_\text{elec}(3, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(3, 4) / \text{AND}_\text{elec}(3, 2) \cdot L \cdot \text{KOW}_\text{all}(4) / ACD2 \ldots
+\text{AND}_\text{elec}(3, 5) / \text{AND}_\text{elec}(3, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(3, 5) / \text{AND}_\text{elec}(3, 2) \cdot L \cdot \text{KOW}_\text{all}(5) / ACD1 \ldots
+\text{AND}_\text{elec}(3, 6) / \text{AND}_\text{elec}(3, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(3, 6) / \text{AND}_\text{elec}(3, 2) \cdot L \cdot \text{KOW}_\text{all}(6) / ACD1 \ldots
+\text{AND}_\text{elec}(3, 7) / \text{AND}_\text{elec}(3, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(3, 7) / \text{AND}_\text{elec}(3, 2) \cdot L \cdot \text{KOW}_\text{all}(7) / ACD2 \ldots
+\text{AND}_\text{elec}(3, 8) / \text{AND}_\text{elec}(3, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(3, 8) / \text{AND}_\text{elec}(3, 2) \cdot L \cdot \text{KOW}_\text{all}(8) / ACD2 \ldots
\}

FD1L = \text{AND}_\text{elec}(3, 3) / \text{AND}_\text{elec}(3, 2) \cdot \text{FNL};
FD2L = \text{AND}_\text{elec}(3, 4) / \text{AND}_\text{elec}(3, 2) \cdot \text{FNL};
FD3L = \text{AND}_\text{elec}(3, 5) / \text{AND}_\text{elec}(3, 2) \cdot \text{FNL};
FD4L = \text{AND}_\text{elec}(3, 6) / \text{AND}_\text{elec}(3, 2) \cdot \text{FNL};
FD5L = \text{AND}_\text{elec}(3, 7) / \text{AND}_\text{elec}(3, 2) \cdot \text{FNL};
FD6L = \text{AND}_\text{elec}(3, 8) / \text{AND}_\text{elec}(3, 2) \cdot \text{FNL};

\text{FNM} = (W / ACN + L \cdot \text{KOW}_\text{all}(2) / ACN \ldots
+\text{AND}_\text{elec}(4, 3) / \text{AND}_\text{elec}(4, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(4, 3) / \text{AND}_\text{elec}(4, 2) \cdot L \cdot \text{KOW}_\text{all}(3) / ACD2 \ldots
+\text{AND}_\text{elec}(4, 4) / \text{AND}_\text{elec}(4, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(4, 4) / \text{AND}_\text{elec}(4, 2) \cdot L \cdot \text{KOW}_\text{all}(4) / ACD2 \ldots
+\text{AND}_\text{elec}(4, 5) / \text{AND}_\text{elec}(4, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(4, 5) / \text{AND}_\text{elec}(4, 2) \cdot L \cdot \text{KOW}_\text{all}(5) / ACD1 \ldots
+\text{AND}_\text{elec}(4, 6) / \text{AND}_\text{elec}(4, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(4, 6) / \text{AND}_\text{elec}(4, 2) \cdot L \cdot \text{KOW}_\text{all}(6) / ACD1 \ldots
+\text{AND}_\text{elec}(4, 7) / \text{AND}_\text{elec}(4, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(4, 7) / \text{AND}_\text{elec}(4, 2) \cdot L \cdot \text{KOW}_\text{all}(7) / ACD2 \ldots
+\text{AND}_\text{elec}(4, 8) / \text{AND}_\text{elec}(4, 2) \cdot W / ACD2 + \text{AND}_\text{elec}(4, 8) / \text{AND}_\text{elec}(4, 2) \cdot L \cdot \text{KOW}_\text{all}(8) / ACD2 \ldots
\}

FD1M = \text{AND}_\text{elec}(4, 3) / \text{AND}_\text{elec}(4, 2) \cdot \text{FNM};
FD2M = \text{AND}_\text{elec}(4, 4) / \text{AND}_\text{elec}(4, 2) \cdot \text{FNM};
FD3M = \text{AND}_\text{elec}(4, 5) / \text{AND}_\text{elec}(4, 2) \cdot \text{FNM};
FD4M = \text{AND}_\text{elec}(4, 6) / \text{AND}_\text{elec}(4, 2) \cdot \text{FNM};
FD5M = aND_elec(4,7)/aND_elec(4,2)*FNM;
FD6M = aND_elec(4,8)/aND_elec(4,2)*FNM;

Frac = [FNMED,FD1MED,FD2MED,FD3MED,FD4MED,FD5MED,FD6MED;...
FNC,FD1C,FD2C,FD3C,FD4C,FD5C,FD6C;...
FNL,FD1L,FD2L,FD3L,FD4L,FD5L,FD6L;...
FNM,FD1M,FD2M,FD3M,FD4M,FD5M,FD6M];

% define all other parameters (RAT)
a= [4.6e-10 9.62e-11 4.9e-11 0.4e-6 1.13e-15 8.87e-17 3.22e-17 1 1 1];
% surface area: cell, lys, mit; volumes: media, cell, lys, mit; number: cell, lys, mit
% human parameters
a= [1.868e-9 1.83e-10 9.24e-11 0.4e-6 2.99e-15 2.33e-16 8.36e-17 1 1 1];
% surface area: cell, lys, mit; volumes: media, cell, lys, mit, number: cell, lys, mit
% using parameters in non-smokers(4/7/14)
a= [1.85e-9 3.37e-10 1.7e-10 0.4e-6 7.46e-15 5.82e-16 2.1e-16 1 1 1];
% surface area: cell, lys, mit; volumes: media, cell, lys, mit, number: cell, lys, mit
% using parameters in smokers(4/7/14)

%% Call solver script and run simulations (output Kp values of cytosol, lysosomes and total cell to medium)
initcond = [1 0 0 0 0]; % condition 1 refers to media concentration; can be change to any value
nstates = length(initcond);
t = 3600; % 3600s == 1h; can be freely changed to any time
% t = 0:15:3600;
Y = Solver_cellmodel_auto(t,a,aND_elec,Frac,initcond,nstates);

Kpc = Y(:,2)./Y(:,1);
maxKpc = max(Kpc);
[maxKpc,index1] = max(Kpc);
pkaKpcmax = (pKa_b1(index1));
Kpl = Y(:,3)./Y(:,1);
maxKpl = max(Kpl);
[maxKpl,index1] = max(Kpl);
pkaKplmax = (pKa_b1(index1));
Kpm = Y(:,4)./Y(:,1);
maxKpm = max(Kpm);
[maxKpm,index1] = max(Kpm);
pkaKpmmax = (pKa_b1(index1));
Kptot=Y(:,5)./Y(:,1);

7.1.1.2. Mass-balance equations

function dR=Cell_model_auto(t,R,a,aND_elec,Frac)

% cell specific parameters
SACELL = a(1);
SAL = a(2);
SAM = a(3);
VMED = a(4); % 0.4.e-6 m3 i.e., 400µL
VCELL = a(5);
VL = a(6);
VM = a(7);
NCELL = a(8); % currently 1 (single cell)
NL = a(9); % surface area and volume of the lysosomes has been
        calculated for a single cell (therefore NL = 1)
NM = a(10); % surface area and volume of the mitochondria has been
        calculated for a single cell (therefore NM = 1)

%% electrostatic interaction
NC1 = aND_elec(8,3);
NC2 = aND_elec(8,4);
NC3 = aND_elec(8,5);
NC4 = aND_elec(8,6);
NC5 = aND_elec(8,7);
NC6 = aND_elec(8,8);
NL1 = aND_elec(9,3);
NL2 = aND_elec(9,4);
NL3 = aND_elec(9,5);
NL4 = aND_elec(9,6);
NL5 = aND_elec(9,7);
NL6 = aND_elec(9,8);
NM1 = aND_elec(10,3);
NM2 = aND_elec(10,4);
NM3 = aND_elec(10,5);
NM4 = aND_elec(10,6);
NM5 = aND_elec(10,7);
NM6 = aND_elec(10,8);

%% drug specific parameters
PN = aND_elec(7,2);
PD1 = aND_elec(7,3);
PD2 = aND_elec(7,4);
PD3 = aND_elec(7,5);
PD4 = aND_elec(7,6);
PD5 = aND_elec(7,7);
PD6 = aND_elec(7,8);

%% ionisation and unspecific binding
FNMED = Frac(1,1);
FD1MED = Frac(1,2);
FD2MED = Frac(1,3);
FD3MED = Frac(1,4);
FD4MED = Frac(1,5);
FD5MED = Frac(1,6);
FD6MED = Frac(1,7);
FNC = Frac(2,1);
FD1C = Frac(2,2);
FD2C = Frac(2,3);
FD3C = Frac(2,4);
FD4C = Frac(2,5);
FD5C = Frac(2,6);
FD6C = Frac(2,7);
FNL = Frac(3,1);
FD1L = Frac(3,2);
FD2L = Frac(3,3);
FD3L = Frac(3,4);
FD4L = Frac(3,5);
FD5L = Frac(3,6);
FD6L = Frac(3,7);
FNM = Frac(4,1);
FD1M = Frac(4,2);
FD2M = Frac(4,3);
FD3M = Frac(4,4);
FD4M = Frac(4,5);  
FD5M = Frac(4,6);  
FD6M = Frac(4,7);  

dR=zeros(5,1);  


%% Set of differential equations solved numerically  
% rate equations have units of [kg/m3/s = g/L/s]; any unit for amount  
can be input (no modification required)  
dR(1) = (  
- (R(1)*FNMED*PN*SACELL*NCELL* (NC1/(exp(NC1)-1))) ... % from  
with three positive charges  
- (R(1)*FD2MED*PD2*SACELL*NCELL* (NC2/(exp(NC2)-1))) ... % from  
with two positive charges  
- (R(1)*FD3MED*PD3*SACELL*NCELL* (NC3/(exp(NC3)-1))) ... % from  
with one positive charge  
- (R(1)*FD4MED*PD4*SACELL*NCELL* (NC4/(exp(NC4)-1))) ... % from  
with one positive charge  
- (R(1)*FD5MED*PD5*SACELL*NCELL* (NC5/(exp(NC5)-1))) ... % from  
with two positive charges  
- (R(1)*FD6MED*PD6*SACELL*NCELL* (NC6/(exp(NC6)-1))) ... % from  
with three positive charges  
+ (R(2)*FNC*PN*SACELL*NCELL* ... % as above  
+ (R(2)*FD1C*exp(NC1)*PD1*SACELL*NCELL* (NC1/(exp(NC1)-1))) ... % from  
as above  
+ (R(2)*FD2C*exp(NC2)*PD2*SACELL*NCELL* (NC2/(exp(NC2)-1))) ... % as above  
as above  
+ (R(2)*FD3C*exp(NC3)*PD3*SACELL*NCELL* (NC3/(exp(NC3)-1))) ... % as above  
as above  
+ (R(2)*FD4C*exp(NC4)*PD4*SACELL*NCELL* (NC4/(exp(NC4)-1))) ... % as above  
as above  
+ (R(2)*FD5C*exp(NC5)*PD5*SACELL*NCELL* (NC5/(exp(NC5)-1))) ... % as above  
as above  
+ (R(2)*FD6C*exp(NC6)*PD6*SACELL*NCELL* (NC6/(exp(NC6)-1))) ... % as above  
as above  
) / (VMED); % media concentration  
dR(2) = (  
+ (R(1)*FNMED*PN*SACELL*NCELL* ... % as above  
+ (R(1)*FD1MED*PD1*SACELL*NCELL* (NC1/(exp(NC1)-1))) ... % as above  
+ (R(1)*FD2MED*PD2*SACELL*NCELL* (NC2/(exp(NC2)-1))) ... % as above  
+ (R(1)*FD3MED*PD3*SACELL*NCELL* (NC3/(exp(NC3)-1))) ... % as above  
+ (R(1)*FD4MED*PD4*SACELL*NCELL* (NC4/(exp(NC4)-1))) ... % as above  
+ (R(1)*FD5MED*PD5*SACELL*NCELL* (NC5/(exp(NC5)-1))) ... % as above  
+ (R(1)*FD6MED*PD6*SACELL*NCELL* (NC6/(exp(NC6)-1))) ... % as above  
- (R(2)*FNC*PN*SACELL*NCELL* ... % as above  
- (R(2)*FD1C*exp(NC1)*PD1*SACELL*NCELL* (NC1/(exp(NC1)-1))) ... % from  
as above  
- (R(2)*FD2C*exp(NC2)*PD2*SACELL*NCELL* (NC2/(exp(NC2)-1))) ... % as above  
as above  
- (R(2)*FD3C*exp(NC3)*PD3*SACELL*NCELL* (NC3/(exp(NC3)-1))) ... % as above  
as above  
- (R(2)*FD4C*exp(NC4)*PD4*SACELL*NCELL* (NC4/(exp(NC4)-1))) ... % as above  
as above  
- (R(2)*FD5C*exp(NC5)*PD5*SACELL*NCELL* (NC5/(exp(NC5)-1))) ... % as above  
as above  
- (R(2)*FD6C*exp(NC6)*PD6*SACELL*NCELL* (NC6/(exp(NC6)-1))) ... % as above  
as above  
% exchange media-cytosol  
- (R(2)*FNC*PN*SAL*NCELL*NL* ... % as above  
- (R(2)*FD1C*PD1*SAL*NCELL*NL* (NL1/(exp(NL1)-1))) ... % as above
\[ \begin{align*}
-R(2) & \cdot FD2C \cdot PD2 \cdot SAL \cdot NCELL \cdot NL \cdot (NL2/(\exp(NL2) - 1)) \quad \% \text{as above} \\
-R(2) & \cdot FD3C \cdot PD3 \cdot SAL \cdot NCELL \cdot NL \cdot (NL3/(\exp(NL3) - 1)) \quad \% \text{as above} \\
-R(2) & \cdot FD4C \cdot PD4 \cdot SAL \cdot NCELL \cdot NL \cdot (NL4/(\exp(NL4) - 1)) \quad \% \text{as above} \\
-R(2) & \cdot FD5C \cdot PD5 \cdot SAL \cdot NCELL \cdot NL \cdot (NL5/(\exp(NL5) - 1)) \quad \% \text{as above} \\
-R(2) & \cdot FD6C \cdot PD6 \cdot SAL \cdot NCELL \cdot NL \cdot (NL6/(\exp(NL6) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FNLC \cdot PD2 \cdot SAL \cdot NCELL \cdot NL \quad \% \text{as above} \\
+R(3) & \cdot FD1L \cdot (NL1) \cdot PD1 \cdot SAL \cdot NCELL \cdot NL \cdot (NL1/(\exp(NL1) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD2L \cdot (NL2) \cdot PD2 \cdot SAL \cdot NCELL \cdot NL \cdot (NL2/(\exp(NL2) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD3L \cdot (NL3) \cdot PD3 \cdot SAL \cdot NCELL \cdot NL \cdot (NL3/(\exp(NL3) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD4L \cdot (NL4) \cdot PD4 \cdot SAL \cdot NCELL \cdot NL \cdot (NL4/(\exp(NL4) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD5L \cdot (NL5) \cdot PD5 \cdot SAL \cdot NCELL \cdot NL \cdot (NL5/(\exp(NL5) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD6L \cdot (NL6) \cdot PD6 \cdot SAL \cdot NCELL \cdot NL \cdot (NL6/(\exp(NL6) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FNC \cdot PD1 \cdot SAM \cdot NCELL \cdot NM \quad \% \text{as above} \\
+R(3) & \cdot FD1C \cdot PD1 \cdot SAM \cdot NCELL \cdot NM \cdot (NM1/(\exp(NM1) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD2C \cdot PD2 \cdot SAM \cdot NCELL \cdot NM \cdot (NM2/(\exp(NM2) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD3C \cdot PD3 \cdot SAM \cdot NCELL \cdot NM \cdot (NM3/(\exp(NM3) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD4C \cdot PD4 \cdot SAM \cdot NCELL \cdot NM \cdot (NM4/(\exp(NM4) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD5C \cdot PD5 \cdot SAM \cdot NCELL \cdot NM \cdot (NM5/(\exp(NM5) - 1)) \quad \% \text{as above} \\
+R(3) & \cdot FD6C \cdot PD6 \cdot SAM \cdot NCELL \cdot NM \cdot (NM6/(\exp(NM6) - 1)) \quad \% \text{as above} \\
+R(4) & \cdot FNLM \cdot PD1 \cdot SAM \cdot NCELL \cdot NM \quad \% \text{as above} \\
+R(4) & \cdot FD1M \cdot (NM1) \cdot PD1 \cdot SAM \cdot NCELL \cdot NM \cdot (NM1/(\exp(NM1) - 1)) \quad \% \text{as above} \\
+R(4) & \cdot FD2M \cdot (NM2) \cdot PD2 \cdot SAM \cdot NCELL \cdot NM \cdot (NM2/(\exp(NM2) - 1)) \quad \% \text{as above} \\
+R(4) & \cdot FD3M \cdot (NM3) \cdot PD3 \cdot SAM \cdot NCELL \cdot NM \cdot (NM3/(\exp(NM3) - 1)) \quad \% \text{as above} \\
+R(4) & \cdot FD4M \cdot (NM4) \cdot PD4 \cdot SAM \cdot NCELL \cdot NM \cdot (NM4/(\exp(NM4) - 1)) \quad \% \text{as above} \\
+R(4) & \cdot FD5M \cdot (NM5) \cdot PD5 \cdot SAM \cdot NCELL \cdot NM \cdot (NM5/(\exp(NM5) - 1)) \quad \% \text{as above} \\
+R(4) & \cdot FD6M \cdot (NM6) \cdot PD6 \cdot SAM \cdot NCELL \cdot NM \cdot (NM6/(\exp(NM6) - 1)) \quad \% \text{as above} \\
\end{align*} \]

\( \begin{align*}
\text{dR}(3) &= \frac{R(2) \cdot FNC \cdot PN \cdot SAL \cdot NCELL \cdot NL \cdot (NL/(VL \cdot NL \cdot NCELL))}{(VL \cdot NL \cdot NCELL)}; \quad \% \text{cytosolic concentration} \\
\text{dR}(4) &= \frac{R(2) \cdot FNC \cdot PN \cdot SAM \cdot NCELL \cdot NM \cdot (NM/(VL \cdot NM \cdot NCELL))}{(VL \cdot NM \cdot NCELL)}; \quad \% \text{lysosome concentration} \\
\end{align*} \)
\[ +R(2) \cdot FD2C \cdot PD2 \cdot SAM \cdot NCELL \cdot NM \cdot (NM2 / (\exp(NM2) - 1)) \ldots \% \text{ as above} \]
\[ +R(2) \cdot FD3C \cdot PD3 \cdot SAM \cdot NCELL \cdot NM \cdot (NM3 / (\exp(NM3) - 1)) \ldots \% \text{ as above} \]
\[ +R(2) \cdot FD4C \cdot PD4 \cdot SAM \cdot NCELL \cdot NM \cdot (NM4 / (\exp(NM4) - 1)) \ldots \% \text{ as above} \]
\[ +R(2) \cdot FD5C \cdot PD5 \cdot SAM \cdot NCELL \cdot NM \cdot (NM5 / (\exp(NM5) - 1)) \ldots \% \text{ as above} \]
\[ +R(2) \cdot FD6C \cdot PD6 \cdot SAM \cdot NCELL \cdot NM \cdot (NM6 / (\exp(NM6) - 1)) \ldots \% \text{ as above} \]
\[ -R(4) \cdot FN1 \cdot PN \cdot SAM \cdot NCELL \cdot NM \ldots \% \text{ as above} \]
\[ -R(4) \cdot FD1M \cdot \exp(NM1) \cdot PD1 \cdot SAM \cdot NCELL \cdot NM \cdot (NM1 / (\exp(NM1) - 1)) \ldots \% \text{ as above} \]
\[ -R(4) \cdot FD2M \cdot \exp(NM2) \cdot PD2 \cdot SAM \cdot NCELL \cdot NM \cdot (NM2 / (\exp(NM2) - 1)) \ldots \% \text{ as above} \]
\[ -R(4) \cdot FD3M \cdot \exp(NM3) \cdot PD3 \cdot SAM \cdot NCELL \cdot NM \cdot (NM3 / (\exp(NM3) - 1)) \ldots \% \text{ as above} \]
\[ -R(4) \cdot FD4M \cdot \exp(NM4) \cdot PD4 \cdot SAM \cdot NCELL \cdot NM \cdot (NM4 / (\exp(NM4) - 1)) \ldots \% \text{ as above} \]
\[ -R(4) \cdot FD5M \cdot \exp(NM5) \cdot PD5 \cdot SAM \cdot NCELL \cdot NM \cdot (NM5 / (\exp(NM5) - 1)) \ldots \% \text{ as above} \]
\[ -R(4) \cdot FD6M \cdot \exp(NM6) \cdot PD6 \cdot SAM \cdot NCELL \cdot NM \cdot (NM6 / (\exp(NM6) - 1)) \ldots \% \text{ cell mitochondria concentration} \]
\[ dR(5) = (dR(2) \cdot (VCELL - VL \cdot NL - VM \cdot NM) \cdot NCELL + dR(3) \cdot VL \cdot NL \cdot NCELL + dR(4) \cdot VM \cdot NM \cdot NCELL) / (VCELL \cdot NCELL) \; \% \text{ total cell concentration} \]
Appendix Table 7-8: Literature reported octanol-water partition coefficients of neutral and ionised forms of 4 drugs and their calculated lipophilicity difference ($\Delta \text{LogP}$).

<table>
<thead>
<tr>
<th>Drug</th>
<th>LogP$^N$</th>
<th>LogP$^I+$</th>
<th>$\Delta \text{LogP}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>4.39</td>
<td>0.47</td>
<td>3.90</td>
<td>Avdeef, 2012</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>3.24</td>
<td>-0.37</td>
<td>3.61</td>
<td>Goldman et al., 1994</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>-0.08</td>
<td>-1.97</td>
<td>1.89</td>
<td>Avdeef, 2012</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-1.08</td>
<td>-1.69</td>
<td>0.61</td>
<td>Avdeef, 2012</td>
</tr>
</tbody>
</table>

N, I, + subscripts represent neutral, ionised and cation, respectively.

Appendix Table 7-9: Physicochemical properties of 5 drugs predicted by the ADMET Predictor$^\text{TM}$ v7.0 software to be used in in silico cell model.

<table>
<thead>
<tr>
<th>Drug</th>
<th>LogP</th>
<th>pK$_{a,a}$</th>
<th>pK$_{a,b}$</th>
<th>Acid-Base Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>2.26</td>
<td>-</td>
<td>8.70</td>
<td>Base</td>
</tr>
<tr>
<td>Imipramine</td>
<td>4.87</td>
<td>-</td>
<td>8.96</td>
<td>Base</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>0.12</td>
<td>8.80, 9.76</td>
<td>10.7</td>
<td>Base</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2.54</td>
<td>6.3, 8.01</td>
<td>10.7</td>
<td>Zwitterion</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-0.81</td>
<td>5.78</td>
<td>8.90</td>
<td>Zwitterion</td>
</tr>
</tbody>
</table>

pK$_{a,a}$: acidic pK$_a$; pK$_{a,b}$: basic pK$_a$
Appendix Figure 7-7: Impact of changing the penalty applied to lipophilicity of ionised drug species in predicted accumulation of imipramine in cytosol, lysosomes, mitochondria and the cell. The lipophilicity penalty impacted on both membrane partitioning which showed reduction with increasing penalty and on membrane permeability which led to increased trapping in the lysosomal compartment. Applied penalty values in log-unit were: -2 ( ); -2.5 ( ); -3 ( ); -3.5 ( ); -4 ( ); -4.5 ( ).
Appendix Figure 7-8: *In silico* cell model predicted $K_p$ values (expressed relative to medium) at acidic lysosomal pH and comparison of predicted $K_{p,\text{cell}}$ with observed $K_{p,\text{NR8383}}$ for all drugs. Predictions were performed with the revised model in terms of the lipophilicity penalty for drugs indicated (*). Observed $K_{p,\text{NR8383}}$ (•); Predicted $K_{p,\text{cell}}$ (■); $K_{p,\text{mitochondria}}$ (□); $K_{p,\text{lysosome}}$ (▲) and $K_{p,\text{cytosol}}$ (□). Observed $K_{p,\text{NR8383}}$ data are from in vitro lysosomal trapping experiments and represent mean ± SD of at least 3 experiments.
**Appendix Table 7-10:** *In silico* cell model predicted cell-to-medium concentration ratio (K<sub>p</sub>) of imipramine and clarithromycin in subcellular compartments of non-smoker and smoker human AMs and comparison with experimental K<sub>p</sub> data (K<sub>p,hAM</sub>). Experimental data represent mean ± SD of at least 3 separate experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Predicted K&lt;sub&gt;p&lt;/sub&gt; (Non-smoker)</th>
<th>Predicted K&lt;sub&gt;p&lt;/sub&gt; (Smoker)</th>
<th>Observed K&lt;sub&gt;p,hAM&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Lysosome</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Imipramine</td>
<td>24.1</td>
<td>80.0</td>
<td>26.8</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>3.69</td>
<td>83.6</td>
<td>2.83</td>
</tr>
</tbody>
</table>
Appendix Figure 7-9: The impact of increasing lipid content of AMs from 5 to 28% of the cell volume in the in silico cell model on predicted $K_p$ of imipramine and clarithromycin. W and L represent water and lipid fractions, respectively. (■) cytosol; (□) lysosome; (■■) mitochondria; (■■■) total cell.