
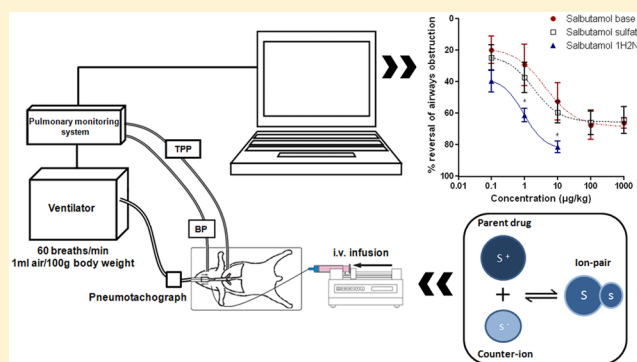


Using Salt Counterions to Modify  $\beta_2$ -Agonist Behavior *in Vivo*Aateka Patel,<sup>\*,†,‡</sup> Sandra D. Keir,<sup>†,‡</sup> Marc B. Brown,<sup>§,||</sup> Robert Hider,<sup>‡</sup> Stuart A. Jones,<sup>‡</sup> and Clive P. Page<sup>†,‡</sup><sup>†</sup>Sackler Institute of Pulmonary Pharmacology, Faculty of Life Sciences & Medicine, King's College London, 150 Stamford Street, London SE1 9NH, U.K.<sup>‡</sup>Institute of Pharmaceutical Science, Faculty of Life Sciences & Medicine, King's College London, 150 Stamford Street, London SE1 9NH, U.K.<sup>§</sup>MedPharm Ltd, Units 1 and 3, Chancellor Court, 50 Occam Road, Surrey Science Park, Guildford, Surrey GU2 7AB, U.K.<sup>||</sup>School of Pharmacy, University of Hertfordshire, College Lane, Hatfield, Hertfordshire AL10 9AB, U.K. Supporting Information

**ABSTRACT:** There is a paucity of data describing the impact of salt counterions on the biological performance of inhaled medicines *in vivo*. The aim of this study was to determine if the coadministration of salt counterions influenced the tissue permeability and airway smooth muscle relaxation potential of salbutamol, formoterol, and salmeterol. The results demonstrated that only salbutamol, when formulated with an excess of the 1-hydroxy-2-naphthoate (1H2NA) counterion, exhibited a superior bronchodilator effect ( $p < 0.05$ ) compared to salbutamol base. The counterions aspartate, maleate, fumarate, and 1H2NA had no effect on the ability of formoterol or salmeterol to reduce airway resistance *in vivo*. Studies using guinea pig tracheal sections showed that the salbutamol:1H2NA combination resulted in a significantly faster ( $p < 0.05$ ) rate of tissue transport compared to salbutamol base. Furthermore, when the relaxant activity of salbutamol was assessed *in vitro* using electrically stimulated, superfused preparations of guinea pig trachea, the inhibition of contraction by salbutamol in the presence of 1H2NA was greater than with salbutamol base (a total inhibition of 94.13%,  $p < 0.05$ ). The reason for the modification of salbutamol's behavior upon administration with 1H2NA was assigned to ion-pair formation, which was identified using infrared spectroscopy. Ion-pair formation is known to modify a drug's physicochemical properties, and the data from this study suggested that the choice of counterion in inhaled pharmaceutical salts should be considered carefully as it has the potential to alter drug action *in vivo*.

**KEYWORDS:** salbutamol, ion-pair, airways, counterion, pharmaceutical salt, 1H2NA



## 1. INTRODUCTION

A pharmaceutical salt is particularly susceptible to the composition of body fluids into which it dissolves because there is the potential that the drug can partake in a number of different noncovalent physical interactions. For example, if the pharmaceutical salt dissolves into a fluid that allows the drug to ionize, ion-pairs can be formed in solution.<sup>1</sup> Ion pairs usually arise from the drug interacting with coadministered salt counterions, which can in turn modify a drug's physicochemical behavior.<sup>2</sup>

Not every pharmaceutical salt will be susceptible to ion-pair formation upon dissolution in body fluids.<sup>3</sup> The formation of an ion-pair complex between the ionized drug and coadministered ionized counterion is dependent upon the physicochemical properties of the drug, the physicochemical properties of the ion-pair, the affinity of the two molecules, and the route of administration.<sup>1</sup> For example, when a pharmaceutical salt is administered orally, the drug is exposed to a relatively large

volume of liquid, and hence, if a labile, noncovalent bond is formed between the drug and the counterion, it will most probably be broken when faced by rapid dilution and competition from charged species in the gut fluids, unless the ion-pair binding is very strong. Such a scenario has led to the regulatory approval of several salt forms of the same drug for a single therapeutic indication when dosed orally because the different salt forms appear to be bioequivalent.<sup>4</sup> However, this does not mean that all salts are bioequivalent. The biological consequences of delivering therapeutic agents as pharmaceutical salts via routes which do not expose the molecules to such a large volume of fluid, e.g., the skin and the airways, may be more significant compared to oral administration.

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Previous literature has suggested that changes in drug transmembrane permeation as a result of ion-pair formation has a major influence on the ability of salt counterions to modify the parent drug's behavior.<sup>5–7</sup> In addition, data generated *in vitro* has suggested that ion-pairs can remain intact upon crossing biological barriers,<sup>8</sup> and therefore it may be possible that a parent drug's behavior is influenced by a salt counterion, even after absorption into the body. However, there appears to be very limited information about the consequence of ion-pair formation *in vivo*.<sup>9</sup> This is surprising given the number of pharmaceutical salts that are used clinically, and it suggests that further research into the manner of how coadministered counterions influence the biological behavior of pharmaceutical salts is required.

The primary aim of this study was to investigate the potential of a coadministered counterion to alter the biological behavior of a drug, which forms a pharmaceutical salt, *in vivo*. The inhaled route was chosen for this study due to the low volume of liquid in which the drug and counterion dissolve and the fact that the ability of an ion-pair to alter the physicochemical properties of an inhaled drug has already been established.<sup>10</sup> The  $\beta_2$ -adrenoceptor (AR) agonists salbutamol, salmeterol, and formoterol were selected as the drug molecules in this work because they are all commercially available as pharmaceutical salts and are widely used clinically for the treatment of asthma and chronic obstructive pulmonary disease (COPD).<sup>11</sup> In addition, salbutamol has already been studied *in vitro* with a number of counterions including sulfate,<sup>10,12</sup> adipate, stearate,<sup>10</sup> succinate,<sup>12</sup> acetate monomethanolate,<sup>13</sup> and hydrochloride,<sup>14</sup> and this provided vital information upon which to base the *in vivo* work. As the physical and chemical properties of the salbutamol salts have already been characterized<sup>10,12,15</sup> this study initially focused on the pharmacodynamic consequences of drug-counterion coadministration. However, to better understand the drug-counterion mixtures that elicited changes in pharmacodynamics responses, an attempt was made to characterize the strength of the drug-counterion interactions, to elucidate the effects of the counterions upon tissue permeation, and to understand the influence of the counterions upon the drug's pharmacological action. The counterions aspartate, maleate, fumarate, and 1H2NA were selected to represent a series of compounds that were used clinically, displayed different physicochemical properties, and because they had the potential to form ion-pairs with the  $\beta_2$ -AR agonists. The drug-counterion combinations used in the study were based upon the molecules' compatibility in the solution state at the concentrations that were used in the work. As it was anticipated that the interaction affinity between the drug and counterions would be relatively weak, the therapeutic agents were administered in all the studies with an excess of counterions to maximize the interactions between the  $\beta_2$ -AR agonist and the counterions. While it was accepted that this does not necessarily mimic the use of a pharmaceutical salt *in vivo*, as they are typically presented to the body with lower counterion concentrations, the experiments were designed in this manner to continually present the test agents to different *in vitro* and *in vivo* systems in a similar state, even when the fluids used in assays varied. This was an essential experimental design trait of this work that allowed the study data to be directly compared across the test systems. In addition, to mimic the inhaled administration of "pharmaceutical salts", which typically are produced as powders, a production method for each drug-counterion combination would be required to produce the

lowest energy salt form in the solid state. This would be a considerable piece of work and the reporting of the physical data, which would be needed to support the use of these compounds, would detract from the *in vivo* aspect of the study which was our focus. Therefore, simple solutions of the drugs and the counterions were used as the test samples in this work.

## 2. MATERIALS AND METHODS

**2.1. Materials and Reagents.** Salbutamol base (BN. H80619) was a kind gift from Cipla Ltd., Mumbai, India. Salbutamol sulfate (BN. B027798) was obtained from GlaxoSmithKline Research and Development, Ware, U.K. Salmeterol xinafoate (BN. SX-0081010) was purchased from Vamsi Laboratories, India. Salmeterol base and formoterol base were synthesized by Tocris Cookson Ltd., Avonmouth, Bristol, U.K. Formoterol fumarate dihydrate (BN. 2700M0 A0031421) was obtained from Arena Pharmaceuticals, Buckingham, U.K. Phosphate buffered saline (PBS) tablets (0.15 M, pH 7.3) were purchased from Oxoid, Basingstoke, U.K. Whatman nylon filters (pore size 0.2  $\mu\text{m}$ , diameter 47 mm) were purchased from Fisher Scientific Ltd., Loughborough, U.K. Methanol (Fisher Scientific Ltd., U.K.) and ammonium acetate (Sigma-Aldrich Company Ltd., Poole, Dorset, U.K.) were of high performance liquid chromatography (HPLC) grade. Indomethacin, sodium hydroxide, hydrochloric acid, 1-hydroxy-2-naphthoate (1H2NA), bombesin acetate, maleic acid, L-aspartic acid, sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium sulfate, sodium bicarbonate, potassium dihydrogen phosphate, and D-glucose were all purchased from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K., and were of analytical grade.

**2.2. Animals.** Experiments were conducted under a project license issued by the United Kingdom Home Office in accordance with the United Kingdom Animal Scientific Procedures Act, 1986. Protocols were approved by the Local Ethical Review Committee of King's College London. All *in vivo* experiments were performed with male Dunkin-Hartley guinea pigs (250–500 g, Charles River). Guinea pigs were housed in animal holding facilities maintained at 18–19 °C with a standard 12 h light–dark cycle. A maximum of six guinea pigs were housed per cage, containing sawdust and hay with cardboard tunnels in order to provide environmental enrichment. Animals were fed *ad libitum* on a FD1 guinea pig diet (Special Diets Services, Witham, Essex, U.K.) with free access to water.

**2.3. Lung Function Studies.** Male Dunkin-Hartley guinea pigs (250–500 g) were anesthetized with urethane (25% w/v, 7 mL  $\text{kg}^{-1}$ , ip), and a neuromuscular blocker was administered to induce skeletal muscle paralysis (succinylcholine chloride, 1 mg  $\text{kg}^{-1}$ , im). The trachea, carotid artery, and both jugular veins were cannulated for measurement of airway obstruction and systemic blood pressure and for iv administration of test substances, respectively. An iv infusion was used to dose the ion-pairs, as this minimized the potential of nonequivalent doses reaching the tissue as a consequence of differences in deposition and absorption via the inhaled route. The tracheal cannula was attached to a pneumotachograph that was in turn connected to a pressure transducer ( $\pm 2$  cm  $\text{H}_2\text{O}$ , model MP-45-14-871, Validyne Engineering). Changes in airflow were measured using a lung function recording system (version 9.2; Mumed Systems, London, U.K.) and were displayed in real time on a personal computer. The flow signal was integrated to give a measure of tidal volume. A cannula was inserted into the

thoracic cavity between the third and fifth ribs and connected to the negative side of the pressure transducer ( $\pm 20$  cm H<sub>2</sub>O, Validyne). The positive side of the pressure transducer was connected to the side of the pneumotachograph proximal to the animal to obtain a measure of transpulmonary pressure (difference between mouth and thoracic pressure). The lung function parameter, total airway resistance ( $R_{L}$ ; cm of water per L per s), was derived from each measure of flow, tidal volume, and transpulmonary pressure by integration. Animals were allowed to acclimatize for 10 min prior to the start of the study.

For bronchodilator experiments, bronchoconstriction was induced by means of continuous iv administration of bombesin ( $2\text{--}4$   $\mu\text{g kg}^{-1}$ ), which elicited sustained submaximal increases in total airway resistance, as described elsewhere.<sup>16</sup> After stable bronchoconstriction had been obtained, salbutamol was formulated with sulfate as it was supplied (2:1 ratio) at 0.1, 1, 10, 100, or 1000  $\mu\text{g kg}^{-1}$ , and 1H2NA at a molar ratio of 1:10. Formoterol fumarate and salmeterol xinafoate were used as supplied at 0.1, 1, 10, or 100  $\mu\text{g kg}^{-1}$ . Formoterol base and salmeterol base were formulated with maleate or aspartate at a ratio of 1:10. The dose was increased when the effect of the previous dose had reached a plateau. Bronchodilation was calculated as the percentage reversal of the increase in total lung resistance induced by bombesin. Data were expressed as arithmetic means  $\pm$  standard error of the mean (SEM).

**2.4. Salbutamol Counterion Interaction Studies.** For the analysis of salbutamol in the presence of the hydrophobic counterion 1H2NA a UV/vis spectrophotometer (PerkinElmer Ltd., Beaconsfield, U.K.) was used. Standard stock solutions of salbutamol base and 1H2NA were prepared in deionized water (pH 6.5 measured using a pH meter) and adjusted using 0.1 M sodium hydroxide and/or hydrochloric acid. Solutions of salbutamol base and 1H2NA were mixed to achieve a concentration range of 0.005–0.84 mM for 1H2NA and a constant salbutamol base concentration of 0.42 mM, made up to volume using deionized water. The final pH of the samples was checked and adjusted to pH 6.5. Samples were scanned between 190 and 400 nm using a UV quartz cuvette (path length 10 mm). A background reading of a “blank” solution was preceded by three replicate readings of each sample. The spectral data was converted into an Excel file for analysis.

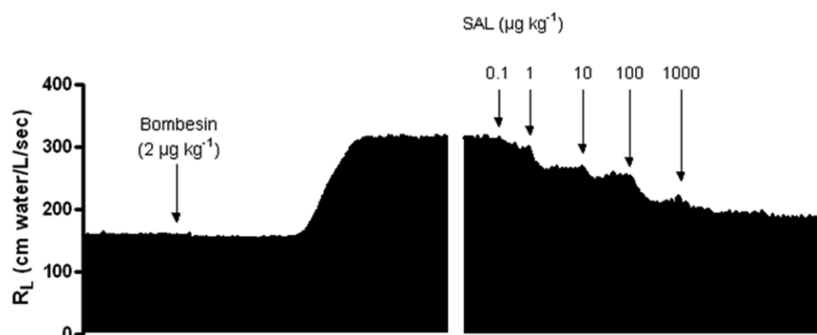
For the infrared (IR) studies, solutions of salbutamol base and salbutamol in the presence of sulfate were prepared in deuterated water and deuterated methanol (80:20). Solutions of salbutamol base and salbutamol in the presence of 1H2NA were prepared in a 70:30 ratio of deuterated methanol to deuterated water. All samples were adjusted to pH 6.5 using 0.1 M sodium hydroxide and/or hydrochloric acid, and checked again at the end of the study. Spectra were obtained with the aid of a universal Omni-Cell system (Specac Ltd., Orpington, Kent, U.K.) for the analysis of liquids equipped with calcium fluoride (CaF<sub>2</sub>) windows (Mylar spacer at 0.025 mm path length). A double solvent subtraction procedure was employed for all spectra. Samples were scanned on average 32 times, with a resolution of 4  $\text{cm}^{-1}$  over the 550–4000  $\text{cm}^{-1}$  range. Data was recorded using a Spectrum One spectrometer, and spectral analysis was performed with Spectrum version 5.3.1 software (PerkinElmer Ltd., Beaconsfield, U.K.).

**2.5. Lung Tracheal Transport Studies.** Transport experiments were carried out in unjacketed, individually calibrated, upright Franz diffusion cells (MedPharm Ltd., U.K.). Guinea pigs were killed by a blow to the head, followed by exsanguination; the trachea was excised and placed in cold

physiological saline (0.9% sodium chloride, pH 5.5). The adherent connective tissue was dissected away and the lumen gently flushed with saline. The trachea was dissected into rings consisting of 5–6 cartilage bands and mounted between the donor and receiver chambers, which were sealed together using parafilm. Small magnetic bars were inserted into each of the receiver compartments to ensure adequate mixing. The receiver compartments were filled with physiological saline (0.9% sodium chloride, pH adjusted to 7.4), and each cell was checked by inversion for leaks. The assembled Franz cells were placed in a 37 °C water bath and allowed to equilibrate for 60 min prior to addition of 300  $\mu\text{L}$  of the donor solution (salbutamol base, salbutamol sulfate (commercial salt), or salbutamol in the presence of 1H2NA). The salbutamol base concentration was fixed at 0.00178 M for all test systems. An excess of 1H2NA was presented at a concentration of  $17.8 \times 10^{-3}$  M to mimic the *in vivo* studies. All samples were made up to volume using deionized water (pH 7.4), and the final pH was adjusted to 7.4 using 0.1 M sodium hydroxide or hydrochloric acid. Samples (0.2 mL) were taken for reverse phase high performance liquid chromatography (RPHPLC) analysis (method I or II detailed below) at 0 (prior to addition of the donor solution), 15, 30, 45, 60, 120, 180, 240, 300, 360, 1320, 1380, and 1440 min after the donor solution was added to the cells. Following sampling each receiver cell compartment was replaced with fresh prewarmed physiological saline (0.9% sodium chloride, pH adjusted to 7.4) of equal volume. The cumulative amount of drug ( $\mu\text{g}$ ) transported through the trachea per unit area ( $\text{cm}^2$ ) was corrected for sample removal and plotted against time (min). Steady-state flux was calculated using the line of best fit over a minimum of five time points with a linearity of  $R^2 \geq 0.97$  and displayed as mean  $\pm$  standard deviation (SD). Where appropriate, a two-tailed, unpaired, Student's *t*-test was used for statistical analysis with a 95% confidence interval.

**2.6. Reverse Phase High Performance Liquid Chromatography (RPHPLC).** **2.6.1. Method I.** Separation of salbutamol base and salbutamol sulfate was achieved using a SphereClone ODS2 (250  $\times$  4.6 mm, particle size: 5  $\mu\text{m}$ ) column (Phenomenex Ltd., Cheshire, U.K.) maintained at 50 °C using a thermostated column compartment, TCC-100 (Dionex Corporation, Sunnyvale, CA, USA). The mobile phase was a mixture of aqueous ammonium acetate solution 0.1% and methanol (80:20) at pH 4.5, filtered through a 0.2  $\mu\text{m}$  nylon membrane and degassed by sonication. The mobile phase flow rate was 1.0  $\text{mL min}^{-1}$ , the injection volume was 20  $\mu\text{L}$ , and detection was set at 272 nm. Quantitative determination of the active substance was performed using a RPHPLC system consisting of a Dionex P680 pump, a Dionex PDA-100 photodiode array detector, and a Dionex ASI-100 automated sampler injector with Chromeleon Client version 6.60 for analysis (Dionex Corporation, Sunnyvale, CA, USA).

**2.6.2. Method II.** Separation of salbutamol base and salbutamol in the presence of 1H2NA was achieved using a SphereClone ODS2 column, maintained at 50 °C using a thermostated column block heater TCC-100. The mobile phase was a mixture of aqueous ammonium acetate solution 0.1% and methanol (80:20) at pH 6.0, filtered through a 0.2  $\mu\text{m}$  nylon membrane and degassed by sonication. The stationary phase was used with a 1.2  $\text{mL min}^{-1}$  flow rate and a 20  $\mu\text{L}$  injection volume. The compounds were analyzed at a wavelength of 276 nm.



**Figure 1.** Representative trace of changes in total lung resistance following iv administration of bombesin ( $2 \mu\text{g kg}^{-1}$ ), followed by iv administration of salbutamol base (SAL) at regular intervals (0.1, 1, 10, 100, and  $1000 \mu\text{g kg}^{-1}$ ).

Both RPHPLC methods were shown to be fit for purpose in terms of precision ( $\leq 2\%$ ), accuracy ( $\geq 99\%$ ), linearity ( $R^2 > 0.9999$ ), and sensitivity (limit of detection  $< 0.53 \mu\text{g mL}^{-1}$ ; limit of quantification  $< 1.78 \mu\text{g mL}^{-1}$ ). A two-tailed, unpaired, Student's *t*-test determined no significant ( $p > 0.05$ ) differences of the average retention times between salbutamol base assayed alone or in the presence of counterions.

**2.7. In Vitro Contractility of Isolated Guinea Pig Trachea.** Superfusion of guinea pig tracheal rings was performed according to a previously described method.<sup>17,18</sup> Guinea pigs were sacrificed by cervical dislocation, and the tracheas were excised and cut into rings. Each tracheal ring (3–5 cartilage bands) was opened by sectioning the ring opposite the smooth muscle and sectioned to form strips. A cotton thread was attached to the cartilage at one end of the strip for attachment to the tension gauge, and a cotton loop to the other end for anchoring the tissue to a fixed stainless steel hook. The tissue was then suspended between two platinum electrodes under 1 g tension and superfused at a rate of  $3 \text{ mL min}^{-1}$  (Watson-Marlow Sci-Q 323S peristaltic pump and a 318MC eight roller five channel micro pump head, Cornwall, U.K.) through a jacketed, glass heat exchanger with Krebs–Henseleit (K–H) solution ( $37^\circ\text{C}$ ,  $5\% \text{ CO}_2$ ,  $95\% \text{ O}_2$ ) containing the cyclooxygenase inhibitor indomethacin ( $5 \mu\text{M}$ ) to inhibit the release of endogenous prostaglandins and reduce spontaneous tone of the airway smooth muscle.

K–H solution (pH 7.2–7.4) was composed of 118 mM sodium chloride, 4.7 mM potassium chloride, 2.5 mM calcium chloride dihydrate, 1.2 mM magnesium sulfate, 25 mM sodium bicarbonate, 1.2 mM potassium dihydrogen phosphate, and 11.1 mM D-glucose made up to volume in deionized water.

The tracheal preparations were equilibrated for 40 min before commencement of electrical field stimulation (EFS) delivered as 10 s trains of square wave pulses at 3 Hz, 0.1 ms duration, and 30 V generated every 100 s by means of a physiological square wave stimulator (Digitimer MultiStim System-D330, Digitimer Ltd., Herts, U.K.). Changes in tension detected by the tension gauge were recorded using a Powerlab/8sp (AD Instruments, Castle Hill, Australia). Ten minutes after the initiation of airway smooth muscle stimulation, the trachea was superfused with K–H solution containing the drug of interest by slow infusion. The drug was delivered at a rate of  $0.275 \text{ mL min}^{-1}$  using a syringe pump (Original-Perfusor-Spritze, B. Braun Medical, Melsungen, Germany) to which a butterfly-25G short winged infusion tube (Abbott, Sligo, Republic of Ireland) was attached for 20 min, after which superfusion with drug was terminated. The tissues were then superfused with drug free K–H solution, and tension was

monitored for a further 6 h. The maximum–minimum (g) peak height for the period of contraction was calculated offline by using LabChart 7 Reader software (AD Instruments, Hastings, U.K.).

All EFS data are expressed as percentage inhibition of the contractile response obtained prior to the administration of the drug and displayed as mean  $\pm$  standard error of the mean (SEM). Onset time ( $\text{OT}_{50}$  and  $\text{OT}_{75}$ ) was expressed as the time taken from administration of the drug to achieve 50% or 75% control (predrug) value (minutes) and was determined by interpolation from the plot of percentage against time to attain 50% or 75% inhibition of the response. Likewise, the recovery (or offset) time ( $\text{RT}_{50}$  and  $\text{RT}_{75}$ ) was expressed as the time taken from stopping the administration of the drug to achieve 50% or 75% recovery of the contractile response (predrug) value (minutes). It was determined by interpolation from a plot of percentage response (% control) against time to attainment of 50% or 75% recovery from that response.

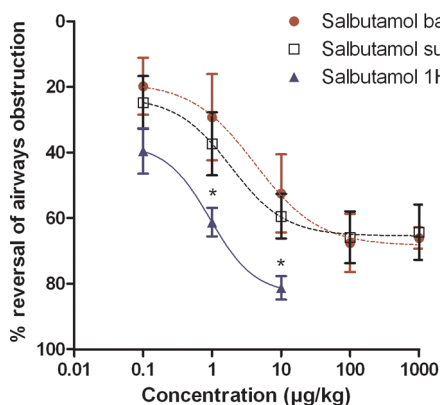
Data was analyzed using a Kruskal–Wallis nonparametric one way analysis of variance (ANOVA) with post hoc Dunn's multiple comparison test. The final concentration of salbutamol base on the tissue remained constant at  $1 \mu\text{M}$ . The hydrophobic counterion, 1H2NA, was added to salbutamol base ( $1 \mu\text{M}$ ) in excess of 10-fold ( $10 \mu\text{M}$ ). Salbutamol sulfate was at a total concentration of  $1 \mu\text{M}$ . All samples were made up to volume in deionized water and adjusted to pH 7.4 with 1 M sodium hydroxide and/or hydrochloric acid.

**2.8. Data and Statistical Analysis.** All data analysis was performed using Graphpad Prism (Prism, version 5; GraphPad Software Inc., San Diego, CA). Data was analyzed for normality using the Anderson–Darling test. Where appropriate, a two-tailed, unpaired, Student's *t*-test was used and statistically analyzed at the 95% confidence level. *In vitro* data was analyzed using a one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test. *In vivo* data was analyzed using a one way analysis of variance (ANOVA) with a post hoc Tukey's multiple comparison test or a two way ANOVA with post hoc Bonferroni test and considered significant at a *p* value less than 0.05.

### 3. RESULTS

**3.1. The Effect of Salbutamol Base, Salbutamol Sulfate, and Salbutamol in the Presence of 1H2NA on Lung Function *in Vivo*.** An iv infusion of the peptide bombesin induced an increase in airway resistance in all animals (Figure 1). A 100% increase in resistance was desired, and the dose of bombesin was adjusted accordingly. Bombesin induced airway obstruction by 85–123% in the animals.

Salbutamol base (0.1, 1, 10, 100, and 1000  $\mu\text{g kg}^{-1}$ ), salbutamol sulfate (0.1, 1, 10, 100, and 1000  $\mu\text{g kg}^{-1}$ ), and salbutamol in the presence of 1H2NA (0.1:1, 1:10, and 10:100  $\mu\text{g kg}^{-1}$ ) all elicited a dose-related reduction in the bombesin-induced resistance (Figure 2). These data seem to suggest that



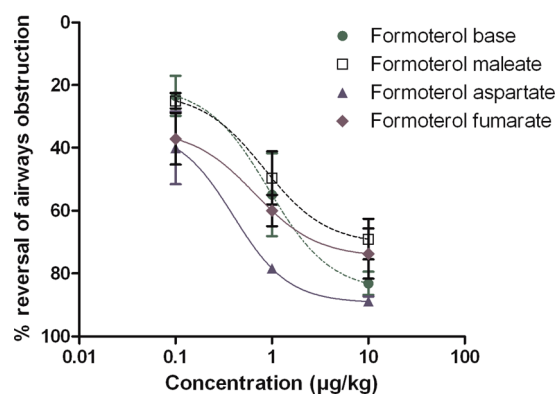
**Figure 2.** Bronchodilating effect of salbutamol base (0.1, 1, 10, 100, and 1000  $\mu\text{g kg}^{-1}$ , pH 7.4), salbutamol sulfate (0.1, 1, 10, 100, and 1000  $\mu\text{g kg}^{-1}$ , pH 7.4), and salbutamol in the presence of 1H2NA (0.1:1, 1:10, and 10:100  $\mu\text{g kg}^{-1}$ , pH 7.4) on bombesin-induced increase in total lung resistance. Points represent mean  $\pm$  SEM;  $n = 3-7$ . Two way ANOVA with post hoc Bonferroni test, (\*)  $p < 0.05$  vs salbutamol base.

the bronchodilator effect of the different salbutamol products differed between the treatment groups (Figure 2, Figure S1). Salbutamol in the presence of 1H2NA at 10  $\mu\text{g kg}^{-1}$  reversed peak resistance by  $81.30 \pm 3.59\%$  ( $n = 7$ ) ( $p < 0.05$ , two way ANOVA with post hoc Bonferroni test vs salbutamol base), whereas at a similar dose, salbutamol sulfate only reversed peak resistance by  $59.44 \pm 6.78\%$  ( $n = 5$ ), and salbutamol base by only  $52.47 \pm 11.92\%$ .

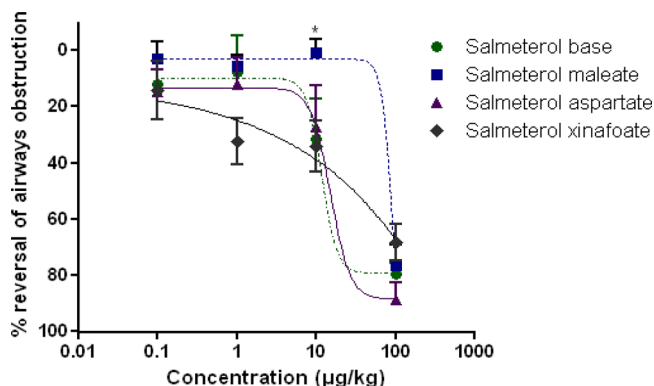
**3.2. The Effect of Counterions on Lung Function upon Administration of Formoterol and Salmeterol.** Formoterol base (0.1, 1, and 10  $\mu\text{g kg}^{-1}$ ), formoterol fumarate (0.1, 1, and 10  $\mu\text{g kg}^{-1}$ ), formoterol in the presence of maleate, and formoterol in the presence of aspartate (0.1:1, 1:10, and 10:100  $\mu\text{g kg}^{-1}$ ) all elicited a dose-related reduction in resistance induced by bombesin (Figure 3, Figure S2). However, the percentage reversal of airway obstruction of the different formoterol products was very similar (Figure 3).

Salmeterol base appeared to have a very different dose-response profile compared to salbutamol and formoterol; for example, salmeterol displayed very little relaxation of the tissue at 0.1 and 1  $\mu\text{g kg}^{-1}$  unlike the other bronchodilators (Figure 4, Figure S3). However, as with formoterol, the presence of counterions did not seem to influence the performance of salmeterol, i.e., all the dose response profiles were similar both with and without the salt counterions (Figure 4). Only salmeterol maleate showed a significantly slower effect at reversing peak resistance compared to salmeterol xinafoate, but only at 10  $\mu\text{g kg}^{-1}$  ( $p < 0.05$ , two-tailed unpaired, Student's  $t$ -test) (Figure 4).

**3.3. Salbutamol Counterion Interaction Studies.** Using Fourier transform infrared spectroscopy (FT-IR) measurements the salbutamol base sample showed a peak at  $1606 \text{ cm}^{-1}$  in solution, which was assigned to the secondary amine (Figure 5). A significant shift in this secondary amine bend was observed for salbutamol base ( $1606 \text{ cm}^{-1}$ ) compared to

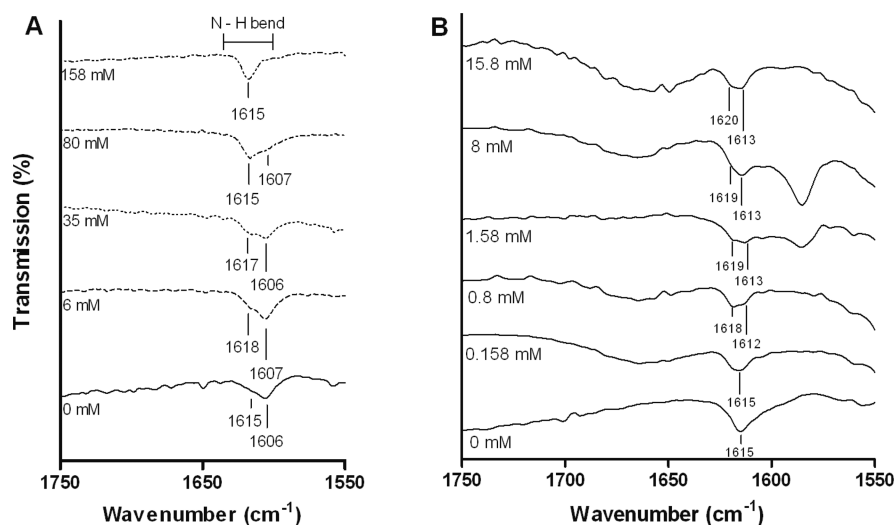


**Figure 3.** Bronchodilator effect of formoterol base (0.1, 1, and 10  $\mu\text{g kg}^{-1}$ , pH 7.4), formoterol fumarate (0.1, 1, and 10  $\mu\text{g kg}^{-1}$ , pH 7.4), formoterol in the presence of maleate, and formoterol in the presence of aspartate (0.1:1, 1:10, and 10:100  $\mu\text{g kg}^{-1}$ , pH 7.4) on bombesin-induced increases in total lung resistance. Points represent mean  $\pm$  SEM;  $n = 3-4$ .



**Figure 4.** Bronchodilator effect of salmeterol base (0.1, 1, 10, and 100  $\mu\text{g kg}^{-1}$ , pH 7.4), salmeterol xinafoate (0.1, 1, 10, and 100  $\mu\text{g kg}^{-1}$ , pH 7.4), salmeterol in the presence of maleate, and salmeterol in the presence of aspartate (0.1:1, 1:10, 10:100, and 100:1000  $\mu\text{g kg}^{-1}$ , pH 7.4) on bombesin-induced increase in total lung resistance. Points represent mean  $\pm$  SEM;  $n = 3-5$ . Two-tailed unpaired, Student's  $t$ -test. (\*)  $p < 0.05$  vs salmeterol xinafoate.

salbutamol presented as the commercial sulfate ( $1617 \text{ cm}^{-1}$ ) (Figure S4). The N-H bend ( $\sim 1616 \text{ cm}^{-1}$ ) of salbutamol was also modified with increasing concentrations of sulfate (Figure 5). At 35 mM sulfate, a peak height ratio ( $1606$  and  $1617 \text{ cm}^{-1}$ ) change was detected. The peak height ratio continued to change with increasing sulfate concentrations until the  $1606 \text{ cm}^{-1}$  peak disappeared with 158 mM sulfate. The peak height ratio, determined using the peaks at  $\sim 1606 \text{ cm}^{-1}$  and  $\sim 1618 \text{ cm}^{-1}$ , was used to calculate the percentage of salbutamol bound to sulfate in solution, and this was plotted against the free sulfate concentration in order to calculate a log affinity constant of  $0.85 \pm 0.03$ , assuming a 1:1 association (note: we did not consider a 1:2 salbutamol sulfate ratio to be relevant in solution due to the weak binding and the relatively low amount of counterion present). In the presence of 1H2NA the spectral splitting of the  $1615 \text{ cm}^{-1}$  peak indicated that an ion-pair was formed. However, these spectral changes occurred at a 10-fold lower concentration compared to the sulfate counterion. Unlike for the sulfate counterion the overlapping signals made it impossible to calculate the peak ratio and construct a binding curve (Figure 5). Attempts were made to use UV/vis



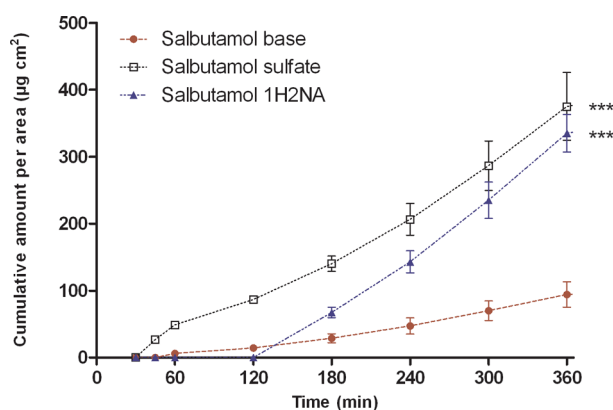
**Figure 5.** FT-IR spectra of (A) salbutamol base in the presence of increasing sulfate and (B) salbutamol base in the presence of increasing 1H2NA concentrations in the liquid state (80% deuterated water and 20% deuterated methanol, pH 6.5). The number denotes the concentration (mM) of counterion present against a fixed salbutamol base concentration (42 mM).

spectroscopy to support the IR data, but the salbutamol base lambda max at 276.43 nm did not shift (Figure S5). To facilitate the subsequent *in vitro* and *in vivo* studies, the HYSS microspeciation software (Protonic Software, U.K.) was used to calculate the optimum ratio for salbutamol:counterion that would generate ion-pairs at pH 7.4 employing the measured affinity constant for salbutamol sulfate (given that the 1H2NA binding was ca. 10-fold stronger). These results suggested that at a 1:10 drug to counterion molar ratio with the ion-paired species would be formed (data not shown).

**3.4. Salbutamol Transport Across Biological Membranes.** The flux of the commercial salbutamol sulfate mixture through the tracheal tissue was  $1.17 \pm 0.27 \mu\text{g}/\text{cm}^2/\text{min}$  (Figure 6). This flux for salbutamol sulfate was similar to that for salbutamol in the presence of 1H2NA ( $1.49 \pm 0.14 \mu\text{g}/\text{cm}^2/\text{min}$ ), but both systems containing the counterions displayed a significantly greater flux than that of salbutamol base alone at  $0.34 \pm 0.07 \mu\text{g}/\text{cm}^2/\text{min}$  ( $p < 0.0001$ , two-tailed, unpaired, Student's *t*-test). The cumulative mass of salbutamol

base (0.00178 M) that transported into the receiver solution was  $97.30 \pm 18.94 \mu\text{g}/\text{cm}^2$  at 360 min (Figure 6). The cumulative mass of salbutamol (0.00178 M) that transported across the guinea pig tracheal tissue when presented with an excess of the hydrophobic counterion, 1H2NA (0.0178 M), at 360 min was  $335.08 \pm 28.16 \mu\text{g}/\text{cm}^2$ , and this was significantly greater ( $p < 0.0001$ , two-tailed, unpaired, Student's *t*-test) than that of salbutamol base alone (Figure 6). The lag time to steady-state flux of salbutamol in the presence of 1H2NA was significantly higher than that of the base ( $138.91 \pm 8.27$  min with 1H2NA vs  $87.60 \pm 5.54$  min for the base;  $p < 0.0001$ , two-tailed, unpaired, Student's *t*-test), whereas the lag time for salbutamol sulfate crossing the lung tracheal tissue was lower than that for the base at  $28.97 \pm 6.52$  min ( $p < 0.0001$ , two-tailed, unpaired, Student's *t*-test). The apparent permeability ( $P_{\text{app}}$  (cm/s)) of salbutamol base in the presence of 1H2NA and in the presence of sulfate was also significantly greater than that of the base alone ( $p < 0.0001$ , two-tailed, unpaired, Student's *t*-test) (Table 1).

**3.5. The Inhibitory Actions of Salbutamol, Salbutamol Sulfate, and Salbutamol in the Presence of 1H2NA on EFS-Induced Contractions of Isolated Guinea Pig Tracheal Smooth Muscle.** The superfusion of salbutamol base (1  $\mu\text{M}$ ) in deionized water (pH 7.4) caused an  $84.24 \pm 0.64\%$  inhibition of the tissues with an  $\text{OT}_{50}$  of  $5.00 \pm 0.00$  min and an  $\text{OT}_{75}$  of  $8.75 \pm 1.03$  min. Both the  $\text{OT}_{50}$  and  $\text{OT}_{75}$  for salbutamol sulfate were significantly faster compared to salbutamol base alone ( $p < 0.01$  and  $p < 0.05$  respectively, one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test) (Table 2). However, the total percentage inhibition of the tissue superfused with salbutamol sulfate was similar to that of the base ( $p > 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). The tissue recovery indices of the base and sulfate forms of salbutamol were also similar ( $p > 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). All of the tissues recovered to 100% of the predrug value, and the tissues that had been treated with salbutamol were not significantly different ( $p > 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test) compared to the drug free controls (Table 2).



**Figure 6.** Transport of salbutamol base (0.00178 M, pH 7.4), salbutamol sulfate (0.00178 M:0.00037 M, pH 7.4), and salbutamol in the presence of excess 1H2NA (0.00178:0.0178 M, pH 7.4) across guinea pig lung tracheal tissue into saline (pH 7.4). Each point represents the mean cumulative mass per area  $\pm$  SD ( $n = 5-12$ ). Two-tailed, unpaired, Student's *t*-test, (\*\*\*)  $p < 0.0001$  vs salbutamol base.

**Table 1. Flux ( $\mu\text{g}/\text{cm}^2/\text{min}$ ) and Apparent Permeability ( $P_{\text{app}}$ ) of Salbutamol Base, Salbutamol Sulfate, and Salbutamol in the Presence of Excess 1H2NA, through Guinea Pig Tracheal Tissue into Saline pH 7.4 at 37 °C<sup>a</sup>**

drug	detection (min)	slope vs time (min)	flux ( $\mu\text{g}/\text{cm}^2/\text{min}$ )	$R^2$	$P_{\text{app}}$ (cm/s)	$n$
salbutamol base	60	120–360	$0.34 \pm 0.07$	0.9992	$1.31 \pm 0.28 \times 10^{-5}$	5
salbutamol sulfate (commercial)	30	60–360	$1.17 \pm 0.27$	0.9717	$4.57 \pm 1.08 \times 10^{-5}$	12
salbutamol 1H2NA	180	180–360	$1.49 \pm 0.14$	0.9964	$5.84 \pm 0.57 \times 10^{-5}$	5

<sup>a</sup>The concentration of salbutamol remained constant at 0.00178 M. Each value of the flux represents the mean  $\pm$  standard deviation.

**Table 2. Percentage Control (%) of Tissues after Drug Addition, and Percentage Tissue Recovery at 360 min Post Dosing, Onset Time ( $OT_{50}$ ,  $OT_{75}$ ) To Achieve 50% or 75% Control (Predrug) Value and Recovery Time ( $RT_{50}$ ,  $RT_{75}$ ) to Return to 50% or 75% of Control (Predrug) Value (min) for Salbutamol Base (SAL, 1  $\mu\text{M}$ ), Salbutamol Sulfate (SAL Sulfate, 1  $\mu\text{M}$ ), and Salbutamol in the Presence of 1H2NA (1  $\mu\text{M}$ :10  $\mu\text{M}$ ) at pH 7.4 in Isolated Guinea Pig Trachea<sup>a</sup>**

	test substance				
	control	water	SAL	SAL sulfate	SAL + 1H2NA
$OT_{50}$ (min)			$5.00 \pm 0.00$	$2.51 \pm 0.00^{**}$	$4.37 \pm 0.62$
$OT_{75}$ (min)			$8.75 \pm 1.03$	$3.89 \pm 0.97^*$	$4.38 \pm 0.84^*$
control (%)	$108.19 \pm 5.90$	$106.04 \pm 4.97$	$15.79 \pm 0.64$	$17.98 \pm 2.33$	$5.87 \pm 1.96^{**}$
$RT_{50}$ (min)			$53.34 \pm 2.64$	$53.34 \pm 0.00$	$51.25 \pm 2.08$
$RT_{75}$ (min)			$84.17 \pm 2.50$	$74.17 \pm 0.00$	$61.67 \pm 0.00^{**}$
control (360 min; %)	$108.19 \pm 3.74$	$104.88 \pm 4.89$	$101.97 \pm 2.13$	$99.07 \pm 2.11$	$102.64 \pm 2.98$
N	5	3	5	3	4

<sup>a</sup>Results expressed as mean  $\pm$  SEM. A one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test was used for statistical comparison: (\*)  $p < 0.05$  vs salbutamol base; (\*\*\*)  $p < 0.01$  vs salbutamol base.

Salbutamol (1  $\mu\text{M}$ ) in the presence of excess 1H2NA (10  $\mu\text{M}$ ) had an  $OT_{50}$  similar to that of salbutamol base ( $p > 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). However, the  $OT_{75}$  for salbutamol in the presence of 1H2NA was significantly faster with an onset time of  $4.38 \pm 0.89$  min compared to  $8.75 \pm 1.03$  min for the base ( $p < 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). The inhibition of contraction by salbutamol in the presence of 1H2NA was also greater than that by salbutamol base alone, with a total inhibition of 94.13% ( $p < 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). The time for the tissues to reach 50% recovery compared to the predrug values, for both salbutamol base and salbutamol in the presence of 1H2NA, was approximately 50 min ( $p > 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). However, the average time taken for the tissues to recover to 75% of the predrug value for salbutamol in the presence of 1H2NA was faster ( $p < 0.01$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test). All of the tissues recovered to 100% of the predrug value and were not significantly different from both controls ( $p > 0.05$ , one way ANOVA Kruskal–Wallis test with post hoc Dunn's multiple comparison test) (Table 2).

#### 4. DISCUSSION

Of the different drug–counterion combinations assessed only the salbutamol 1H2NA ion-pair had an effect on airway smooth muscle relaxation (ASM) when compared with salbutamol base. It could be argued that the enhanced smooth muscle relaxation of salbutamol in the presence of 1H2NA could have been a consequence of this molecule having an independent action on ASM as it was presented to the tissue in a molar excess compared to salbutamol base. There is little information in the literature regarding the effect of 1H2NA on biological tissues, but Groeben and Emala<sup>19</sup> did evaluate the effect of xinafoic acid (1H2NA) on ASM, in particular its ability to bind to the  $\beta_2$ -AR.

In this previous work it was concluded that 1H2NA had no direct airway irritant effects, had no direct bronchodilating effects *in vivo*, and it was thought that it did not interfere with  $\beta_2$ -AR binding *in vitro*, nor impair the binding of salmeterol to  $\beta_2$ -AR. The data from the present study, which showed an effect of 1H2NA with salbutamol, but not with salmeterol, concurred with the previous suggestions that the 1H2NA effects were not dependent on any direct interaction with  $\beta_2$ -receptors.

The ability of drug–ion pair formation to enhance drug absorption has been reported in the literature.<sup>1,6,20</sup> In addition, it has already been established in previously published work that a number of anionic drugs can form complexes with 1H2NA. However, there seems to be an absence of studies that determine the ion-pair association using direct spectroscopy and then go on to understand their pharmacological effects. This is presumably because the ionic and other noncovalent interactions (i.e., hydrogen bonding) that occur between the ionic drug and the counterion during ion-pair formation are relatively weak,<sup>21,22</sup> and in aqueous solvents the water signals make the detection of any spectral shifts difficult to observe.<sup>23,24</sup> Despite these issues, in the present study, we attempted to generate direct spectroscopy evidence of ion-pair formation, but as a simple aqueous solution was not capable of dissolving salbutamol and the test counterions, methanol was added to aid solubility. It was known that the presence of methanol would influence the absolute values for the affinity constant compared to a pure water system, but it was believed that by keeping a constant solvent system across the measurements it would not detract from the comparative measurements made in the current study. In addition, there are methods available to predict affinity constants in water from experimental data with water–methanol solvents.<sup>25</sup>

The FT-IR spectroscopy data generated in this study substantiated that salbutamol formed an ion-pair with sulfate and 1H2NA. However, a full binding curve could only be obtained for the salbutamol sulfate salt. The spectral shifts for

1H2NA occurred at a 10-fold lower concentration compared to salbutamol, and despite not being able to generate an absolute value for the binding affinity between salbutamol and 1H2NA, the data allowed us to predict with some confidence that the salbutamol–1H2NA ion-pair would be formed at the 1:10 ratio of drug:counterion that was used across all the experiments.<sup>26</sup>

For salbutamol, which is thought to enter the tissue primarily via the paracellular route,<sup>27</sup> the formation of the ion-pairs could have modified tissue uptake by making the transcellular route more favorable since the formation of a complex would have to some extent reduced the charge and hydrophilicity of the salbutamol molecule.<sup>1</sup> The comparative effects of this counterion enhanced drug transport process would be much less for formoterol and salmeterol because it is thought that these molecules are naturally transported by the transcellular route, even without the addition of a counterion, due to their greater hydrophobicity.<sup>28</sup> It has been hypothesized that once deposited into the airways formoterol moves efficiently across the airway epithelium and into the lamina propria, where it diffuses toward ASM by partitioning into the lipid bilayer and then progressively leaches out to interact with the  $\beta_2$ -AR active site.<sup>29</sup> Salmeterol contains a longer side chain than the shorter acting  $\beta_2$ -agonist salbutamol that makes it >10000 times more lipophilic, and so it has also been hypothesized to move readily through the epithelial cell membrane.<sup>30</sup>

Although superfusion studies were not completed in this work for salmeterol or formoterol, previous studies have shown that both formoterol and salbutamol display a faster onset time compared to salmeterol.<sup>31,32</sup> Furthermore, formoterol is known to be more potent than salmeterol in both *in vitro* and *in vivo* studies.<sup>31,32</sup> This information from the literature compliments the lung function studies in the current work where formoterol seemed to have a greater degree of inhibition compared to salmeterol alone and in the presence of counterions across the concentration range (0.1–10  $\mu\text{g kg}^{-1}$ ).<sup>33</sup> This suggested that the *in vivo* systems used in this study were working well. The lack of a significant difference observed when the counterions were combined with formoterol (Figure 3) or salmeterol (Figure 4) may be due to the choice of counterions. The ability to form an ion-pair that will remain complexed for long enough to influence its pharmacological behavior is related to the chemical structure and physicochemical properties of the parent drug and the counterions present. The curve for salmeterol in the presence of the maleate counter ion was significantly different from that for salmeterol xinafoate, and the dose response profiles for salmeterol base and salmeterol aspartate were very different from that of salmeterol xinafoate (Figure 4). These small differences could be exploited by investigating alternative counterions which may have a strong affinity for the parent drug.

As the 1H2NA counterion has been previously shown to form ion-pairs with a wide range of drug molecules,<sup>1</sup> it was thought that it would be worthwhile to further understand the transport of salbutamol 1H2NA into lung tissue using an *in vitro* model. As the influence of ion-pairs on a range of cell lines has previously been established *in vitro*,<sup>1,34</sup> in this work we wanted to use *ex vivo* tissue such that we could understand not only passage through the epithelial barrier, but also drug permeation across through pulmonary tissue as this has relevance to both locally administered and systemically administered agents. Although we accept that only a limited amount of drug is absorbed through the tracheal tissue, this section of the respiratory tract provided significant rigidity to

allow drug transport to be assessed. A 60 min equilibration time was needed to ensure that the Franz cells were secure, free of bubbles, and at a temperature of 37 °C.<sup>35,36</sup> As these conditions remained constant throughout the work, it was unlikely that the application of the liquid, which can change apical ion channel abundance, and hence cause increased fluxes due to activation of fluid homeostasis mechanisms, had an effect on the data set trends.<sup>37</sup>

For the *in vitro* lung trachea transport studies the hypothesis was that ion-pair formation enhanced the ability of salbutamol to move into the tissue. Salbutamol base at pH 7.4 is very hydrophilic with a  $\text{cLogD}_{7.4}$  of  $-1.32$  (MarvinSketch, ChenAxon Ltd.), and it is fully ionized (calculated amine  $\text{pK}_a = 9.02 \pm 0.028$ ) from pH 8 and below; therefore there is not a pH dependent change in the transport of salbutamol across the physiological pH range.<sup>38</sup> Although lung lining fluid is slightly acidic (pH 6.9),<sup>39</sup> the transport studies were conducted at pH 7.4 to prevent a pH gradient across the cells as this could influence transport. Furthermore, a pH of 7.4 is typically used in cell culture models when characterizing salbutamol transport.<sup>40</sup> These physicochemical properties enable fast dissolution of the parent drug to occur when it is administered as a solid, but salbutamol's polarity results in only a moderate affinity with the cellular epithelium of tracheal tissue. Evidence for the transcellular route being the main path of entry into the pulmonary tissue has been generated through transport inhibition studies using other charged molecules such as amino acids.<sup>27</sup> More recent studies have also suggested the involvement of organic cation transporters (OCTs) in tissue accumulation and cellular uptake of inhaled drugs.<sup>41,42</sup> As salbutamol base carries a net positive charge at physiological lung pH,<sup>43</sup> OCTs may be involved in the absorption and clearance processes for this drug in the lung without the formation of an ion-pair,<sup>42,43</sup> but the OCT's capacity to take up a neutral ion-pair would presumably be diminished.

The association of 1H2NA with salbutamol would generate a larger, more neutral molecule, which would be inherently more hydrophobic than the parent.<sup>34,44</sup> It is these changes in physicochemical properties that would most likely drive the increase in affinity of the ion-pair for the lung tissue compared to the parent drug.<sup>45,46</sup> The increase in transport lag time, which traditionally occurs as a consequence of an increase in molecular size of a compound,<sup>47</sup> and the higher  $P_{\text{app}}$  of salbutamol in the presence of 1H2NA support this hypothesis. The  $P_{\text{app}}$  values reported in the literature for salbutamol range by 2 orders of magnitude from  $1.5 \times 10^{-8}$  (porcine tissue)<sup>48</sup> to  $5.2 \times 10^{-6}$  cm/s (Calu-3 cells).<sup>49</sup> The salbutamol base  $P_{\text{app}}$  of  $1.31 \pm 0.28 \times 10^{-5}$  cm/s reported in the current work was approximately double the highest of these previously reported values, and this was considered to be due to the different permeability of guinea pig trachea. No previous work has used this tissue to test drug transport, and our transport data suggested that the barrier was confluent.

The sulfate counterion could also have a similar effect to 1H2NA on salbutamol transport, but the charging on the sulfate at pH 7.4 would mean that a neutral complex is unlikely to be formed and the transport of the salbutamol sulfate may be a little more complex than the 1H2N salbutamol ion pair. However, it was noteworthy that the sulfate counterion displayed the ability to alter the transport into the tissue, even at the 1:2 drug:counterion ratio. The  $\text{cLogD}_{7.4}$  of 1H2NA is predicted to be  $-0.53$  (MarvinSketch software (ChemAxon)), making it a more lipophilic ion-pairing agent compared to



the sulfate,  $c\text{LogD}_{7.4} -5.54$  (MarvinSketch software (Chem-Axon)). Assuming that salbutamol and 1H2NA are forming an ion-pair in solution, the overall complex would be more hydrophobic compared to the sulfate counterion complex, and thus one may expect the former to show greater affinity to tissue and a greater propensity to be transported via the transcellular route.<sup>46</sup> Ion-pair transport across the membrane will not be hindered by charged proteins in the ECM as may be the case for weak bases such as salbutamol,<sup>50</sup> and this could aid the transport rates across the tissue.

The *in vitro* studies that investigated the relaxant activity of salbutamol in the presence of the counterions in isolated guinea pig tracheal tissue showed only a marginal effect of the salbutamol:1H2NA complex. The small magnitude of the response in the isolated tissue model suggested that the pharmacology of the drug action was not dramatically modified as a consequence of ion-pair formation. Therefore, it was proposed that the ion-pair broke down once taken up into the tissue. This was supported by the fact that the weaker of the two ion pairs, the salbutamol sulfate complex, did not show any effect. It was interesting however that the delayed lag time shown in the transport studies did not have any consequences on the pharmacological indices measured both *in vitro* and *in vivo*. In fact the salbutamol-1H2NA ion-pair had a significantly faster onset rate ( $\text{OT}_{75}$  only) in addition to a greater degree of inhibition compared to the base alone, which would not be predicted when analyzing drug transport across lung trachea. It was also surprising that the faster onset of pharmacological action was coupled with a significantly faster recovery rate, not observed with salbutamol sulfate. It was expected that the 1H2NA counterion would provide a slower recovery rate due to an increase in the hydrophobicity of the complex, but perhaps if the ion-pair complex was dissociating in the tissue, this resulted in a complex relationship between the transport and pharmacological activity.

## 5. CONCLUSIONS

The results from this study provide evidence that the counterions included in pharmaceutical salts are important in determining the biological behavior of the parent drug molecule when delivered by the inhaled route. These results need confirmation using pharmaceutical salts in the solid state in humans, but if these results are replicated in such studies it would have significant implications for the choice of inhaled drug counterions. It is recommended, based on the data generated to date, that when counterions are switched in generic products, careful characterization of the pharmaceutical alternative's properties must be undertaken prior to medicine approval.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.molpharmaceut.6b00448](https://doi.org/10.1021/acs.molpharmaceut.6b00448).

Bronchodilating effects, FT-IR spectra, and UV scans (PDF)

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## Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

1H2NA, 1-hydroxy-2-naphthoate;  $\beta_2$ -AR,  $\beta_2$ -adrenoceptor; COPD, chronic obstructive pulmonary disorder; im, intramuscular; iv, intravenous; SEM, standard error of the mean; RPHPLC, reverse phase high performance liquid chromatography; SD, standard deviation; K-H, Krebs-Henseleit; EFS, electrical field stimulation; ANOVA, analysis of variance; SAL, salbutamol base; FT-IR, Fourier transform infrared;  $P_{app}$ , apparent permeability; ASM, airway smooth muscle; OCTs, organic cation transporters

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