Pharmacological characterisation of indacaterol, a novel once-daily inhaled β₂-adrenoceptor agonist, on small airways in human and rat precision-cut lung slices

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Abstract

Indacaterol is a novel once-daily inhaled β₂ adrenoceptor agonist in clinical development. This study compared the properties of indacaterol with salmeterol, formoterol and albuterol on small airways in precision-cut lung slices from human and rat contracted with carbachol and serotonin, respectively. In human lung slices, the rank order of potency was formoterol ≥ salmeterol > indacaterol > albuterol, respectively. Indacaterol had similar intrinsic efficacy to formoterol, followed by albuterol and salmeterol. The onset of action was fast for albuterol, formoterol and indacaterol whereas it was significantly slower for salmeterol. The duration of action ranking was indacaterol > salmeterol > formoterol > albuterol. When compared to human lung slices, in the rat lung slices, similar potency, intrinsic efficacy and onset of action were observed for indacaterol, formoterol and salmeterol. Albuterol had an increased potency when compared to human lung slices and a slower onset of action. In conclusion, our results show that the human lung slice system seems to be a good model to study the clinical properties of inhaled long acting β₂ adrenoceptor agonists and that caution is needed extrapolating from rat model to humans. Finally, using the human lung slice model, we have characterized indacaterol as a fast acting compound with a longer duration of action than salmeterol and formoterol.
Introduction

The small conducting airways (defined in man as less than 2 mm in diameter) are now recognized as the major site of airflow limitation in chronic obstructive pulmonary disease (COPD) and are also involved in severe asthma (Hasegawa et al., 2006; Davidson and Bai, 2005). Small airway function has been studied by classical organ bath pharmacology using parenchymal strips and to a lesser extent by myography. However, in the case of the former technique, it is not possible to isolate responses of the airway smooth muscle from those of other contractile elements within the lung (e.g. vascular smooth muscle), whilst in the latter, the airway is isolated from interaction with parenchymal cells and is limited to airways down to a minimum of 100-200 microns in diameter. Videomicroscopy of perpendicularly sliced airways in precision-cut lung slices can be used to quantify changes in airway area in response to contractile and relaxant stimuli. The technique has the advantage that very small airways (down to approximately 50 µm diameter) can be studied. In addition, since the slices are viewed under a microscope, it is possible to study only the response of the small airways in the presence of parenchymal attachments without the contributions from vascular smooth muscle (Wohlsen et al., 2003).

Agents that act as agonists of the β₂ adrenoceptor are effective in the management of COPD and asthma, primarily through their bronchodilatating properties. These drugs induce bronchodilatation by causing direct relaxation of airway smooth muscle through activation of adenylate cyclase, which in turn increases intracellular 3′,5′-cyclic adenosine monophosphate levels. Although, the pharmacological properties of β₂ adrenoceptors on isolated human bronchus have been reported several times in the past (Nials et al., 1993; Naline et al., 1994; Schmidt
et al., 2000), it is not known whether this class of compounds has the same properties 
on human small conducting airways.

Indacaterol (QAB149, Novartis) (Battram et al., 2006), is a novel inhaled once-daily 
$\beta_2$ adrenoceptor agonist that completed Phase II clinical trials for the treatment of 
COPD and asthma and is currently in Phase III. The present study utilized human 
precision-cut lung slices to study the potency, efficacy, onset and duration of action of 
indacaterol and compared its properties with the marketed $\beta_2$ adrenoceptor agonists: 
albuterol, that is used as rescue medication and has a duration of action of 
approximately 4-h and formoterol and salmeterol used a maintenance therapy, both 
having a duration of action of approximately 12-h. In addition, given the limited 
availability of human tissue, we have assessed whether the rat small airways could be 
used as a good surrogate for the pharmacological characterization of $\beta_2$ adrenoceptor 
agonists by comparing the responses to the compounds in human and rat precision-
cut lung slices.
Methods

Materials

Indacaterol maleate and formoterol fumarate were synthesized by the Department of Chemistry (Novartis, Horsham, UK). Albuterol hemisulfate was purchased from Sigma-Aldrich (Poole, UK) and salmeterol from Tocris Cookson Ltd (Bristol, UK). Stock solutions of compounds were prepared at a concentration of $10^{-2}$ M in dimethyl sulfoxide, serial dilutions made in dimethyl sulfoxide in half log increments and the solutions stored at $-20^\circ$C until required. Unless otherwise stated all other reagents were purchased from Sigma Aldrich (Poole, UK).

Preparation of precision-cut lung slices from resected human lung

The experiments described in this report were approved by the local ethics committee (Royal Brompton & Harefield Ethics Committee). Macroscopically normal human lung (3-10 g) was obtained from subjects undergoing resection usually for carcinoma of the lung. The tissue was weighed and then washed 3 times with 3 volumes of ice-cold slicing buffer (Hanks balanced salt solution containing 100 units/ml penicillin and 0.1 mg/ml streptomycin). Tissue supplied prior to 2:00 pm on the day of resection was processed immediately as described below. Tissue received after 2:00 pm was kept overnight in ice-cold slicing buffer supplemented with 1 g/l of D-glucose and bubbled with O$_2$/CO$_2$ (95% / 5%). There were no significant differences between the responses to spasmogen or $\beta_2$ adrenoceptor agonists of small airways prepared on the day of resection and those kept on ice overnight.

Immediately prior to preparation, the tissue was submerged in a reservoir containing ice-cold slicing buffer and 3 % (w/v) ultra low melting point agarose (Type IXA) injected into the tissue using a fine gauge needle (NR18; Microlance).
Injection aliquots (approximately 20 µl) were applied as evenly as possible throughout the tissue and were continued until the total agarose volume was 3-4 ml/g wet weight of tissue.

The inflated tissue was then left for a further 15 min at 0°C at which point it was cut into slices of approximately 1 cm thickness in several different planes using a microtome blade. The cut faces of these slices were then inspected by eye for the presence of vascular or airway features. Cylindrical cores of 8 mm diameter were then prepared containing these features oriented longitudinally along the core. These were further processed to precision-cut lung slices using a Krumdieck tissue slicer (TSE Systems GmbH, Bad Homburg, Germany) with the slice thickness set on 260-280 µm. Slices were transferred, in order of preparation, to wells of a 12-well tissue culture plate containing 1 ml per well of incubation buffer (RPMI 1640 containing 100 units/ml penicillin, 0.1 mg/ml streptomycin and 4 mM L-glutamine.). After warming to 37°C in a humidified air/CO₂ (95 % / 5 %) incubator the slices were inspected under a dissecting microscope. Those containing only blood vessels (identified by the absence of beating cilia) were discarded. Slices containing airways were further inspected under the microscope and only included in the study if: a) the airway was approximately circular b) beating cilia indicating an intact epithelium were observed and c) the airway wall and all parenchymal attachments were intact. Slices containing such airways were washed, at 37°C, every 30 min with 1 ml of incubation buffer for 2 h and then left overnight at 37°C in an air/CO₂ (95 % / 5 %) incubator on a rotating platform (1 rpm). The following morning slices were again washed with 1 ml incubation buffer and returned to the incubator. Several sample airways were monitored using the image analysis equipment described below to ensure that a stable baseline area had been attained. In those experiments where these
showed evidence of instability further washes with incubation buffer were performed until the airway area remained constant over a 5 min period. During the course of these experiments, human small airways with a diameter range of 30-800 µm were studied.

**Preparation of precision-cut lung slices from rat lungs.**

The studies reported here conform to the UK Animals (Scientific Procedures) Act 1986. Precision-cut lung slices were prepared from the lungs of male Brown Norway rats (250-350 g) essentially as described previously (Martin et al., 1996) except that the pulmonary circulation was not perfused prior to inflation and the concentration of agarose used for inflation was 2 % w/v. Slices, from tissue cores generated in the same way as for the human lung slices, (260-280 µm thickness) were transferred individually to 1 ml of incubation buffer (Dulbecco’s Modified Eagle Medium containing 100 units/ml penicillin, 0.1mg/ml streptomycin, 4 mM L-glutamine and 2.5 µg/ml amphotericin B) at 4°C in a 12-well tissue culture plate and then placed in an air/CO₂ (95 % / 5 %) incubator at 37°C. Slices were then washed, at 37°C, with 1 ml of incubation buffer every 30 min for 3 h before overnight incubation at 37°C on the rotating incubator. The following morning slices were washed again with 1 ml of incubation buffer and left at 37°C for a further 1 h to stabilise prior to functional studies described below.

**Onset of action, potency and intrinsic efficacy determinations**

The spasmogens used were serotonin and carbachol for rat and human small airways, respectively. In the series of experiments used to determine potency and intrinsic efficacy or onset times, serotonin (rat) concentrations were increased from 0.3 μM in
half log increments until either 70% contraction of the airway was achieved or the concentration reached 30 µM. The β₂ adrenoceptor agonist under test was then added at the concentrations indicated. An identical procedure was followed for the corresponding experiments with human airways except that carbachol was used as a spasmogen; the range of concentrations used was again 0.3-30 µM. There was no significant difference in final concentration of spasmogen used for assessment of the four β₂ adrenoceptor agonists under test.

Precision-cut lung slices containing airway sections were positioned under a Nikon SMZ-U dissecting microscope (magnification 75 x) on which was mounted a JVC TK-1280E video camera. The video camera was connected to a Matrox Meteor 2 frame-grabber card and image capture and analysis were performed using KS 300 image analysis software (Image Associates Ltd, UK). All measurements were performed at 37°C.

For the onset of action protocol, a single precision-cut lung slice was positioned in a purpose built lung slice chamber (Harvard Instruments, UK) containing 0.4 ml of assay medium (incubation buffer containing 20 mM HEPES, pH 7.4). The slice was held in place by a “U” shaped platinum weight to which were attached nylon threads of diameter 50 microns. Baseline images were captured before adding the lowest dose of spasmogen at which time images were collected (usually every 30 s unless otherwise indicated) until completion of the contractile response. This procedure was repeated, replacing the chamber solution with increasing concentration of spasmogen at half log intervals up to a concentration inducing about 70% closure of the airway under study or until a concentration of 30 µM was reached. The chamber contents were then replaced with 0.4 ml of assay medium containing both the β₂ adrenoceptor agonist under test at the concentration indicated...
(or vehicle; 0.001 % dimethyl sulfoxide) and the final concentration of spasmogen. Images were then collected every 15 s for formoterol, salbutamol and indacaterol and every 30s for salmeterol up to the maximal relaxation. Preliminary experiments have shown that at this concentration, dimethyl sulfoxide did not interfere with the assay. Bronchodilatation was calculated by expressing the pixels of dilation at each time point as a percentage of the maximum closure for each airway.

For determination of potency and intrinsic efficacy, precision-cut lung slices were positioned in a 12-well tissue culture plate containing 1 ml of incubation medium per well. Baseline images of the airway under study in each well were taken at 5 min intervals until the baseline area was stable. A cumulative dose response curve to the spasmogen was then performed by the addition of 10 µl of a 100 times solution of spasmogen increasing in half-log increments until either > 70 % contraction of the airways was achieved or a concentration of 30 µM spasmogen was reached. Each airway on the tissue culture plate was then used to assess the effect of a single concentration of β₂ adrenoceptor agonist by adding 10 µl of a 100 times solution of the test compound (final dimethyl sulfoxide concentration of 0.001 %). Images were collected immediately after addition, and again at 5, 10, 15, min subsequently. Bronchodilatation was calculated by expressing the pixels of dilation at each time point as a percentage of the maximum closure for each airway.

**Duration of action studies**

These studies were only performed on precision-cut lung slices prepared from human tissue samples. Tissue slices were positioned in a 12-well plate each well containing 1 ml of incubation medium. Baseline images were collected at 5 min intervals and then 10 µl of 10⁻⁵ M carbachol was added to each well. Images were captured at 5, 10 and
15 min after addition of spasmogen. This procedure was repeated at increasing (half-log intervals) concentrations of carbachol until the majority of the airways under study contracted to about 50% of the baseline area. Slices containing airways with either poor or complete contractile responses were discarded. Medium was aspirated off the remaining slices which were then washed with 1 ml of incubation buffer. This was then replaced with 1 ml of incubation buffer containing test compound or vehicle and returned to the incubator for 1 h. At the end of this treatment the medium was removed and each slice washed with 1 ml of incubation buffer before suspending in 1 ml of incubation buffer and returning to the incubator. At different times after the removal of test compound or vehicle the contraction phase was repeated using a protocol identical to that used for the baseline contraction phase. Sensitivity to carbachol was determined at: 1, 2, 4, 6 and 24 h after the end of the incubation with test compound or vehicle. Each slice was used to determine the carbachol sensitivity at 2 time points after test compound incubation. After determination of the first time point, slices were washed with 1 ml of incubation medium and returned to the incubator until required for the second time point.

The maximum contraction induced by carbachol at each time point after test compound or vehicle treatment was expressed as a percentage of the maximum contraction induced before the addition of test compound or vehicle. In order to strengthen the data for comparison of indacaterol and salmeterol, these two treatments were always tested on adjacent segments of the same airway.

**Data analysis**

Airway areas (in pixels$^2$) at baseline and after treatments were calculated using a computer algorithm written within the KS 300 environment. For potency, efficacy and
onset times the bronchodilatation (in pixels$^2$) was expressed as a percentage of the total closure (in pixels$^2$). In the experiments to determine onset time, the time taken to reach 50% of the maximum bronchodilatation was calculated using the following equation: Percent bronchodilatation at time $t = \frac{(\text{pixels at time } t - \text{pixels at maximum closure})}{(\text{pixels uncontracted-pixels at maximum closure})} \times 100\%$. The maximum value for this was then set to 100% and all other time points expressed as a percentage of this. These values are then plotted versus time and the value for 50% of the maximum determined by interpolation.

Statistical analysis to determine the differences between onset times of the compounds was carried out using a Student’s unpaired t-test.

For the duration of action experiments, the maximum percentage contraction to carbachol at different times after incubation with β-adrenoceptor agonist was expressed as a percentage of the maximum contraction (to the same concentration of carbachol) prior to treatment. Two or three airways from each subject were used for vehicle control and for each β-adrenoceptor agonist; these were averaged to give a “subject mean”. The data were then expressed as a mean of the different subjects. The differences between compounds were then analysed statistically using a Student’s t-test. In the case of comparisons between salmeterol and indacaterol, as noted above, in all experiments adjacent segments from the same airway were used to compare duration of action of the two compounds. Preliminary studies (data not shown) revealed that adjacent segments of the same airway gave virtually identical responses to contractile agonists and more importantly equivalent bronchodilatation to isoproterenol. For this reason statistical analysis of the relative duration of action of these compounds was performed using a Student’s paired t-test. IC$_{50}$ values were calculated using the non-linear regression protocol for a sigmoidal curve with variable
slope within the Graphpad Prism 4 software package. The fitting procedure was constrained such that the bottom of the bronchodilator curve was equal to zero while the top was constrained to be less than or equal to 100%.
Results

Human small airways

In human small airways, all compounds reversed the carbachol-induced contraction in a concentration-dependent manner. Indacaterol and formoterol had the highest intrinsic efficacy (73 ± 27 % and 67 ± 11 %, respectively), followed by albuterol (48 ± 4 %) and salmeterol (35 ± 5 %). Formoterol and salmeterol had subnanomolar potency with IC$_{50}$ values of 0.3 ± 0.1 and 0.7 ± 0.2 nM, respectively. Indacaterol had an IC$_{50}$ value of 37 ± 8 nM and albuterol was of lower potency (125 ± 11 nM) (Fig. 1).

The onset of action for each of the compounds was derived using equi-efficacious concentration inducing about 30 to 40 % relaxation – 3 nM for formoterol and 30 nM for indacaterol, salmeterol and albuterol. The onset was fast for albuterol (1.6 ± 0.3 min), formoterol (2.0 ± 0.3 min) and indacaterol (3.0 ± 0.2 min) whereas it was significantly slower for salmeterol (6.6 ± 0.3 min, p < 0.05) (Fig. 2).

Albuterol had a short duration of action and no inhibition of the baseline response to carbachol was evident at the first time point studied, 1 h. Formoterol, was still active at 1 h but no significant inhibition of contraction was observed at subsequent time points, 2, 4 and 24 h. Indacaterol and salmeterol were active up to the 6 h time points and lost their activity by 24 h. The inhibition of carbachol induced bronchoconstriction by salmeterol and indacaterol was identical at the 1 and 2 h time points after incubation with these compounds. However, at both the 4 and 6 h time points, the inhibition by indacaterol was significantly greater than that by salmeterol. The control response to carbachol remained constant over the time frame of the study, indicating the viability of the slices (Fig. 3).
**Rat small airways**

In rat small airways, all compounds concentration-dependently reversed the serotonin-induced contraction. Formoterol was the most potent compound, with an IC$_{50}$ value of 0.6 ± 0.1 nM. Salmeterol (2.0 ± 0.2 nM) had similar potency to albuterol (2.5 ± 0.3 nM) and indacaterol was of lower potency (10.5 ± 2.3 nM). Formoterol had the highest intrinsic efficacy (62 ± 7 %) followed by indacaterol (53 ± 19 %), albuterol (42 ± 12 %) and salmeterol (36 ± 5 %) (Fig. 1). The onset of action for each of the compounds were derived using equi-efficacious concentration inducing about 35 % relaxation – 1 nM for formoterol and 30 nM for indacaterol, salmeterol and albuterol. The onset of action was fast for formoterol (1.7 ± 0.3 min) and indacaterol (3.5 ± 0.8 min) whereas it was significantly slower for salmeterol (7.6 ± 0.6 min, $p < 0.05$) and albuterol (8.8 ± 1.0 min, $p < 0.05$) (Fig. 2).
Discussion

In this report, we described for the first time the pharmacological profile of indacaterol, a new once-daily inhaled β₂ adrenoceptor agonist that completed Phase II clinical trials for the treatment of COPD and asthma and is currently in Phase III, in human and rat precision-cut lung slices and compare its properties with those of marketed drugs.

The human lung slice model used in the present study appears to be a useful model for the clinical situation. In this preparation, our results show that indacaterol had a fast onset of action similar that of albuterol and formoterol. This is in contrast to the slower onset observed with salmeterol. In the clinical situation, it is recognized that albuterol and formoterol have fast onsets of action, whereas salmeterol has a slower onset (Wegener et al., 1992; van Noord et al., 1996) and results from a number of recent clinical studies have shown that a statistically significant beneficial effect for indacaterol is observed within the first 5 min after inhalation (Beeh et al., 2007; Tarral et al., 2005). Regarding the duration of action, although the drugs tested have a shorter duration of action in our model when compared to the clinical situation, the rank order obtained (indacaterol > salmeterol > albuterol) is in line with their clinical properties. As observed previously in other systems, when studied in vitro, formoterol behaves as a short-acting drug (Nials et al., 1993; Nials et al., 1994; Battram et al., 2006). A number of theories have been put forward to explain this inconsistency but none of them are really satisfactory. In addition to being a good model for the clinical properties (onset and duration of action) of the inhaled β₂ adrenoceptor agonists, the human lung slice appears to be a good quality model that can be used to determine the pharmacological properties of these compounds. Indeed, both the potency and intrinsic efficacy values reported in the present manuscript are in very good...
agreement with our previous results using Chinese hamster ovary cells stably transfected with human \( \beta_2 \) adrenoceptors (Battram et al., 2006) and isolated human bronchus (Naline et al., 2007).

Given the limited availability of human tissue, one of the goals of this study was to assess whether tissue obtained from laboratory animals could be used as a surrogate for human lung tissue in studies to characterize the small airway relaxant effect of \( \beta_2 \) adrenoceptor agonists. In previous work, we have shown that the isolated guinea pig trachea was a good predictor for human isolated bronchus to determine the potency, efficacy, and onset of action for the compounds used in the present study (Battram et al., 2006). Therefore we started this work by assessing the appropriateness of precision-cut lung slices from guinea pigs and found that it was difficult to obtain good quality slices from this species. This is probably due to post mortem bronchoconstriction, reported previously (Lai et al., 1984). During the course of this work, it was demonstrated that this phenomenon could be overcome by the inclusion of a \( \beta_2 \) adrenoceptor agonist, isoproterenol, in the media used for preparation of guinea pig precision-cut lung slices (Ressmeyer et al., 2006). However, since the goal of our study was to characterize the pharmacology of \( \beta_2 \) adrenoceptor agonists, the presence of isoproterenol in the preparation buffer could have introduced confusing factors such as desensitization or other interaction at the \( \beta_2 \) adrenoceptor. Therefore we utilized rat tissues in the current study.

Our results obtained with long acting \( \beta_2 \) adrenoceptor agonists (indacaterol, salmeterol and formoterol) on the rat lung slices, suggest that this species seems to be a useful model for human tissue for determination of their potency, efficacy, and onset of action. Indeed, in both rat and human precision-cut lung slices all three compounds had similar potency. In addition, indacaterol and formoterol behave as compounds
with a fast onset of action and a high intrinsic efficacy whereas salmeterol had a slow onset of action with a lower intrinsic efficacy when compared with indacaterol and formoterol. In contrast, although the intrinsic efficacy of albuterol was similar in rat and human precision-cut lung slices, this compound was about 25 times more potent in the rat when compared to human and had a fast onset in the human but a slow onset in the rat precision-cut lung slices. The reason for these discrepancies is not clear and deserves further studies.

Although it is well established that the relaxant effect of β₂ adrenoceptor agonists in the airways is mainly at the levels of the tracheal and bronchial smooth muscle, the precise site of action for this class of compounds in the lower airways is still a matter of debate. Autoradiographic studies have shown that in both human and rat lungs, β₂ adrenoceptors are present in the smooth muscle of large and small airways, the epithelium, the vascular smooth muscle and to a lesser extend in the alveolar wall (Carstairs et al., 1985; Mak et al., 1995), suggesting that the site of action for the β₂ adrenoceptor agonists in the lower part of the respiratory tract could include the airway smooth muscle, the vascular smooth muscle or the structural cells within the airway wall. Studies trying to differentiate the site of action for the relaxant effect of β₂ adrenoceptor agonist have generated controversial results (Vettermann et al., 1989; Kaczka et al., 1999; Petak et al., 1999). Although, we cannot conclude on the possible role of vascular smooth muscle and cells within the airway parenchyma, our data show that the small airway smooth muscle could contribute to the relaxant effect induced by the β₂ adrenoceptors in the lower respiratory tract.

In conclusion, our results show that the human lung slice seems to be a useful model to study the clinical and pharmacological properties of inhaled β₂ adrenoceptor agonists. Although, the pharmacological properties for the long acting β₂
adrenoceptor agonists were similar in the human and rat lung slice models, this was not true for albuterol. Caution is therefore needed in extrapolating from this model to humans. Finally, using the human lung slice model, we have characterized indacaterol as a fast acting compound with a longer duration of action than salmeterol and formoterol.
References


Footnotes

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Legends for Figures

Figure 1. Potency and intrinsic efficacy for the compounds in the human (left panel) and rat (right panel) small airways contracted to 70% of the maximal closure with carbachol and serotonin, respectively. Data are represented as percentage relaxation of the maximum closure and shown as mean ± S.E.M. of 3 to 8 patients or 3 to 10 rats.

Figure 2. Onset of action for the compounds in the human (left panel) and rat (right panel) small airways contracted to 70% of the maximal closure with carbachol and serotonin, respectively. Data are represented as percentage of the maximum relaxation obtained for a given compound and shown as mean ± S.E.M. of 3 to 4 patients or 6 to 10 rats.

Figure 3. Duration of action for the compounds in the human small airways contracted to 50% of the maximal closure with carbachol. Data are represented as percentage of the maximum contraction induced before the addition of test compound and shown as mean ± S.E.M. of 3 to 7 patients or means of 2 patients. Significance, $p < 0.05$, indicated by * and #, is against the respective time-matched, vehicle control slices and salmeterol-treated slices, respectively.
Figure 1

![Graph showing relaxation from maximal closure for human and rat tissues with different compounds at different concentrations.](image-url)