**In Vitro Assessment of Uptake and Lysosomal Sequestration of Respiratory Drugs in Alveolar Macrophage Cell Line NR8383**

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**ABSTRACT**

**Purpose** To assess accumulation and lysosomal sequestration of 9 drugs used in respiratory indications (plus imipramine as positive control) in the alveolar macrophage (AM) cell line NR8383.

**Methods** For all drugs, uptake at 5 μM was investigated at 37 and 4°C to delineate active uptake and passive diffusion processes. Accumulation of basic clarithromycin, formoterol and imipramine was also assessed over 0.1–100 μM concentration range. Lysosomal sequestration was investigated using ammonium chloride (NH₄Cl), monensin and nigericin. Impact of lysosomal sequestration on clarithromycin accumulation kinetics was investigated.

**Results** Both cell-to-medium concentration ratio (Kᵢ) and uptake clearance (CLuptake) ranged >400-fold for the drugs investigated. The greatest Kᵢ was observed for imipramine (391) and clarithromycin (82), in contrast to no accumulation seen for terbutaline. A concentration-dependent accumulation was evident for the basic drugs investigated. Imipramine and clarithromycin Kᵢ and CLuptake were reduced by 59–85% in the presence of NH₄Cl and monensin/nigericin, indicating lysosomal accumulation, whereas lysosomal sequestration was not pronounced for the other 8 respiratory drugs. Clarithromycin uptake rate was altered by NH₄Cl, highlighting the impact of subcellular distribution on accumulation kinetics.

**Conclusions** This study provides novel evidence of the utility of NR8383 for investigating accumulation and lysosomal sequestration of respiratory drugs in AMs.

**KEY WORDS** alveolar macrophages • lysosomal sequestration • NR8383 • respiratory drugs

**ABBREVIATIONS**

- ABT 1-aminobenzotriazole
- AM(s) Alveolar macrophage(s)
- BSA Bovine serum albumin
- CAD Cationic amphiphilic drug
- CGM Complete growth medium
- CLdiff Passive diffusion clearance
- CLuptake Total uptake clearance
- DPBS Dulbecco’s phosphate buffered saline
- Kᵢ Cell-to-medium concentration ratio
- LC-MS/MS Liquid chromatography with tandem mass spectrometry
- LTR LysoTracker Red
- NH₄Cl Ammonium chloride

**INTRODUCTION**

Understanding drug disposition in the lungs remains a challenging task in the development of new medications for asthma, chronic obstructive pulmonary disease and bacterial infections (1). Several factors may affect the therapeutic efficacy of inhaled drugs including delivery devices, formulation and dose, drug physicochemical properties, metabolism and lung clearance mechanisms and disease state (2–4). Phagocytic alveolar macrophages (AMs) represent important components...
of clearance mechanism of drugs from the respiratory airways. While accumulation of certain drugs in AMs is required for their therapeutic effect (e.g., macrolide antibiotics), this process could affect the efficacy and duration of drugs targeting submucosal compartments such as β₂-agonists and antimuscarinics. In addition, pronounced accumulation of basic drugs (often cation amphiphilic drugs, i.e., CADs) in lysosomes of AMs may lead to drug induced phospholipidosis and the presence of foamy macrophages in the airways [5]. Lysosomal sequestration of CADs has been attributed to the pH differences between cytosol (pH=7.2) and lysosomes, as the most acidic cell organelles (pH=4.7–4.8) [6]. The unionised form of basic drugs can rapidly diffuse into lysosomes where they become predominantly ionised and ‘trapped’ as a result of the limited permeability of lysosomal membrane to ionised drugs. Lysosomal sequestration may lead to significantly higher intracellular drug concentrations which may have important therapeutic or toxicological consequences [7,8]. In addition, drug–drug interactions involving lysosomotropic drugs have been proposed as an important implication of lysosomal drug sequestration [8,9]. In vitro methods employed to investigate lysosomal sequestration include both direct approaches such as measuring drug concentrations in isolated lysosomes following homogenisation [10–12] and indirect methods based on the use of chemical agents to abolish lysosome–cytosol pH gradient (e.g., ammonium chloride (NH₄Cl), monensin and nigericin) [12,13]. Reduction in drug accumulation in the presence of these indirect agents relative to control provides the extent of lysosomal accumulation (reference studies provided in the Supplementary Material, Table S1). In addition, assays involving the use of lysosome specific fluorescent dye, LysoTracker Red (LTR), have also been reported for semiquantitative investigation of lysosomal drug sequestration [14–16].

NR8383 is an AM cell line originally derived by bronchoalveolar lavage procedure of an adult male Sprague-Dawley rat; this cell line was shown to exhibit several characteristics and functions of freshly isolated rat AMs [17,18]. Previous uptake studies in NR8383 focused on a single drug per study and mainly on antibiotics used for respiratory infections [19,20]. To date, there are no studies investigating the accumulation in AMs of a substantial number of respiratory drugs targeting other disease areas including asthma and chronic obstructive pulmonary disease.

The aim of this study was to systematically assess the extent of intracellular accumulation in AMs and the contribution of lysosomal sequestration for nine selected drugs. All drugs in the dataset are designed or used in respiratory indications, cover a wide range of physicochemical properties and have limited in vitro accumulation data reported in AMs. The selected drugs and their respective physicochemical properties are summarised in Table I, together with the location of the drug target (intra- or extracellular). The corresponding chemical structures are shown in the Supplementary Material Figure S1. Drug uptake into AMs was investigated at a single substrate concentration at 37°C and 4°C to determine the dependence of cellular accumulation on active uptake and passive diffusion processes, respectively. In addition, the accumulation of a number of selected drugs in AMs was investigated over a range of substrate concentrations. In order to assess its contribution to uptake into AMs, lysosomal sequestration of the drugs was investigated in the presence of agents that abolish lysosome–cytosol pH gradient; lysosomotropic drug imipramine was included as a positive control in the studies. Cell-to-medium concentration ratio (Kᵥₑ) was determined in the presence and absence of these agents to evaluate the extent of lysosomal accumulation. The in vitro data were supported by staining of the cells with LTR under control condi-
conditions and in the presence of a number of basic drugs and the above mentioned chemical agents. Recommendations of the application of NR8383 for the assessment of accumulation and lysosomal sequestration of respiratory drugs are provided.

MATERIALS AND METHODS

Chemicals and Reagents

1-Aminobenzotriazole, ammonium chloride, ciprofoxacin, clarithromycin, dimethyl sulphoxide, Imipramine, lactate dehydrogenase activity assay kit, monensin sodium salt, nigericin, 1-Aminobenzotriazole, ammonium chloride, ciprofoxacin, clarithromycin, dimethyl sulphoxide, Imipramine, lactate dehydrogenase activity assay kit, monensin sodium salt, nigericin, and lysosomal sequestration of respiratory drugs are provided.

MATERIALS AND METHODS

Drug Uptake in NR8383

The cellular uptake method was adapted from Ménachet et al. (2012) (31) and optimised for NR8383, as outlined herein. The studies were carried out in a 24-well plate format and the in vitro conditions were optimised in terms of cell detachment method from culture flasks, coating type of 24-well plates, seeding density, culturing time and drug incubation time points. Briefly, for cell detachment, gentle scraping versus Mg²⁺-Ca²⁺ free DPBS treatment of cells in culture flasks were tested. Considering the semi-adherent nature of AMs, cell seeding into non-coated, collagen-I and poly-D-lysine coated 24-well plates was investigated for cell attachment, distribution in the wells and detachment following DPBS washes during the uptake experiments. In addition, seeding densities of 0.36 and 0.72×10⁶ cells per well were assessed with respect to cell attachment, confluency following culture (plating efficiency) and potential cell detachment following DPBS washes. Culturing time ranging from 4 to 24 h was investigated for optimal cell attachment before carrying out uptake experiments. Uptake rate and Kᵢₐ data were normalised by cell number determined indirectly by measuring the amount of protein in samples by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Loughborough, UK). Following the optimisation, detachment of NR8383 from cell culture flasks was performed by gentle scraping prior to cell counting and seeding into collagen-I coated 24-well plates at 0.5×10⁶ cells per well. Cells were cultured for 16 h at 37°C, 5% CO₂ (CO₂ incubator, MCO-17AIC, Sanyo Biomedical, Loughborough, UK) before uptake studies performed over 1 to 10 min period. The details of method optimisation outlined above can be found in the Supplementary Material, Figure S2.

Initially, uptake experiments in NR8383 were carried out for all drugs at single substrate concentration of 5 μM at 37 and 4°C in order to delineate contribution of active uptake and passive diffusion processes. The drug uptake in NR8383 corresponding to these conditions was described by total uptake (CLuptake) and passive diffusion (CLdiff) clearance terms, respectively. For a subset of selected drugs, accumulation was assessed over a range of drug concentrations (0.1–100 μM for imipramine, 1–100 μM for formoterol and clarithromycin) at 37°C. In addition, clarithromycin uptake kinetics was evaluated over this concentration range. Substrate solutions were prepared by dilution of the stock solutions in DPBS resulting in...
in final DMSO content <1%. Uptake studies were performed in the presence of 1 mM ABT, a nonspecific P450 inhibitor, in order to avoid any potential impact of phase I metabolism. Currently there is no evidence of the presence of any conjugation enzymes in this cell line. Following microscopy, 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 min. 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) in each well. Each substrate was incubated with the cells for 1, 2, 5 and 10 min and each time point at 37°C and 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 stopped by removal of the media with drug solution and washing the monolayers three times with 800 μL ice-cold DPBS. The back-diffusion of the drug from the cell into the medium was considered to be negligible under these conditions. The removed incubation media were retained for the measurement of drug medium concentrations and subsequent determination of $K_p$ for each drug. The cells were lysed by addition of 200 μL ice-cold deionised water; cell lysates were kept at −20°C overnight and then processed further for LC-MS/MS analysis. Experiments were performed on three or more separate occasions for each drug investigated. Clarithromycin uptake at 5 μM was used as a control to assess the performance of individual experiment. The mean uptake of the 5 μM clarihromycin across a large number of experiments performed (n=59) was 5.50±2.96 μL/min/10^6 cells. The amount of protein per well was measured using a BSA calibration curve and absorbances read at 562 nm (S22 Boeco UV-Visible Spectrophotometer, Hamburg, Germany). Determination of cell number from measured protein was reproducible across experiments (1×10^6 cells =0.25±0.06 mg protein, n=66).

Assessment of Lysosomal Sequestration in NR8383 Cells with LysoTracker Red

Qualitative assessment of the localisation of lysosomes in NR8383 and lysosomal targeting of drugs investigated was made using LTR (Life Technologies, Paisley, UK). The protocol used for staining of the cells with LTR was adapted from a previous study (32). 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, BD Biosciences, Oxford, UK). NR8383 cells (250,000 cells/well) were plated in the slides and cultured for 16 h 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. LysoTracker Red was added into the solutions of the chemical agents and the drugs to achieve a final concentration of 200 nM as used previously (14). 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 reaching 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 min 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 (control) or LTR with each of the chemical agents or selected drugs for 1 h 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 5 min at room temperature. Three subsequent washes with ice-cold DPBS were performed before visualisation of LTR with a confocal laser scanning microscope (Zeiss LSM 510, Jena, Germany) using a C-Apochromat 40×/1.2 NA (Numerical Aperture) water-immersion objective and helium/neon laser ($\lambda_{\text{excitation}}$ 543 nm, $\lambda_{\text{emission}}$ 560 nm). Images were collected and processed using Combi LSM-FCS v.3.2 and LSM Image Browser v4.2 software (Jena, Germany). Furthermore, Image J v1.47 software was used for the quantification of the fluorescence intensity of LTR in 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) (33). The CTCF of treated cells was compared to 100% control (LTR only).

Lysosomal Sequestration Studies in NR8383

The extent of accumulation of drugs in lysosomes was assessed using the same uptake methodology, as described above in the absence and presence of NH₄Cl and ionophores, monensin and nigericin. Preliminary investigation of imipramine and clarithromycin uptake was performed across a range of NH₄Cl concentrations (10–50 mM), based on previous studies.
in the literature (12–14,34). In addition, a range of incubation conditions was explored, including pre-incubation and co-incubation alone and combined together. Once the conditions were optimised, further experiments were performed with 20 mM NH₄Cl co-incubated with investigated drugs. The details of this optimisation work can be found in the Supplementary Material, Table S2 and Figure S3. Following the initial assessment with NH₄Cl, confirmation of lysosomal sequestration for a number of selected drugs was further carried out by co-incubation of 5 μM monensin or 10 μM nigericin with the selected drugs. The concentrations of both ionophores used in this study were optimised based on previous reports (13,14,34) and in house solubility observations. Stock solutions of NH₄Cl, monensin and nigericin were prepared as described above, followed by cell lysis in deionised water. All samples were included in the analysis; namely, the incubation media consisting of DPBS alone (negative control), drug alone (e.g., clarithromycin), chemical agents alone (e.g., NH₄Cl), and chemical agent and drug combination (e.g., NH₄Cl+ clarithromycin). For the assessment of the individual chemical agents, same concentrations were used as in the lysosomal sequestration studies. The cytotoxicity in above samples was measured on three separate occasions. 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).

Sample Preparation and LC-MS/MS Analysis

All cell lysates and media samples 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 lysate and media samples, equal volume of methanol/ internal standard mixture (200 μL) was used. Media samples with substrate concentrations of 10 μM and above 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 LC-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 min (Eppendorf Centrifuge 5804, Cambridge, UK). Ten or 20 μL of supernatant 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, 50×4.6 mm) (Phenomenex, Macclesfield, UK), except for fenoterol, terbutaline, ipratropium and tiotropium bromide which were separated on Luna Phenyl Hexyl column (3 μm, 50×4.6 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 the 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/h, respectively. LC-MS/MS conditions for each individual drug and their corresponding internal standards are detailed in Supplementary Material, Table S3. 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 and 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.

**Determination of Uptake Clearance Parameters**

The CL_{uptake} and CL_{diff} were estimated at a single substrate concentration for each drug from the 37 and 4°C data, respectively. Uptake rate was determined from the slope of the linear regression of the cell concentration vs. time plot. Total uptake clearance was then estimated from the ratio of the uptake rate against the substrate concentration. Both CL_{uptake} and CL_{diff} were determined for each drug on a minimum three separate occasions. Uptake clearance attributed to the active process(es) was determined from the difference between CL_{uptake} and CL_{diff} for each drug (36).

**Determination of Drug Accumulation**

Accumulation of drugs in NR8383 was determined by calculating K_p of all drugs at 5 μM at 10 min. K_p was determined by Eq. 1 as reported previously (36,37);

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

(1)

where \(C_{\text{cell}}\) and \(C_{\text{medium}}\) represent concentration of the drug in the cell and medium, respectively. Both represent the measurements made at the final incubation time point (10 min). The K_p represents partition of the drug 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/10^6 cells, CV = 24%) and number of cells measured per well. Cell volume was calculated by multiplying the reported NR8383 volume of 4.2 μL/mg protein (38) with the amount of protein measured experimentally (0.25 ± 0.06 mg/10^6 cells). While K_p was determined at a single substrate concentration for all the drugs investigated, the accumulation of 3 basic drugs including imipramine, clarithromycin and formoterol was also characterised over a concentration range at the same incubation time. The concentration dependence of drug accumulation was investigated by fitting the two-site binding model to the K_p and concentration data in GraFit v6.0.6 software (Erithacus Software Limited, Horley, UK).

The model incorporates a saturable site and a linear function for unsaturated binding as shown in Eq. 2;

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

(2)

where \(K_{p,U1,max}\) is maximum K_p for saturable uptake, \(K_{p,min}\) is K_p for nonsaturable uptake and \(K_{U1}\) is apparent saturable uptake equilibrium constant. K_p for maximum total uptake was calculated as the sum of \(K_{p,U1,max}\) and \(K_{p,min}\), as reported previously (39).

**Determination of Uptake Kinetic Parameters of Clarithromycin**

The estimation of uptake kinetic parameters of clarithromycin was performed using the two-step method (31,36) for analysis in GraFit v6.0.6 (Erithacus Software Limited, Horley, UK). CL_{diff} 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 Eq. 3. When estimating the uptake kinetic parameters in the presence of NH4Cl, CL_{diff} estimated at single substrate concentration was used a constant in the equation. Subsequently, kinetic parameters Km and V_max were obtained by nonlinear regression.

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

(3)

where v is the uptake rate, \(K_m\) is the binding affinity constant and \(V_{\text{max}}\) is the maximum uptake rate associated with an active process mediating uptake of clarithromycin into AM. S_{med} (μM) is the nominal media concentration corrected for fraction unbound in the media (f_{unmed} = 0.93 ± 0.03). The latter was determined from the slope of the linear regression of the unbound concentration extrapolated at time 0 vs. the nominal incubation concentration plot.

**Determination of Lysosomal Sequestration**

K_p and CL_{uptake} were determined as described above for each drug both under control condition and in the presence of a chemical agent affecting the pH gradient between cytosol and lysosomes. The reduction in K_p and CL_{uptake} of each drug in the presence of the chemical agents was expressed as a percentage relative to control and used as an indicator of the extent of lysosomal sequestration. A 50% reduction in both parameters was set as a cut-off for lysosomal accumulation, while also taking into account the physicochemical properties of the compounds (Table 1) and the uncertainty associated with the experimental data.

The in vitro parameters were calculated on at least three separate occasions and the mean data were reported. When assessing the extent of lysosomal sequestration, the control and
chemical agent treated cells were compared using the two-tailed, paired t-test in order to determine statistically significant difference between the two conditions. Values were reported as significant when \( p < 0.05 \).

**RESULTS**

**Drug Accumulation in NR8383 Cell Line**

Accumulation of 10 drugs was assessed in NR8383 at a single substrate concentration (5 \( \mu \text{M} \)) at 37°C. Measured cell and media concentrations of all drugs were used to determine their \( K_p \), as shown in Table II. More than 500-fold range in \( K_p \) was observed in NR8383 for the current dataset. The most extensive accumulation in NR8383 was seen for imipramine and clarithromycin (\( K_p \) of 391 and 82, respectively). Ciprofloxacin, rifampicin, budesonide and formoterol accumulated in NR8383 to a similar extent (\( K_p \) values ranging between 25 and 43). For the remaining drugs, relatively less partitioning into cells was observed, as tiotropium, ipratropium bromide and fenoterol achieved a \( K_p \) between 5 and 9. In the case of terbutaline, the medium concentration of the compound remained higher than its cell concentration throughout the incubation period, resulting in \( K_p \) close to 1.

For a number of basic drugs, accumulation in the NR8383 was assessed further over a range of substrate concentrations. A concentration-dependent accumulation was observed for the drugs investigated, as illustrated in Fig. 1. The two-site binding model was fitted to the experimental data and the estimated uptake parameters \( K_{p,U1,max} \), \( K_{p,min} \) and \( K_{U1} \) are shown in Table III. In contrast to the \( K_p \) value obtained at 5 \( \mu \text{M} \), imipramine maximum \( K_p \) of 1166 was estimated by the modeling approach (corresponds to concentration <0.1 \( \mu \text{M} \)).

The analysis indicated that half of maximal \( K_p \) associated with saturable uptake would be reached at concentrations below 2 \( \mu \text{M} \) for these three drugs (Table III). The fold difference between \( K_{p,min} \) and \( K_{p,max} \) ranged between 3 and 8 for clarithromycin and imipramine, respectively.

Uptake of 10 drugs was also assessed in NR8383 at 5 \( \mu \text{M} \) at 37 and 4°C in order to delineate contribution of active and passive diffusion processes to the overall uptake into AMs (Table II). The trends seen in the intracellular accumulation data were reflected in the uptake clearances, with over 400-fold range in \( C_{\text{U,uptake}} \) in NR8383 observed for the current dataset (0.04–16.7 \( \mu \text{L/min}/10^6 \text{cells} \)). Imipramine showed the highest uptake clearance in NR8383 (16.7 \( \mu \text{L/min}/10^6 \text{cells} \)) with maximal contribution of the passive process to the total uptake of approximately 30%. Negligible \( C_{\text{diff}} \) was observed for clarithromycin, suggesting a large contribution of an active process to its accumulation in NR8383. The same trend was observed for the majority of drugs with the exception of terbutaline and budesonide where contribution of passive diffusion to accumulation in NR8383 was >50% (Table II). Fenoterol, terbutaline, ipratropium and tiotropium bromide were marginally taken up into NR8383 cells with a \( C_{\text{U,uptake}} \) of <1 \( \mu \text{L/min}/10^6 \text{cells} \).

**Confocal Microscopic Examination of NR8383 Cells and Lysosomal Sequestration**

The imaging of NR8383 cells was performed in order to confirm localisation of functional lysosomes in NR8383 and assess the effect of the chemical agents on LTR accumulation in these organelles. In addition, the lysosomal targeting of selected drugs was investigated. A differential contrast image was taken every time prior to excitation of the cells for LTR detection. A representative of this image is shown in Fig. 2a, demonstrating the presence of cytoplasmic vesicular structures likely to represent acidic organelles including lysosomes. The

| Table II | Drug Uptake Clearance and Cell-to-Medium Concentration Ratio (\( K_p \)) of 10 Drugs in NR8383 at 5 \( \mu \text{M} \) Substrate Concentration. Data Represent Mean \( \pm \) SD of at Least 3 Separate Experiments. \( C_{\text{U,uptake}} \) and \( C_{\text{U,diff}} \) are Total Uptake and Passive Diffusion Clearances, Respectively |
|----------|---------------------------------------------------------------|
| Drug     | \( C_{\text{U,uptake}} \) (\( \mu \text{L/min}/10^6 \text{cells} \)) | \( C_{\text{U,diff}} \) (\( \mu \text{L/min}/10^6 \text{cells} \)) | Contribution of passive (%) | \( K_p \) |
| Imipramine | 16.7 \( \pm \) 4.3 | 5.06 \( \pm \) 1.7 | 30.2 | 391 \( \pm \) 108 |
| Clarithromycin | 6.0 \( \pm \) 3.7 | 0.03 \( \pm \) 0.03 | 0.53 | 81.6 \( \pm \) 22.8 |
| Formoterol | 2.0 \( \pm \) 0.7 | 0.08 \( \pm \) 0.08 | 3.71 | 24.5 \( \pm \) 8.71 |
| Budesonide | 1.5 \( \pm \) 0.7 | 1.5 \( \pm \) 0.9 | 97.2 | 37.6 \( \pm \) 11.4 |
| Rifampicin | 1.4 \( \pm \) 0.5 | 0.03 \( \pm \) 0.01 | 1.87 | 38.6 \( \pm \) 18.1 |
| Ciprofloxacin | 1.3 \( \pm \) 0.4 | 0.3 \( \pm \) 0.2 | 22.3 | 43.0 \( \pm \) 21.1 |
| Tiotropium bromide | 0.4 \( \pm \) 0.3 | 0.06 \( \pm \) 0.04 | 16.3 | 8.74 \( \pm \) 7.24 |
| Fenoterol | 0.3 \( \pm \) 0.2 | 0.03 \( \pm \) 0.01 | 8.03 | 5.69 \( \pm \) 0.98 |
| Ipratropium bromide | 0.2 \( \pm \) 0.2 | 0.01 \( \pm \) 0.01 | 5.19 | 5.42 \( \pm \) 3.38 |
| Terbutaline | 0.04 \( \pm \) 0.01 | 0.02 \( \pm \) 0.01 | 47.6 | 0.69 \( \pm \) 0.16 |
corresponding fluorescent image of the same cell is illustrated in Fig. 2b where LTR was localised in lysosomes, confirming the presence of these organelles in NR8383 cells. Treatment of NR8383 with LTR in the presence of NH₄Cl (20 mM), monensin (5 μM) and nigericin (10 μM) showed reduced fluorescent signal up to 85% for all agents. The reduced accumulation of LTR in the presence of these agents (Fig. 2c, e, g) confirmed lysosomal targeting of this basic probe in NR8383.

Assuming the competition of basic drugs for lysosomal sequestration, LTR was co-incubated with 5 μM of clarithromycin, imipramine and formoterol. Clarithromycin and imipramine decreased LTR accumulation by 86 and 72%, respectively (Fig. 2d, f), confirming indirectly the lysosomotropic properties of these two drugs. In contrast, the localisation of LTR was still evident in the presence of formoterol (Fig. 2h) which caused only 25% reduction in LTR accumulation, suggesting its marginal targeting of NR8383 lysosomes.

### Cytotoxicity Assessment

Analysis of all three agents either on their own or when co-incubated with clarithromycin or imipramine showed minimal cytotoxicity (less than 2% compared to 100% positive control). At all times, the viability of the control (i.e., untreated) cells was more than 95%. The measured cytotoxicity was less than 1% for imipramine at both 50 and 100 μM.
concentrations. For formoterol, the cytotoxicity was less than 5% compared to the positive control, whereas in the case of clarithromycin, the cytotoxicity levels were <3% and 10% for 50 μM and 100 μM concentrations, respectively.

Assessment of Lysosomal Sequestration in NR8383

Lysosomal sequestration 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 drug at 37°C for 10 min. The pH of the incubation medium showed a minimal reduction in the presence of NH₄Cl (<0.06 unit) relative to control conditions. The effect of NH₄Cl on the intracellular accumulation of individual drugs is shown in Fig. 3. Of all the drugs investigated, the most pronounced reduction in intracellular accumulation was observed for clarithromycin, as Kₚ obtained at 5 μM decreased by 6-fold in the presence of NH₄Cl relative to control conditions, whereas in the case of imipramine 3.4-fold lower Kₚ was seen (Table IV). The presence of NH₄Cl in the incubation did not significantly alter the intracellular accumulation of the remaining drugs (Table IV and Supplementary Material, Table S4). Even though Kₚ was reduced for some of the drugs (e.g., by 46% for ipratropium bromide), the large uncertainty associated with the data (both control and +NH₄Cl data) resulted in no significant difference between the two conditions. For the majority of drugs, the% reduction in Kₚ in the presence of NH₄Cl was within 20% at both substrate concentrations.

Lysosomal sequestration of imipramine, clarithromycin, formoterol and fenoterol was investigated further by monensin and nigericin; total Kₚ of these drugs in the absence and presence of these agents are shown in Fig. 4. The Kₚ of imipramine was significantly reduced in the presence of both monensin (by 66%) and nigericin (by 75%). The reduction of clarithromycin Kₚ was more pronounced in comparison to imipramine (77 and 82% by monensin and nigericin, respectively), in agreement with the effect of NH₄Cl. In contrast, minor reduction in fenoterol and formoterol Kₚ in the presence of these agents was observed, corroborating findings from the studies with NH₄Cl. The observations from confocal imaging studies confirmed the results outlined above, with regards to both the effectiveness of the chemical agents and the extent of lysosomal accumulation of clarithromycin, imipramine and formoterol (Fig. 2). In addition to Kₚ, CLuptake of each drug was estimated in the presence of these chemical agents; the effect of NH₄Cl, monensin and nigericin on CLuptake of the drugs investigated followed the same trends seen for Kₚ data (details are shown in the Supplementary Material, Table S5, Figures S4 and S5).

Among all drugs, the accumulation of clarithromycin in NR8383 in the presence of NH₄Cl was further characterised over a range of clarithromycin concentrations (1–100 μM). Compared to control conditions, a marked reduction in clarithromycin Kₚ was observed at all drug concentrations (Fig. 1c). Estimated Kₚ, U1,max and Kₚ,min values in the presence of NH₄Cl were 9.7 and 4.7, respectively, resulting in a mean maximum fitted Kₚ of 14.4. Assessment of uptake kinetics of clarithromycin under the control conditions showed

![Fig. 2](image-url) Qualitative assessment of lysosomal sequestration of LysoTracker Red (LTR) and three drugs studied in NR8383 by confocal microscopy: (a) differential interference 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 presence of 20 mM NH₄Cl (c), 5 μM monensin (d), 10 μM nigericin (e), 5 μM clarithromycin (f) and 5 μM imipramine (g), and showed minor changes in presence of 5 μM formoterol (h).
Table IV  Cell-to-Medium Partition Coefficients ($K_p$) Estimated Under Control and NH$_4$Cl Treatment Conditions for 10 Drugs Investigated at 5 μM in NR8383. Percent Reduction in $K_p$ in the Presence of NH$_4$Cl (20 mM), monensin (5 μM) and nigericin (10 μM) is shown as an indicator of the extent of lysosomal sequestration. Data Represent Mean±SD of at Least 3 Experiments

| Drug          | $K_p$ Control | $K_p + NH_4$Cl | NH$_4$Cl | % Reduction in $K_p$ by Monensin | % Reduction in $K_p$ by Nigericin |
|---------------|---------------|----------------|----------|---------------------------------|---------------------------------|
| Imipramine    | 347±59.8      | 102±17.1       | 71±2.6** | 75±11**                         | 66±11**                         |
| Clarithromycin| 82.7±27.3     | 13.3±6.50      | 84±6.6** | 77±8.5**                        | 82±9.6**                        |
| Formoterol    | 20.8±3.6      | 15.5±1.16      | 24±9.0   | 40±7.5                          | 27±5.1                          |
| Fenoterol     | 4.25±2.64     | 2.91±2.11      | 31±23    | 27±25                           | 30±12                           |
| Terbutaline   | 0.92±0.39     | 0.72±0.31      | 21±5.0   | n/a                             | n/a                             |
| Rifampicin    | 27.9±3.54     | 33.2±11.9      | NR       | n/a                             | n/a                             |
| Tiotropium bromide | 4.39±2.90 | 3.87±2.61      | 12±1.8   | n/a                             | n/a                             |
| Ipratropium bromide | 4.94±0.30 | 2.69±1.09      | 46±21    | n/a                             | n/a                             |
| Budesonide    | 31.5±8.46     | 28.1±7.12      | 10±7.8   | n/a                             | n/a                             |
| Ciprofloxacin | 43.0±21.1     | 37.6±20.5      | 15±13    | n/a                             | n/a                             |

n/a not available, NR no reduction in $K_p$. *, p<0.05; **, p<0.01 by t-test.

Fig. 3 $K_p$ ($C_{cell}/C_{medium}$) estimated in absence (a) and presence (b) of 20 mM ammonium chloride (NH$_4$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<0.01 by t-test).

Table IV  Cell-to-Medium Partition Coefficients ($K_p$) Estimated Under Control and NH$_4$Cl Treatment Conditions for 10 Drugs Investigated at 5 μM in NR8383. Percent Reduction in $K_p$ in the Presence of NH$_4$Cl (20 mM), monensin (5 μM) and nigericin (10 μM) is shown as an indicator of the extent of lysosomal sequestration. Data Represent Mean±SD of at Least 3 Experiments
nonsaturating conditions up to 100 μM and therefore, the kinetic parameters could not be determined. In contrast, in the presence of NH₄Cl, the uptake rates of clarithromycin were reduced markedly at all substrate concentrations compared to the control condition (Fig. 1d) and showed a saturable uptake kinetic profile (despite highly variable rates at higher concentrations, CV > 30%). In the presence of NH₄Cl, Kₘ′ and Vₘₐₓ associated with the active uptake of clarithromycin into NR8383 were 28.5 μM and 12.4 pmol/min/10⁶ cells, respectively; the corresponding clearance was 0.43 μL/min/10⁶ cells. Considering estimated marginal contribution of the passive process, total clarithromycin CL uptake (0.46 μL/min/10⁶ cells), was in agreement with the value obtained at a single concentration in the presence of NH₄Cl (Supplementary Material, Figure S4).

**DISCUSSION**

Many clinically used drugs are basic and lipophilic, with a high propensity to accumulate in lysosome rich tissues, including the lungs. Depending on the location of intracellular drug targets the consequence of lysosomal sequestration varies, and could affect the efficacy of respiratory drugs targeting airway submucosal compartments (e.g., β₂-agonists and antimuscarinics). Drug accumulation in AMs may lead to formation of foamy macrophages (e.g., in drug induced phospholipidosis) and may raise safety concerns. Currently, there is a paucity of *in vitro* studies investigating the accumulation of respiratory drugs and potential lysosomal distribution in either primary human AMs or macrophage cell lines. In the current study, comprehensive analysis of uptake and lysosomal sequestration of 10 drugs was performed in NR8383. This rat alveolar macrophage cell line was selected as a representative AM system due to its ease of culturing and reported comparable characteristics to freshly isolated rat AMs (17,18).

Assessment of Drug Accumulation and Membrane Partitioning in NR8383

Following the optimisation of the methodology, the uptake of 10 drugs was investigated in NR8383 at a single concentration (5 μM) limited by the analytical quantification of the cellular concentrations of the drugs investigated. The analysis showed pronounced differences in the extent of accumulation illustrated by over a 400-fold range in both CLuptake (0.04–17 μL/min/10⁶ cells).
min/10^6 cells) and Kp (0.7–391) for the drugs in the dataset. Imipramine and clarithromycin, as the two most lipophilic basic drugs, showed the highest intracellular accumulation in NR8383, in agreement with previous studies reporting accumulation of clarithromycin and a number of CADs in AMs in vitro (12,40–42). A number of studies also reported substantial imipramine accumulation in isolated perfused lungs of rats (estimated Kp of 17–44 depending on concentration), or other cell systems including rat hepatocytes (Kp,max 360) (37,43,44).

Recently, clarithromycin Kp of ~16 was reported in NR8383; the estimate is based on measurements at 10 min at 37°C and is approximately 5-fold lower than the Kp obtained here (12). This discrepancy can be explained by the use of much higher initial clarithromycin concentration (50 μM) in the study by Togami et al. (2013) (12) compared to the current analysis. For the remaining respiratory drugs investigated, relatively lower intracellular accumulation in AMs was observed (Table II) reflective of their physicochemical properties. Most drugs have limited plasma membrane permeability due to being largely ionised and relatively hydrophilic at physiological pH, and are non-amphiphilic in structure, supporting no extensive partitioning into membranes (Table I).

In this present study, the mechanisms driving the uptake of respiratory drugs in AMs were investigated at both 37 and 4°C. Although the reliability of the 4°C approach to accurately determine passive diffusion is often questioned because of the reduced fluidity of the cellular membrane (45), this method was used as the presence of any membrane transporters in NR8383 is currently unknown, hence eliminating the possibility to assess the process by the use of specific transporter inhibitors. Uptake of most drugs into NR8383 was driven by an active process, reflected in <25% contribution of the passive diffusion to the total uptake for 7/10 drugs investigated. The largest contribution of active uptake process(es) to overall intracellular accumulation in NR8383 was observed for clarithromycin. This finding is in agreement with Togami et al. study (12) that showed a reduction in clarithromycin Kp by 90% in NR8383 at 4°C compared to its value at 37°C. In the case of rifampicin, formoterol, ipratropium and tiotropium bromide, the non-amphiphilic and relatively ly less lipophilic nature of cellular plasma membrane is not surprising considering the non-amphiphilic and relatively lipophilic nature of clarithromycin and formoterol relative to imipramine.

Assessment of Lysosomal Sequestration of Drugs in NR8383

Lysosomal sequestration is an important active process that may contribute to intracellular drug accumulation. The qualitative assessment of this process was performed with LTR staining of NR8383 in the absence and presence of NH4Cl, monensin and nigericin, agents known to abolish lysosome-cytosol pH gradient. In the quantitative analysis, the reduction in total drug uptake clearance and accumulation by the mentioned chemical agents was assessed. This indirect method provides an advantage over methods requiring cell homogenisation and differential centrifugation to isolate lysosomes, as it is simple, rapid and allows a larger number of compounds to be assessed. In addition, it avoids issues of poor recovery, potential contamination of lysosomes and drug diffusion from these organelles during sample preparation. The extent of reduction in intracellular accumulation of investigated drugs in NR8383 obtained in the presence of chemical agents differed substantially for the drugs in the dataset. No or minimal reduction in Kp (~15%) was observed for zwitterionic rifampicin and ciprofloxacin, neutral budesonide and permanently cationic tiotropium bromide. Among basic drugs, the reduction in Kp was in the range of 20–40% for formoterol, fenoterol and terbutaline. Out of all drugs, significant lysosomal accumulation in NR8383 was observed only for basic imipramine and clarithromycin (reduction in Kp of 65%
and above). 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 sequestration, as demonstrated in numerous different in vitro systems either in the presence of the chemical agents (Supplementary Material, Table S1) or LTR (14,55). Although clarithromycin is cationic, it is not an amphiphilic drug due to the absence of the typical hydrophobic moiety (an aromatic and/or aliphatic ring structure) present in many CADs (56). However, macrolides have been indicated to be one of the few exceptions which lack the typical CAD structure yet cause phospholipidosis linked to lysosomal sequestration (5).

Lysosomal accumulation of clarithromycin was observed for the first time across a concentration range, supported by the reduction in its $K_p$ in the presence of NH$_4$Cl at all drug concentrations including 100 μM. These findings suggest that even at high concentrations, its intracellular accumulation was not driven solely by membrane partitioning but also by the lysosome-cytosol pH gradient. Therefore, the true membrane partitioning (i.e., $K_{p,\text{min}}$) of clarithromycin is likely to be closer to the estimate obtained in the presence of NH$_4$Cl. No such data currently exist in the literature to allow direct comparison.

Togami and colleagues previously showed that treatment of NR8383 with NH$_4$Cl reduced the amount of clarithromycin in the cells by approximately half and the fraction of the drug in the granules compartment (containing the lysosomes) by 75% (12), in excellent agreement with the reduction in $K_p$ obtained in the current study.

The inclusion of basic drugs in this study was driven by 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 (predicted $K_{p,\text{lysosome}}$ based on pH partitioning ranged between 315 and 443 for terbutaline and imipramine, respectively). However, under the experimental conditions used herein, 3 out of 5 basic drugs with $\log P>3$ failed to show substantial lysosomal accumulation, suggesting that both sufficient lipophilicity and ionisation are required for lysosomal accumulation within relatively short incubation times. This finding was in agreement with a number of studies reporting lysosomal sequestration for drugs with $\log P>2$ and $pK_a$ between 6.5 and 11 (14,55). However, it is also evident that the mentioned physicochemical space is not definitive and that a number of drugs with these properties may fail to accumulate in the lysosomes. The discrepancy may be explained by the previous reports showing that drugs with similar octanol-water partition coefficients in unionised and ionised forms did not accumulate in the lysosomes to the same extent (mitochondrial accumulation was observed instead). This finding was in contrast to drugs for which partition coefficients of the ionised form were much lower relative to neutral (all have the above mentioned physicochemical properties) (13).

An important finding of the current lysosomal sequestration studies was the altered accumulation kinetics of clarithromycin in NR8383 when its lysosomal sequestration was significantly reduced in the presence of NH$_4$Cl. While it was not possible to determine the uptake kinetic parameters for clarithromycin under control incubation conditions, the initial uptake rates were substantially reduced in the presence of NH$_4$Cl and the kinetic parameter estimates could be obtained. It is uncertain whether these estimates truly reflect the existence of a membrane transporter in NR8383 and further investigation of transporters expressed in this cell line is required. The existing evidence for clarithromycin being a substrate for membrane transporters is conflicting. Although it was previously shown that it was subject to active hepatic uptake (36), a more recent study demonstrated that clarithromycin was not a substrate for OATPs and OCT1 in vitro (transfected HEK293 cells) and in vivo in OATP and OAT gene knockout mice (57). One potential explanation for the more saturable kinetics under NH$_4$Cl condition is the saturation of lysosomal accumulation with increasing clarithromycin concentration. These results further suggest that for CADs, macrolides and other basic lipophilic drugs which accumulate in the lysosomes, the uptake kinetic parameter estimates obtained may not represent the true transporter affinities and uptake rates without adequate consideration of the lysosomal sequestration. This is particularly important if in vitro generated uptake kinetic parameters are to be used in physiologically-based pharmacokinetic models to predict in vivo transporter-mediated disposition of these drugs (58–61). 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.

In conclusion, the current study is the first to provide a comprehensive analysis of the extent of accumulation of a range of respiratory drugs in alveolar macrophages and the mechanisms contributing to the observed intracellular drug concentrations. The current data suggest minimal sequestration of inhaled drugs in lysosomes of alveolar macrophages; however, formulation and particle size also need to be taken into consideration in the translation of the findings to the in vivo setting. The study has highlighted the importance of both lysosomal sequestration and binding to acidic phospholipids for extensive cellular accumulation in AMs, which are distinct to transporter-mediated cellular uptake. Clarithromycin example clearly emphasizes the relevance of lysosomal sequestration on the understanding of the cellular accumulation kinetics and interplay with other active processes affecting intracellular drug concentrations. The abundance of lysosomes in NR8383, together with the data presented here, indicate potential application of this cell line in preclinical drug development as a routine system for investigation of lysosomal sequestration of new drug candidates. In addition, NR8383 may be used to predict the extent of respiratory drug accumulation in human AMs and to validate mechanistic in silico models.
developed to predict subcellular drug distribution. Therefore, this study provides a considerable scope for future research in the area of the prediction of respiratory drug accumulation in human alveolar macrophages.

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