



# **King's Research Portal**

DOI: 10.1021/acs.molpharmaceut.7b00715

Document Version Peer reviewed version

Link to publication record in King's Research Portal

Citation for published version (APA):

Benaouda, F., Jones, S. A., Chana, J., Dal Corno, B. M., Barlow, D. J., Hider, R. C., Page, C. P., & Forbes, B. (2018). Ion-Pairing with Spermine Targets Theophylline to the Lungs via the Polyamine Transport System. *Molecular Pharmaceutics*, *15*(3), 861-870. https://doi.org/10.1021/acs.molpharmaceut.7b00715

#### Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

#### General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

•Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research. •You may not further distribute the material or use it for any profit-making activity or commercial gain •You may freely distribute the URL identifying the publication in the Research Portal

#### Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Ion-pairing with spermine targets theophylline to the lungs via the polyamine transport system

Faiza. Benaouda<sup>1</sup>, Stuart. A. Jones<sup>1\*</sup>, Jasminder. Chana<sup>1</sup>, Benedetta. M. Dal Corno<sup>1</sup>, Dave. J. Barlow<sup>1</sup>, Robert. C. Hider<sup>1</sup>, Clive. P. Page<sup>1,2</sup>, Ben Forbes<sup>1</sup>

<sup>1</sup>Institute of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH.

<sup>2</sup>Sackler Institute of Pulmonary Pharmacology, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE 1 9NH.

\***Corresponding author:** Dr. S. A. Jones. King's College London, Institute of Pharmaceutical Science, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH. Tel: +44 (0)207 848 4843. Fax: +44 (0)207 848 4800. E-mail: <u>stuart.jones@kcl.ac.uk</u>

## **Graphical Abstract**



### Abstract

Certain xenobiotics, such as paraquat, are sequestered into the lungs from the systemic circulation by the polyamine transporter system (PTS). The aim of this study was to investigate whether ion-pairing a drug (theophylline) with a PTS substrate (spermine) provides a means of using this active transport mechanism to target drug delivery to the lungs. Fourier transform infrared spectroscopy showed that two of the amines of spermine interact with C-N7 and C6=O of theophylline, leaving two free amines to interact with the PTS. In A549 cells, which possess a functional PTS (spermidine K<sub>m</sub> and V<sub>max</sub>,  $0.6 \pm 0.3 \mu$ M and  $1.8 \pm 0.3 \text{ pmol.min}^{-1}$  per 10<sup>5</sup> cells, respectively), uptake of the theophylline-spermine ion-pair was increased 1.8-fold compared to free theophylline at 37°C, but not at 4°C. In an isolated perfused rat lung model (IPL) a 3.6fold increase in lung theophylline concentration was observed after vascular administration of the ion-pair compared to free theophylline. Theophylline was cleared from the IPL with similar kinetics irrespective of whether it was delivered as the free drug or an ion-pair, although lung levels remained elevated after washout following delivery as an ion-pair. In vitro simulation of the theophylline-spermine break down demonstrated that a drop in pH from 9.6 to 7.4, such as that undergone by the ion-pair in biological matrices, induces rapid and almost complete dissociation of the ion-paired species. However, infusion of the ion-pair formulations via the vasculature provides almost immediate delivery to the pulmonary capillary bed permitting PTS-mediated active sequestering of ion-paired theophylline into the lungs.

Keywords: Polyamine; ion pair; lung; theophylline; active uptake; isolated perfused lung; A549. drug targeting

#### 1 Introduction

The polyamine transporter system (PTS) has been identified in both prokaryotic and eukaryotic cells <sup>1</sup>. It transports polyamines in a saturable, carrier-mediated, time, temperature, pH, energy and concentration-dependent manner <sup>2</sup>. The PTS has the capacity to sequester certain xeniobiotics from the systemic circulation into the pulmonary system <sup>1,3</sup> and thus offers a potential means by which to target drug delivery to the lungs.

A gene for the mammalian polyamine transporter has not yet been identified, but structure-activity studies have established that good PTS substrates require two or more N-containing moieties that are protonated at physiological pH <sup>4</sup>. As very few therapeutic agents that require delivery into the lungs display these structural characteristics, a number of polyamine drug analogues and covalent polyamine drug conjugates have been developed in an attempt to use the PTS to target delivery to the lungs <sup>5-7</sup>. However, these strategies have had limited success because combining the molecular features required for effective active PTS transport and pharmacological action in a single molecule is problematic <sup>5-7</sup>. An alternative, but untested approach, to utilising the PTS to target drug delivery to the lungs is to form non-covalent polyamine complexes such as ion-pairs. If sufficiently tightly complexed ion-pairs could be transported from the vasculature into the lungs by the PTS, before dissociating to release the drug to elicit its pharmacological effect.

The association of two oppositely charged molecules in the solution state to form ionpairs (A<sup>-</sup>B<sup>+</sup>) has been shown to alter the transport of ionised molecules into the skin <sup>8</sup>, across the nasal mucosa <sup>9</sup> and across the intestinal mucosa <sup>10</sup>. In these previous studies the counter ions used for ion-pair formation altered the drug transport rate through modification of the parent drug's physicochemical characteristics, which affected the molecules absorption. The formation of ion-pairs has also been shown to enable active transport into cells by an energy dependent mechanism <sup>11</sup> and it is possible, therefore, that linking a therapeutic agent to a PTS substrate via ion-pairing may facilitate sequestration of the drug into the lungs.

The aim of the present study was to investigate whether forming a non-covalently bound ion-pair complex between theophylline and spermine can enable active drug uptake into the lungs. Spermine, a tetra-amine, which displays a high affinity for the PTS (K<sub>i</sub> ca. 0.02  $\mu$ M)<sup>2</sup>, was utilised as the ion-pair counter ion and theophylline was used as the model drug.

Theophylline contains an acidic nitrogen with a pKa of 8.6 and hence it has the potential to form an ion-pair with spermine both at the commercial intravenous formulation pH of 9.4 (Phyllocontin<sup>®</sup>), and under physiological conditions, pH 7.4. Although the reduction in theophylline ionisation driven by the pH drop from 9.4 to 7.4 upon administration would theoretically weaken the theophylline-spermine ion-pair it was predicted that the ion-pair stability, which is dependent on association strength <sup>8</sup>, would be sufficient to influence uptake into the lung after intravenous administration.

The PTS function has been characterised in rat lung slices<sup>12-14</sup> and it has been shown to mediate polyamine uptake into type I and type II pulmonary epithelial cells; it is also active in pulmonary artery endothelial cells<sup>15,16</sup>. Although a rat endothelial cell-line with an active polyamine transport system has been reported<sup>17</sup>, comparatively few studies have used this model to assess drug transport<sup>18</sup> and therefore in this work the A549 human alveolar epithelial cell line was selected to study the kinetics of PTS-mediated uptake of ion-paired theophylline. Although the A549 cell line does not model the capillary endothelium, the objective of the cell culture studies was to evaluate whether formulating theophylline as an ion-pair can modify drug transport kinetics. The A549 cell line was used as a robust model, in which PTS activity is well characterised, before the formulation approach was tested in isolated perfused lungs  $(IPL)^2$ . In the IPL the ion-pair formulations were administered to the vasculature in either plasma protein-containing or protein-free perfusate. The ion-pair dissociation kinetics could not be monitored in physiological fluids using commercial analytical apparatus due to solvent signal interference with the spectrum, therefore an *in vitro* model was used to simulate the pH change upon introduction of the ion-pair (pH 9.6) into the perfusate (pH 7.4) and investigate temporal complex association. Ion-pair dissociation rate data were used to interpret theophylline kinetics in the IPL studies, thus determine whether modified lung uptake was consistent with active sequestration of a labile ion-pair.

#### 2. Materials and Methods

#### 2.1 Materials

Theophylline (anhydrous,  $\geq$  99%), propylene glycol (PG, USP grade), spermine ( $\geq$  99%), hydrochloric acid (HCl), and Krebs buffer salts (NaCl, KCl, CaCl<sub>2</sub>, MgSO<sub>4</sub>, NaHCO<sub>3</sub>, KH<sub>2</sub>PO<sub>4</sub>, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and D-glucose), were supplied by Sigma Aldrich, UK. STEEN solution ((human serum albumin, dextran-40, NaCl, glucose, KCl, Na H<sub>2</sub>PO<sub>4</sub>, CaCl<sub>2</sub>H<sub>2</sub>, MgCl, NaHCO<sub>3</sub>, NaOH and water for injection) was obtained from XVIVO Perfusion AB, Sweden. Methanol (high performance liquid chromatography (HPLC) grade) and Optiphase "Safe" scintillation cocktail were obtained from Fisher Scientific International, UK. Heparin was purchased from Movianto UK Ltd, saline (0.9 %w/v) from Baxter, UK and tissue solubilizer Soluene-350, was obtained from PerkinElmer, UK. 8-<sup>14</sup>C theophylline (60 mCi/mmol, > 98%) and 1,4-<sup>14</sup>C spermidine trihydrochloride (3.7 mCi/mmol, > 98 %) were obtained from American Radiochemicals, USA. A549 cells were obtained from ATCC, USA and all reagents for the cell culture (Minimum Essential Medium Eagle, fetal bovine serum (FBS), L-glutamine, non-essential amino acids, gentamicin, trypsin-EDTA (0.25% trypsin, 0.05% EDTA), Hank's Balanced Salt Solution, trypan blue and Triton X), and deuterated water were all purchased from Sigma Aldrich, UK.

#### 2.2 Ion-pair Characterisation

The conditional theophylline-spermine association constants (pK<sub>cond</sub>) were determined using solution-state Fourier transform infrared spectroscopy (FTIR). A universal transmission cell system (Omni-Cell, Specac Ltd., UK) fitted with CaF<sub>2</sub> windows and a 25  $\mu$ m Mylar spacer (Specac Ltd., UK) was used for transmission measurements. Theophylline (3.6 mg.mL<sup>-1</sup>) and spermine (from 0 to 83 mg.mL<sup>-1</sup>) were combined to achieve theophylline:spermine molar ratios of 1:0-1:10 in water and 1:0-1:20 in PG-water solvents. The theophylline ionisation (theophylline pKa = 8.6 at the acidic N<sub>7</sub><sup>19</sup>) and spermine ionisation (spermine pKa's = 10.8(N<sub>1</sub>), 10.2(N<sub>2</sub>), 8.85(N<sub>3</sub>) and 7.95(N<sub>4</sub>)<sup>19</sup>) were maintained constant during the ion-pair affinity measurements by adjusting the final pH of all the solutions to pH 9.6 ± 0.2 using sodium hydroxide or hydrochloric acid when appropriate (at this pH 86% of the theophylline was ionised and 97%, 84%, 26%, 4% of the spermine at the N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub> and N<sub>4</sub> sites, respectively, was ionised). Deuterated water (D<sub>2</sub>O) was employed in the measurements as it allowed accurate detection of theophylline absorption bands in the 1700-1500 cm<sup>-1</sup> range. All infrared spectra were recorded using a Spectrum One spectrometer (Perkin Elmer Ltd., UK) and spectral analysis was performed with Spectrum version 10 software (Perkin Elmer Ltd., UK). The spectra were generated from 32 scans collected at a spectral resolution of 4 cm<sup>-1</sup>. The vehicle spectrum containing the equivalent amount of the spermine (and HCl when appropriate) was subtracted from the sample spectrum. The peak height of the normalised theophylline C-N<sub>7</sub> stretching bands, previously assigned to the uncomplexed (1551 cm<sup>-1</sup>) and complexed (1530 cm<sup>-1</sup>) species <sup>20, 21</sup>, were employed to construct plots of % theophylline-spermine complex formed *vs* –log[spermine]free. These data were fitted with a sigmoidal regression model (SigmaPlot 12.0, Systat Software Inc, UK) to determine the pK<sub>cond</sub>. The pK<sub>cond</sub> was derived on the assumption that theophylline and spermine formed a 1:1 complex, as indicated by in-silico modelling (Chem3D, Perkin Elma, UK) which suggested that such a complex would form 2 hydrogen bonds. The apparent stability constant (pK<sub>app</sub>) of the theophylline-spermine complex was calculated by correcting for the acidity constant of spermine (1.09) which accounted for the incomplete ionisation at the N<sub>1</sub> of spermine, which was directly involved in forming the ion-pair.

The formation of the theophylline-spermine complex was confirmed using a high performance liquid chromatography (HPLC) method based on work by Horvath *et al.*, <sup>22</sup>. The retention time of the theophylline ( $36 \mu g.mL^{-1}$ ) was obtained by injecting 20  $\mu$ L into the HPLC system with a mobile phase consisting of methanol:water (10:90 v/v, pH 9.6  $\pm$  0.3). The HPLC system (HP1050, UK) was a quaternary pump set at a flow rate of 1 mL.min<sup>-1</sup> and a single wavelength UV detector set at 274 nm. The stationary phase was a reversed-phase octadecyl silane (ODS-2) 5  $\mu$ m column (250 × 4.6 mm). The pH of the mobile phase was carefully maintained at pH 9.6  $\pm$  0.3 and checked prior to, and post sample injection.

### 2.3 Cell culture studies

The human alveolar epithelial cell line A549 was used between passages 90-115. The cultures were maintained in a humidified 5%  $CO_2/95\%$  atmospheric air incubator at 37°C. Cell culture medium comprised Minimum Essential Eagle Medium supplemented with FBS (10 % (v/v)), *L*-glutamine (200 mM, 40% (v/v)) and gentamicin (50 mg/ml, 0.1 % (v/v)). Medium was exchanged every 2-3 days and cells were passaged weekly at a 1:3 split ratio using trypsin-EDTA solution. Cells were harvested and seeded in 24-well tissue culture plates (Greiner

cellstar, Sigma Aldrich, UK) at a density of  $2 \times 10^5$  cells/well and used for uptake studies once a confluent cell layer had been established.

To assess the functionality of the PTS in A549 cells, <sup>14</sup>C-spermidine uptake was measured. After the cells were washed with HBSS and immersed in fresh HBSS (0.5 mL), uptake studies were initiated by the addition of 0.5 mL spermidine solutions (in water at pH 7.4 and  $9.6 \pm 0.2$ ), at varying concentrations (final <sup>14</sup>C-spermidine concentrations ranging from 0.12 to 1.7  $\mu$ M. At given time points up to 40 min, the experiments were terminated by aspirating the culture medium. To displace any <sup>14</sup>C-spermidine attached to the cell surface, the cells were washed with ice-cold HBSS. The cells were then lysed by the addition of a 1% Triton X100 solution for 45 min at 37°C. The activity in the culture medium, the cell washings and that associated with the lysed cells was determined by scintillation counting following the addition of scintillation cocktail on a LS6500 multipurpose scintillation counter (Beckman, USA). The uptake kinetics for spermine (K<sub>m</sub> and V<sub>max</sub>) were determined by fitting the data with a non-linear regression model (R<sup>2</sup> > 0.95) (SigmaPlot 13, Systat Software Inc, UK).

To investigate <sup>14</sup>C-theophylline uptake, the culture medium was removed, the cells were washed with HBSS and then immersed in HBSS (0.5 mL). Uptake studies were initiated by the addition of 0.5 mL of either theophylline control (theophylline at 0.06  $\mu$ g.mL<sup>-1</sup> in water at pH 7.4 or at pH 9.6 ± 0.2), theophylline-spermine non-ion-paired (theophylline is 0.06  $\mu$ g.mL<sup>-1</sup> and spermine at 1.34  $\mu$ g.mL<sup>-1</sup> in water, pH 7.4) or ion-paired theophylline:spermine (theophylline at 0.06  $\mu$ g.mL<sup>-1</sup> and spermine at 1.34  $\mu$ g.mL<sup>-1</sup> in water, pH 9.6 ± 0.2). The mixing of the HBSS with the theophylline solutions generated solutions that were tolerated by the cells (pH 8.3, tolerability determined by MTT tests, data not shown), but did not dilute the ion-pairs in the electrolyte solution in advance of the experiments. After an incubation period of 2 min, the experiment was stopped and a full mass balance performed as described for spermidine. Each condition was tested in triplicate using three different passages of cells. All uptake studies were carried out at 37°C and 4°C in order to assess the energy dependence of the uptake process.

#### 2.4 Isolated perfused lung studies

All procedures were performed within the guidelines of the Animals (Scientific Procedures) Act 1986 under the authority of approved Home Office personal and project

licences. The project was also approved by the local ethics committee at King's College London. Male Wistar rats, (7-8 weeks old, 220 - 260 g), were anesthetised by intraperitoneal injection of pentobarbital (280 mg.kg<sup>-1</sup>). The lungs were excised and perfused through the pulmonary artery as previously described <sup>23</sup>. Briefly, the trachea was intubated and the lungs were inflated in situ with 0.5 mL of air. After laparotomy, heparin sodium (500 IU) was injected into the *vena cava* and the animals were exsanguinated. The pulmonary artery was cannulated and perfused with a modified Krebs-Ringer buffer saturated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> (composition in mM: NaCl 11,8 KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 24.9, KH<sub>2</sub>PO<sub>4</sub> 1.2, HEPES 10, D-glucose 11). The buffer (pH 7.4) was thermostated to 37°C and perfused at a constant rate (ca. 8 mL.min<sup>-1</sup>) maintained by a peristaltic pump (Masterflex, UK). The right atrium and ventricles were cut to create an open circuit and allow the unrestricted passage of the effluent buffer. The lungs and heart were then excised and suspended by the trachea. The lungs were inflated with a further 1.5 mL of air, which provided constant inflation throughout the duration of the study. If the lungs maintained viability, determined by the absence of significant oedema, they were allowed to stabilize for 2-3 min prior to the initiation of the uptake studies.

A total of four theophylline formulations (603  $\mu$ g.mL<sup>-1</sup><sup>14</sup>C-theophylline) were assessed in the study: (i) theophylline in water (pH 7.4); (ii) theophylline:spermine, 1:20 molar ratio, in water, equilibrated for > 4 h prior to use (pH 7.4 ± 0.2); (iii) theophylline:spermine 1:20 molar ratio in water (pH 9.6 ± 0.2); and (iv) theophylline:spermine 1:20 molar ratio in PG-water (70:30 v/v, (pH 9.6 ± 0.2)). Formulations (i), (iii) and (iv) were introduced into the perfusate as it entered the pulmonary artery of the IPL using a syringe pump, with rate set at 0.04 mL.min<sup>-1</sup> (Severn Sales, UK) to achieve a theophylline concentration of 3  $\mu$ g.mL<sup>-1</sup> in the perfusate, whereas formulation (ii) was added to the perfusate The formulations were administered via single pass perfusion for 25 min followed by a 10 min washout period (perfusion with Kreb's buffer only). At predetermined time points during the infusion and washout periods, 1 mL aliquots were removed from the perfusion effluent. At the completion of the washout period, the lungs were removed for the measurement of theophylline concentration. The exact perfusate and formulation flow rates were normalised using a control run prior to each study, which measured the theophylline present at the sampling time points after running through the perfusion apparatus in the absence of the lungs. To quantify theophylline in the lung tissue at the end of the infusion, the tissues were homogenised (Ultra-Turrax homogeniser) in PBS (1:2 w/v) and solubilised with Soluene-350 tissue solubilizer (1:5 w/v) (PerkinElmer, UK), under constant shaking for 4 h at 50°C. Theophylline was quantified in samples from the perfusion studies and the tissue extraction studies by mixing an aliquot of the solutions with 20 mL of Optiphase "Safe" scintillation cocktail (Fisher Scientific, UK), acidified with 0.7% v/v glacial acetic acid to eliminate any chemi-luminescence. The samples were vortexed and their radioactivity determined using an LS6500 multipurpose scintillation counter (Beckman, USA). The lung uptake of <sup>14</sup>C-theophylline during the infusion process was determined by assessing the difference in theophylline perfusate concentration in the presence and absence of the lungs. The concentration at steady-state (SS) and time to 50% SS (T<sub>SS50</sub>) were calculated from plots of theophylline uptake vs time. The clearance data, obtained from 0-5 min of the washout period, were fitted with a second order kinetic model (R<sup>2</sup> > 0.95) to determine the elimination rate (K) and half-life (T<sub>1/2</sub>).

To assess the effects of protein binding on the theophylline lung uptake when the theophylline:spermine ion-pair was administered, a STEEN/blood mixture (10:4 v/v) was employed with the IPL system in a re-circulation mode. The perfusion was initiated with the perfusate containing no drug for ca. 5 min, then either theophylline control or theophylline:spermine ion-pairs dissolved in water at pH 9.6 were spiked in the perfusion medium to generate a theophylline concentration in the circulating medium of 3 µg.mL<sup>-1</sup>. After 25 min, the perfusion reservoir was switched over to a Krebs buffer, at a single pass mode, for a 10 min washout period. The amount of theophylline in perfusate samples was quantified as described previously. The concentration at steady-state (SS) and time to 50% SS (T<sub>SS50</sub>) were calculated by applying a one-site saturation model ( $y = \frac{SS. x}{SS_{50}+x}$ ) (SigmaPlot 13.0, Systat Software Inc, UK) to a plot of theophylline uptake *vs* time. The clearance data, obtained from 0-5 min of the washout period, were fitted to a second order kinetic model ( $\frac{1}{[THE]} = \frac{1}{[THE]_0} + kT$ ) (R<sup>2</sup> > 0.95) to determine the elimination rate (K) and half-life (T<sub>1/2</sub>).

#### 2.5 Ion-pair dissociation

To simulate ion-pair dissociation induced by a pH change when introduced into the vascular perfusate, theophylline-spermine formulations were made up in D<sub>2</sub>O and in PG-D2O

at pH 9.6  $\pm$  0.2 at a theophylline concentration of 3.6 mg.mL<sup>-1</sup> and a spermine concentration of 83 mg.mL<sup>-1</sup>. Just prior to measurement, the pH of the test solutions was adjusted to pH 7.4  $\pm$  0.2 using a pre-determined volume of HCl (5 M). The samples were then mounted in a universal transmission cell system (Omni-Cell, Specac Ltd., UK) fitted with CaF<sub>2</sub> windows and a 25  $\mu$ m Mylar spacer (Specac Ltd., UK) mounted on a Spectrum One spectrometer (Perkin Elmer Ltd., UK). A total of 46 s elapsed before the first scan was taken (time needed to mount the cell onto the spectrometer) and the continuous monitoring of the C-N<sub>7</sub> and C<sub>6</sub>=O was carried out for 20 min using TimeBase<sup>TM</sup> software (Perkin Elmer Ltd., UK). The pH was monitored throughout the process.

#### 2.6 Data Analysis

Data were expressed as their mean  $\pm$  standard deviation (SD). Normality (Sapiro-Wilk) and homogeneity of variances (Levene's test) were assessed prior to statistical analysis. Lung uptake results were analysed using one way analysis of variance (one-way ANOVA) tests with post-hoc Tukey analysis where required (Statistical package for social sciences SPSS version 16.0 (SPSS Inc., Chicago, IL. USA)). All other data were analysed using a Student's *t*-test (SPSS version 16.0 (SPSS Inc., Chicago, IL. USA)). Statistically significant differences were assumed when  $p \le 0.05$ .

### 3 Results

#### 3.1 Ion-Pairing

The FTIR spectra of theophylline in D<sub>2</sub>O displayed characteristic peaks for the C<sub>2</sub>=O stretch at 1698 cm<sup>-1</sup>, the C<sub>6</sub>=O at 1647 cm<sup>-1</sup> and the C-N<sub>7</sub> stretch at 1551 cm<sup>-120,21</sup>. The multiple peaks of low intensity around 1530 cm<sup>-1</sup> in the C-N stretch region were assigned to theophylline dimer formation (Fig.1a) <sup>24</sup>.



Figure 1. Characterisation of the theophylline-spermine ion-pair formation; (a) infrared spectra; (b) association curves. THE refers to theophylline alone in D<sub>2</sub>O at pH 9.6, THE-SPE the theophylline:spermine ion-pair in D<sub>2</sub>O at pH 9.6 and THE-SPE PG the ion-pair in a 70/30 D<sub>2</sub>O propylene glycol mixture at pH 9.6 ( $n = 3 \pm SD$ ). pK<sub>app</sub> is the apparent stability constant. In the structure cartoon, H-bonds are shown in green, with atoms coloured as blue = N, dark grey = C, red = O, light grey = H.

When spermine was added to the theophylline at a 1:1 ratio the C<sub>2</sub>=O band split into two peaks (1671, 1698 cm<sup>-1</sup>), the C<sub>6</sub>=O band split into two peaks (1618, 1647 cm<sup>-1</sup>) and the C-N<sub>7</sub> displayed two peaks of similar height (1530, 1551 cm<sup>-1</sup>). When the spermine concentration was increased to a ratio of 1:20 theophylline:spermine the predominant peaks for the carbonyl

were at 1671 cm<sup>-1</sup> (C<sub>2</sub>=O stretching) and 1618 cm<sup>-1</sup> (C<sub>2</sub>=O stretching), whilst the predominant C-N<sub>7</sub> peak was at 1531 cm<sup>-1</sup>. The up-field peak shifts, i.e., lower wave numbers, resulting from the mixing of theophylline with spermine indicated ion-pair formation. The charged amine at C-N<sub>7</sub> was thought to be the primary site for the ion-pair interaction and therefore the peak height ratio at 1530 (ion-paired species) and 1551 cm<sup>-1</sup> (non ion-paired species) was used to determine the percentage of ion-pair formation at different ratios of theophylline to spermine. When the ion-pair was formed in the 70:30 (v/v) PG-D<sub>2</sub>O vehicle similar up-field shifts for the theophylline peaks to those observed in the water vehicle were recorded and so the C-N<sub>7</sub> was again used to determine the percentage of ion-pair species present in solution. In both vehicles, a 1:1 ion-pair complex was thought to be formed between theophylline and spermine, which used two of the amines in spermine in the ion-pair interaction leaving two charged amine groups for the complex to interact with the PTS (Fig. 1b).

The theophylline-spermine association curve, derived from the FTIR data, was sigmoidal ( $R^2 \ge 0.94$ ) in both D<sub>2</sub>O and the PG-D<sub>2</sub>O co-solvent. The association of the theophylline-spermine ion-pair in PG-D<sub>2</sub>O (Fig. 1b) was stronger than in D<sub>2</sub>O alone (pK<sub>app</sub> at 0.91 ± 0.1 (D<sub>2</sub>O) vs 1.12 ± 0.1 (PG- D<sub>2</sub>O)). Using these ion-pair affinity constants, computer simulation of the microspecies present in the formulations at pH 9.6 used for the subsequent IPL studies, predicted that 33% and 44% of the theophylline would be ion-paired to spermine in water and PG:water 70:30 vehicles, respectively (see supporting data Fig. S1). The increase in the C18 column retention of theophylline, when presented as the spermine ion-pair compared to the theophylline 's charge was shielded when the ion-pair complex formed and this reduced the overall polarity of the theophylline to promote stronger interactions with the hydrophobic C18 silica column.

#### **3.2 Cell culture studies**

The spermidine uptake kinetics in the A549 cells suggested a single affinity, saturable PTS transport mechanism was active with a  $K_m$  at  $0.5 \pm 0.1 \mu$ M and  $V_{max}$  at  $1.6 \pm 0.2 \text{ pmol.min}^{-1}$  per 10<sup>5</sup> cells for the pH 7.4-vehicle and  $K_m$  at  $0.6 \pm 0.3 \mu$ M and  $V_{max}$  at  $1.8 \pm 0.3 \text{ pmol.min}^{-1}$  per 10<sup>5</sup> cells for the pH 9.6-vehicle (See supporting data Fig. S2). When the spermidine was studied at pH 9.6, 500  $\mu$ L of the pH 9.6 test formulation was added to 500  $\mu$ L of HBSS (pH

7.4) present on the surface of the cells to try mimic the theophylline studies where ion-pair dissociation was initiated with this mixing process (a final pH of ca. 8.3, note MTT studies showed the cells were viable under both pH 7.4 and pH 8.3 conditions, data not shown). Cellular uptake of spermidine was temperature-dependent with a significant decrease (p < 0.01) in spermidine uptake when the temperature was lowered to 4°C compared to 37°C (See supporting data Fig. S2). The temperature-induced reduction in spermidine uptake (pmol/10<sup>5</sup> cells) ranged from 1.9-fold at 1 min to 5.4-fold at 20 min at pH 7.4 and ranged from 1.5-fold decrease at 1 min to 3.8-fold decrease at 20 min at pH 9.6.

Presentation of theophylline to the A549 cells as the theophylline:spermine ion-pair increased the drug's uptake at 37°C (p < 0.05) compared to the non-ion-paired controls (Fig. 2). Reducing the temperature from 37°C to 4°C negated the ion-pair effect on theophylline transport into the cells. At 4°C the theophylline cellular uptake from all the test systems significantly increased (p < 0.05) compared to that obtained at 37°C, which indicated that theophylline may be subject to active efflux from the cells, which was suppressed at 4°C.



Figure 2. Theophylline (left) uptake by A549 epithelial cells at 37°C and 4°C following the application of theophylline (THE, pH 7.4) (white), theophylline-spermine non-ion-paired (THE-SPE, pH 7.4) (light grey) and theophylline-spermine ion-pair (THE-SPE, pH 9.6) (dark grey) to HBSS-submerged cells for 2 min. \* indicates a statistical difference to the controls (p < 0.05). ( $n = 3-9 \pm SD$ ).

#### 3.3 Isolated perfused lung studies

When theophylline formulations were introduced into the single-pass perfusate upstream of the lungs, the lung uptake profile of the theophylline fitted a typical one-compartment model for continuous infusions, regardless of the formulation used (Fig. 3).



Figure 3. Theophylline uptake in a single-pass isolated perfused lung model following introduction of theophylline into the perfusate. The theophylline was formulated alone at pH 7.4 (control) ( $n = 4 \pm \text{SEM}$ ) ( $\diamond$ ), theophylline-spermine ion-pair in water, pH 9.6 ( $n = 4 \pm \text{SEM}$ ) ( $\diamond$ ) and theophylline-spermine ion-pair in propylene glycol:water 70:30, pH 9.6 ( $n = 3 \pm \text{SEM}$ ).

Formulating the ophylline as the spermine ion-pair increased the extent of lung uptake (p < 0.05) compared to the non-ion-paired control, but the time to establish 50% of the steady-state levels was unchanged (Table 1).

Table 1. The theophylline uptake in the lung tissue at steady-state (SS), the time to 50% SS (T<sub>SS50</sub>), the rate of lung clearance (*K*) and lung tissue half-life (T<sub>1/2</sub>) when theophylline was administered into the isolated perfused lung pulmonary circulation perfusate alone (THE), as the theophylline-spermine ion-pair in water (THE-SPE) and as the THE-SPE ion-pair in a propylene glycol-water vehicle (THE-SPE (PG)) ( $n = 3-4 \pm$  SD). Dash indicates that the data were not collected.\*indicates statistically significant difference (p < 0.05)

Theophylline formulation	SS (µg)	T <sub>SS50</sub> (min)	$\frac{K}{(\mu g^{-1}.min^{-1})}$	T <sub>1/2</sub> (min)
THE (water, pH 7.4)	$25.4\pm7.2$	$3.2 \pm 3.2$	$0.1 \pm 0.1$	$0.9 \pm 0.7$
THE-SPE (water, pH 9.6)	$55.6 \pm 11.6 *$	$5.2\pm 6.1$	$0.1 \pm 0.0$	$1.9 \pm 1.3$
THE-SPE (PG:water 70:30, pH 9.6)	$96.4\pm18.0^{\ast}$	$2.6\pm0.5$	-	-

At steady-state, the ion-pair produced a 2.2-fold increase in theophylline lung uptake when formulated in the water vehicle and a 3.6-fold increase when formulated in the PG-water vehicle compared to the non-ion-paired theophylline (Table 1). Control experiments showed that the enhanced lung uptake of the theophylline-ion-pair was not a consequence of delivering the drug in a vehicle at pH 9.6 (as there was no difference for free theophylline at pH 9.6 compared to pH 7.4, p > 0.05, see supporting data Fig S3) or the 'free' spermine influencing the transport (ion-pair formulation left to dissociate for 4 h in the Krebs vehicle at pH 7.4 showed no change in the lung uptake, p > 0.05, compared to the free theophylline, see supporting data Fig S3).

Upon cessation of the theophylline infusion in the IPL model, the drug cleared from the lung tissue rapidly with a lung tissue half-life ( $T_{1/2}$ ) of ca. 1 min (Fig. 4a, Table 1). There was no statistical difference in the kinetics of theophylline clearance from the lungs when theophylline was administered alone or as a spermine ion-pair (p > 0.05) (Table 1). However, following the 10 min washout period, the mass of theophylline remaining in the lungs was significantly higher when the theophylline-spermine ion-pair was administered compared to when the non-ion-paired control was infused, i.e., a 1.7 ± 1.1-fold increase using the water vehicle and a 2.8 ± 1.3-fold increase using the propylene glycol vehicle compared to the control (Fig. 4b).

Theophylline lung uptake was not significantly altered in the presence of blood proteins compared to Krebs ( $SS_{(Krebs)} = 25.4 \pm 7.2 \ \mu g$  and  $SS_{(blood)} = 25.2 \pm 1.6 \ \mu g$  (p > 0.05)) when theophylline was administered alone. Theophylline is known to be extensively plasma protein bound, hence the presence of the plasma proteins would be expected to deplete the theophylline available for lung uptake and thus reduce the theophylline transport if it was primarily taken up via passive transport. The fact that the plasma proteins did not influence the theophylline transport into the lungs suggested that the drug did not simply enter the lung tissue via a passive process. However, the presence of the plasma proteins did increase the clearance and reduce the half-life of the theophylline in the re-circulatory system (supplementary data table S1).



Figure 4. a) Theophylline (THE) and theophylline-spermine ion-pair in water (THE-SPE) ( $n = 3-4 \pm SD$ ) clearance from the lung (note clearance data not collected for the ion-pair in water and propylene glycol (THE-SPE(PG)). Inset displays the second-order kinetic fit of the data during the first 5 min of the post-perfusion period. (b) THE retained in the isolated perfused lung tissue after the 10 minute washout period ( $n = 3 \pm SEM$ ) \*denotes difference compared to THE control (ANOVA, p < 0.05).



Figure 5. a) Theophylline (THE) lung uptake from a continuous 'recycled' perfusate of blood:steen mixture into which THE (•) and THE-SPE ion-pair ( $\circ$ ) dissolved in water were spiked ( $n = 4-5 \pm \text{SEM}$ ). b) Theophylline clearance from the lung tissue during the washout period following perfusion of THE (•) and THE-SPE ( $\circ$ ) ( $n = 3-4 \pm \text{SD}$ ).

Lung uptake of the theophylline-spermine ion-pair was enhanced in the re-circulating IPL compared to free theophylline, but the magnitude of the enhancement was lower when compared to the single pass IPL system; 2-fold at the start of the experiment (t = 1-6 min) falling to 1.2-fold after 25 min (Fig. 5a). Statistical analysis indicated that only 3 time points (at 8, 10 and 25 min) did not show a statistical difference between the theophylline and the theophylline-spermine ion-paired formulation. The reduction in ion-pair lung uptake enhancement compared to the single pass system was presumably due to dissociation of the ion-pair over time in the re-circulating perfusate. It is also possible that spermine was oxidised

by serum oxidases as we avoided using amino-oxidase inhibitors, such as aminoguanidine, because of their potential to ion-pair with theophylline and confound observations.

#### **3.4 Ion-pair dissociation**

Dissociation studies were carried out to mimic the infusion of the formulation (pH 9.6) into the IPL perfusate (pH 7.4). The pH of the formulation (pH 9.6 at t = 0) was reduced by the addition of HCl, mixed to achieve a final pH of 7.4, then analysed using an FTIR spectrophotometer. The process took 46 s, which was longer than the 5 s required for the ionpair formulation to travel from the site of infusion to the lung tissue in the IPL studies. Unfortunately, 46 s represented the shortest time in which complete mixing could be assured in the static FTIR cell. The FTIR results showed that the theophylline peaks assigned to the ion-pair species (in D<sub>2</sub>O: C<sub>2</sub>=O - 1673; C<sub>6</sub>=O - 1619; C-N<sub>7</sub> - 1535 cm<sup>-1</sup>) had reverted to the peaks assigned to the non-ion-paired species (in  $D_2O$ :  $C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1696$ ;  $C_6=O - 1645$ ;  $C-N_7 - C_2=O - 1645$ ;  $C-N_7 - 164$ 1551 cm<sup>-1</sup>, Figure 6). These data suggested that the pH change induced ion-pair breakdown. However, the shoulder at 1619 cm<sup>-1</sup> in D<sub>2</sub>O spectra confirmed that a small proportion of ionpaired theophylline existed even when the pH was dropped to 7.4. This result was in accordance with the calculations using the pKa of the drug, that ca. 7% of the theophylline was likely to remain ionised at pH 7.4 (Marvin Sketch, ChemAxon Ltd) of which the Hyss calculations predicted 0.4% of the total theophylline dose would remain ion-paired (Figure S1). In the  $D_2O/PG$  vehicle the peak at 1619 cm<sup>-1</sup> could not be seen clearly as all the peaks moved to lower wavenumbers, thus the proportion of ion-pairs that remained in water could not be compared with PG. After 20 min there were several small peaks around the 1530 cm<sup>-1</sup> region of the FTIR spectrum, which suggested that after ion-pair dissociation a small proportion of theophylline dimers were formed. The formation of theophylline dimers has been previously reported to occur in water at the concentrations used in the FTIR work <sup>24</sup>.



Figure 6. Theophylline–spermine ion-pair dissociation kinetics in a)  $D_2O$  and b) 70/30 PG/ $D_2O$ . The ion-pairs were formed in the vehicles at pH 9.6, t=0 and the pH was then dropped to physiological pH 7.4 over 46 s using HCl.

### Discussion

The FTIR data generated in this study showed that theophylline and spermine formed ion-pairs in polar solutions at pH 9.6. In this alkaline environment, the N<sub>1</sub> amine was the most extensively ionised of spermine's 4 amine groups (97% ionised) and therefore it was proposed to preferentially associate with the charged C-N<sub>7</sub> of theophylline. This hydrogen bond would orientate the spermine N<sub>2</sub> and the theophylline C<sub>6</sub>=O such that a second hydrogen bond was possible. The C-N<sub>7</sub> and C<sub>6</sub>=O peak shifts in the theophylline FTIR trace, assigned to conformational restriction caused by ion-pair formation and the C<sub>2</sub>=O peak shifts, assigned to electron distribution changes in the ring of theophylline supported the suggestion that 2 hydrogen bonds were formed<sup>20</sup>. The affinity constant of the theophylline spermine ion-pair also suggested that the complex had two points of interaction because it was held together more strongly than a simple ion-pair that was formed from a single electrostatic interaction such as KCl (K<sub>Ass</sub> is -0.7)<sup>19</sup>. The theophylline-spermine ion-pair supressed the charge on the theophylline compared to the theophylline alone<sup>22</sup>. However, *in silico* modelling of the ion-pair association using the affinity constant demonstrated that the complex was prone to break down rapidly upon a drop in pH or large dilution (i.e., the conditions experienced upon administration to the IPL). The addition of the propylene glycol co-solvent to the water vehicle enhanced the theophylline spermine interactions. These enhanced ion-pair interactions were most likely caused by the weakening the water's supramolecular structure in the solvent, which has previously been shown to promote hydrogen-bond interactions <sup>25</sup>. When evaluated in cell culture and IPL systems it was apparent that the physical stability of ion-pairs was fundamental to their uptake by the lungs and thus control of this aspect will be essential in order to successfully exploit this drug targeting approach.

The  $K_m$  and  $V_{max}$  of the spermidine uptake in the A549 cells in this study was similar to previously reported work where the PTS was shown to be active and this provided evidence that PTS transporter was functional in the A549 cells<sup>2</sup>. Theophylline uptake into the cells was low, approximately 0.05 ng in 2 minutes, after which theophylline concentration did not increase in the cells suggesting that equilibrium conditions were achieved rapidly. The enhancement of transport during the rapid theophylline uptake phase was energy dependent and attributed to PTS which transports spermine and was functional in the A549 cells (Supplementary information S2). The initial enhancement of uptake in the cell culture system with lack of further effect after the first measurement at 2 minutes was consistent with the timeframe for PTS uptake of the complex initially with the effect terminated after the actively transported ion pair dissociated. Definitive confirmation of PTS facilitated uptake was hindered by lack of a specific PTS inhibitor. However, alternative explanations such as active transport by other transporters and/or enhanced passive uptake of the ion-pair or were considered unlikely. Formation of the ion-pair adds two charged amines to the drug complex, which is not favourable for passive membrane transport<sup>26</sup>. In addition, recent studies have shown that the OCT mechanism is 30-fold less efficient at moving amines into cells compared to the PTS<sup>27</sup>. In the cell culture studies, the spermine concentration (SPE at  $3.3 \mu$ M) used exceeded the PTS Km (at  $0.02 \mu$ M), hence, the free spermine, which is required to force ion-pair formation, was competing for the saturated PTS with the ion-paired THE-SPE. However, theophylline

transport was more than 100 times slower than spermine transport and therefore an ion-pair binding ratio of 1 out of every 20 spermine molecules being ion-paired was sufficient to enhance cell uptake<sup>2</sup>.

The data from the IPL experiments supported the suggestion generated by the cell culture work, viz., that ion-pairing with spermine enabled active transport of theophylline into the lung tissue. Control experiments showed that the increased theophylline uptake was not a consequence of using a formulation with pH of 9.6, nor did the presence of unpaired spermine alter the lung uptake of theophylline. The effects of the ion-pair were more pronounced and sustained in the IPL compared to the cell culture model. This may have been a consequence of differences in (i) the method of delivery, i.e. constant replenishment by infusion of ion-paired drug in the IPL, (ii) levels of transporter activity in the cell vs intact organ models, (iii) access to the different cell types and tissue structures in the IPL<sup>28,29</sup>. The current work suggests for the first time that PTS will not only extract polyamines from the circulation into the lung, but can also transport therapeutic agents which can ion-pair to a polyamine. The effectiveness of the ion-pair formulation approach for enhancing theophylline uptake via PTS-mediated drug transport opens up more generalised possibilities to modify drug disposition in the lungs using similar technology. For example, ion-pairing could be used to reduce affinity for a promiscuous transporter such as p-glycoprotein, which would be applicable to a much wider range of drugs and could utilise a much wider range of counter ion candidates.

Theophylline has a narrow therapeutic index in the clinic, particularly when administered systemically for the acute treatment of asthma and chronic obstructive pulmonary disease (COPD). Our lung uptake data showed that the theophylline had a low affinity to the lung tissue (its tissue half-life was ca. 1 min). This supported previous data <sup>30</sup>, and suggested that the low extraction of the drug into the lung tissue was a contributing factor to its unfavourable pharmacokinetic profile <sup>31, 32</sup>. However, when theophylline was delivered as an ion-pair significantly more of the drug remained in the lungs after a 10 min wash out period compared with administration as free drug and this supported the notion that the ion-pair facilitated lung targeting. It is unknown whether this effect was a result of ion-pair persistence in the lungs or targeting to compartments in the lung tissue where the dissociated theophylline formed a reservoir. Even in the re-circulating IPL system in which the ion-pair complex almost completely dissociates after bolus administration, there was increased lung uptake after 25 min recirculation. The dynamics of ion-pair break down have previously been shown to be retarded

in the presence of buffers using the model agents anthracene and tetranitromethane <sup>33</sup>. However, in the absence of excess counter-ions the breakdown for these compounds still occurred rapidly, over  $\mu$ s, rather than the time course of the IPL experiments <sup>33</sup>. This discontinuity between the measurement of pharmaceutical ion-pair stability in model systems and those in biological systems is at present irresolvable because of the complexity of making accurate analytical measurements in the presence of proteins and salts.

#### Conclusion

Results from the cell culture and IPL experiments indicated that therapeutic ion-pairs formed with a PTS substrate as the counter ion can target drug to the lungs via the PTS system. Modifying the ion-pair delivery vehicle to increase the physical stability of the ion-pairs enhanced the uptake into the lung suggesting that formulation selection can modulate the polyamine-based uptake process. Using a non-covalent complex to target the PTS appears to have merit over covalently linked systems because the active agent is presented in its original form upon dissociation, which mitigates a number of pharmacological, toxicology and regulatory issues associated with the clinical use of covalently conjugated drug targeting systems. In the case of theophylline, selective delivery to the lungs would improve its therapeutic window and increase the safety of the drug when used in the treatment of asthma and COPD. However, the pH dependence and dynamics of theophylline ion-pairing means that it could not be used orally and thus it would be limited to intravenous use of theophylline for the treatment of acute asthma and COPD where a rapid selective delivery to lung tissue is required. It is promising however that the same approach can be applied to other therapeutic classes of compounds. For example, there are several anionic anticancer agents, which if ionpaired with polyamines could take advantage of the overexpression of the PTS system in rapidly dividing cells as a means of selective drug delivery.

#### Acknowledgments

The authors wish to acknowledge the funding of FB with a C W Maplethorpe Postdoctoral Fellowship provided by the University of London.

#### **Supporting Information**

Contains ion-pair micro speciation plot, spermidine cell uptake kinetics, the effect of formulation pH upon lung uptake of the ion-pair and clearnance data from the blood-steen infused lung.

#### References

- Hoet, P.H.M.; Nemery, B. Polyamines in the lung: polyamine uptake and polyaminelinked pathological or toxicological conditions. Am. J. Physiol. Lung Cell. Mol. Physiol. 2000, 278:L417-L433.
- Cullis, P.M.; Green, R.E.; Merson-Davies, L.; Travis, N. Probing the mechanism of transport and compartmentalisation of polyamines in mammalian cells. Chem. Bio. 1999, 6:717–729.
- 3) Rose, M.S.; Smith, L.L.; Wyatt, I. Evidence for energy dependent accumulation of paraquat into rat lung. Nature. **1974**, 252:314-315.
- Ross, J.H.; Krieger, R.I. Structure–activity correlations of amines inhibiting active uptake of paraquat (methyl viologen) into rat lung slices. Toxicol. Appl. Pharmacol. 1981, 59:238-249.
- 5) Marton, L.J.; Pegg, A.E. Polyamines as targets for therapeutic intervention. Annu. Rev. Pharmacol. Toxicol. **1995**, 35:55-91.
- 6) Tomasi, S.; Renault, J.; Martin, B.; Duhieu, S.; Cerec, V.; Le Roch, M.; Uriac, P.; Delcros, J.G. Targeting the polyamine transport system with benzazepine- and azepine-polyamine conjugates. J Med. Chem. 2010, 53:7647-7663.
- Wang, C.J.; Delcros, J.G.; Cannon, L.; Konate, F.; Carias, H.; Biggerstaff, J.; Gardner, R.A.; Phanstiel, O. Defining the molecular requirements for the selective delivery of polyamine conjugates into cells containing active polyamine transporters. J Med Chem. 2003, 24:5129-5138.
- 8) Hatanaka, T.; Kamon, T.; Morigaki, S.; Katayama, K.; Koizumi, T.J.J. Ion-pair skin transport of a zwitterionic drug, cephalexin. J. Control. Release. **2000**, 66:63-71.
- 9) Ivaturi, V.D.; Kim, S.K. Enhanced permeation of methotrexate in vitro by ion-pair formation with L-arginine. J. Pharm. Sci. **2009**, 98:3633-3639.
- 10) Neubert, R.; Dittrich, T. Ion-pair transport of ampicillin compared with the transport of other penicillins. Pharmazie. **1989**, 44:67-68.
- 11) Song, I.S.; Choi, M.K.; Shim,W.S.; Shim, C.K. Transport of organic cationic drugs: Effect of ion-pair formation with bile salts on the biliary excretion and pharmacokinetics. Pharmacol. Therap. **2013**, 138:142–154.
- 12) Nemery, B. The lungs as a target for the toxicity of some organophosphorus compounds Toxicology of Pesticides In: Toxicology of Pesticides. NATO ASI Series (Series H: Cell Biology); Costa, L.G.; Galli, C.L.; Murphy, S.D. (eds), Springer, Berlin, Heidelberg, 1987; pp 297-303, vol. 13.
- 13) Smith, L.L.; Wyatt, I.; Cohen, G.M. The accumulation of diamines and polyamines into rat lung slices. Biochem. Pharmacol. **1982**, 31:3029-30331.
- 14) Dinsdale, D., Preston, S.G.; Nemery, B. Effects of injury on [3H]putrescine uptake by types I and II cells in rat lung slices. Exp. Mol. Pathol. **1991**, 54: 218–229.
- 15) Sokol, P.P.; Longenecker, K.L.; Kachel, D.L.; Martin, W.J. Mechanism of putrescine transport in human pulmonary artery endothelial cells. J. Pharmacol. Exp. Ther. 1992, 265: 60–66.

- 16) Ehrhardt, C.; Bäckman, P.; Couet, W., Edwards, C.; Forbes, B.; Fridén, M.; Gumbleton, M.; Hosoya, K.I.; Kato, Y.; Nakanishi, T.; Takano, M.; Terasaki, T.; Yumoto, R. Current Progress Toward a Better Understanding of Drug Disposition Within the Lungs: Summary Proceedings of the First Workshop on Drug Transporters in the Lungs. J. Pharm Sci. 2017, S0022-3549, 17, 30243-5.
- 17) Babal, P.; Ruchko, M.; Olson, J.W.; Gillespie, M.N. Interactions between agmatine and polyamine uptake pathways in rat pulmonary artery endothelial cells. Gen. Pharmacol. 2000, 34, 255-261.
- 18) Van den Bogaard, E.H.; Dailey, L.A.; Thorley, A.J; Tetley, T.D.; Forbes, B. Inflammatory response and barrier properties of a new alveolar type 1-like cell line (TT1). Pharm. Res. **2009**, 26(5), 1172-80.
- 19) Martell, A.E.; Smith, R.M. Critical stability constants. Plenum Press, New York. 1989.
- 20) Tarulli, S. and Baran, E.J. Spectroscopic behavior of the 2 C = O stretching vibrations in free and complexed theophylline. Journal of Raman Spectroscopy. **1993**, 24 (3); 139-141.
- 21) Socrates, G. Infrared Characteristic Group Frequencies. John Wiley & Sons, New York. 1994.
- 22) Horvath, C.; Melander, W.; Nahum, A. Measurement of Association Constants For Complexes By Reversed-Phase High-Performance Liquid-Chromatography. Journal of Chromatography. 1979, 186; 371-403.
- 23) Forbes, B.; Wilson, C.G. and Gumbelton, M. Extraction of peptidase substrates by the isolated perfused rat lung. J. Pharm. Sci. **1995**. 1:569-572.
- 24) Kirschbaum, J. Self- Association of Theophylline in Aqueous Solution. J. Pharm. Sci. **1973**, 62:168.
- 25) Benaouda, F.; Brown, M.B.; Ganguly, S.; Jones, S.A.; Martin, G.P. Discriminating the molecular identity and function of discreet supramolecular structures in topical pharmaceutical formulations. Mol. Pharm. **2012**. 9:2505-2512.
- 26) Tronde, A. Drug Absorption from the Isolated Perfused Rat Lung–Correlations with Drug Physicochemical Properties and Epithelial Permeability. J. Drug Targeting. **2003**, 11:61–74.
- 27) Sala-Rabanal, M.; Li, D.C.; Dake, G.R.; Kurata, H.T.; Inyushin, M.; Skatchkov, S.N., Nichols, C.G. Polyamine Transport by the Polyspecific Organic Cation Transporters OCT1, OCT2, and OCT3. Mol. Pharmaceutics. **2013**, 10:1450–1458.
- 28) Bosquillon, C. Drug Transporters in the Lung—Do They Play a Role in the Biopharmaceutics of Inhaled Drugs? J. Pharm. Sci. **2010**, 99: 2240-2251.
- 29) Hoet, P.H.; Dinsdale, P.; Lewis, C.P. Kinetics and cellular localisation of putrescine uptake in human lung tissue. Thorax. **1993**, 48:1235-1241.
- 30) Kroll, F.; Karlsson, J.A.; Nilsson, E.; Ryrfeldt, A.; Persson, C.G.A. Rapid Clearance of Xanthines From Airway And Pulmonary Tissues. Am. Rev. Respir. Dis. **1990**, 141; 5:1167-1171.
- 31) Mitenko, P.A.; Olgilvie, R.I. Rational intravenous doses of theophylline. New England Journal of Medicine. **1973**, 289:600-603.
- 32) Ogilvie, R.I. Clinical Pharmacokinetics of Theophylline. Clinical Pharmacokinetics, **1978**, 3: 267-293.
- 33) Masnovi, J.M.; Kochi, J.K. Direct Observation of Ion-Pair Dynamics. J. Am. Chem. Soc. **1985**, 107:7893-7897.