Trachea Relaxing Effects and $\beta_2$-Selectivity of SPFF, a Newly Developed Bronchodilating Agent, in Guinea Pigs and Rabbits

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In this paper we evaluated the bronchodilator effects of SPFF [2-(4-amino-3-chloro-5-trifluomethyl-phenyl)-2-tert-butylamino-ethanol chloride], a newly synthesized $\beta_2$ adrenergic agonist in guinea pigs and rabbits, in comparison with other $\beta_2$ adrenergic agonists, isoprenaline or salbutamol. We studied in vitro the bronchodilator effects of SPFF and isoprenaline on isolated guinea pig trachea strips with or without the precontraction of bronchoconstrictors (acetylcholine and histamine). The positive chronotropic effects of SPFF and isoprenaline on isolated guinea pig left atria were also tested in vitro. Potency values (P donors, $pA_2$ and $ED_{50}$) were determined from the cumulative concentration–response curves. The results showed that SPFF and isoprenaline dose-dependently relaxed the isolated guinea pig trachea strips and the $P_D_2$ values of both drugs were 7.66±0.68 and 8.79±0.19, respectively. Moreover, we confirmed that the bronchodilator effect of SPFF was due to the activation of $\beta_2$ adrenoceptor because this effect was easily antagonized by ICI-118551 ($pA_2 8.90±0.01$), a specific $\beta_2$ adrenoceptor antagonist. SPFF also dose-dependently relaxed the isolated guinea pig trachea strip preconstriction with acetylcholine or histamine with $ED_{50}$ values of 10.2±0.7 $\mu$g and 550±38.2 nm, respectively. Furthermore, the positive chronotropic effect of SPFF on isolated guinea pig left atria ($P_D_2 5.41±0.38$) was much weaker than that of isoprenaline ($P_D_2 8.75±0.24$), which implied that SPFF was more selective to airway $\beta_2$ adrenoceptor than isoprenaline; the $\beta_2/\beta_1$ selectivity assay also showed that SPFF was about 162 times more selective to $\beta_2$ adrenoceptor than isoprenaline. A radioligand binding experiment using guinea pig lung and cardiac ventricle as $\beta_1$ and $\beta_2$ adrenoceptor sources, respectively, also demonstrated that SPFF possesses high affinity (27.3 nm) and selectivity (4.6 fold) to $\beta_2$ adrenoceptors. The protective effects of SPFF and salbutamol on bronchospasm induced by bronchoconstrictor aerosol in guinea pigs in vivo were investigated, and the Konzett and Rössler experiment in rabbits in vivo was also carried out. SPFF significantly prolonged the latency time of histamine and acetylcholine induced asphyxiation collapse in guinea pigs: the $ED_{50}$ value of SPFF i.g. was 0.32±0.05 mg·kg$^{-1}$ in this experiment. Meanwhile, the $ED_{50}$ values of salbutamol was 2.37±0.22, which meant that the bronchodilatation effect of salbutamol was about 6 times less potent than that of SPFF. The Konzett and Rössler experiment performed in anesthetized rabbit showed that intraduodenal administration of SPFF exerted action of longer duration than salbutamol. From the results above we suggested that SPFF was a potent, long-acting bronchodilator with relatively higher $\beta_2$ adrenoceptor selectivity.

Key words $\beta_2$ adrenergic agonist; bronchodilator effect; receptor selectivity; 2-(4-amino-3-chloro-5-trifluoromethyl-phenyl)-2-tert-butylamino-ethanol chloride (SPFF)

Asthma is a common respiratory disease characterized by reversible airway obstruction and airway hypersensitivity. $\beta_2$ adrenoceptor agonists have long been widely used as agents for the treatment of asthma, and the use of $\beta_2$ adrenoceptor agonists is based on the fact that bronchial muscles are mainly controlled by $\beta_2$ adrenoceptors, whose stimulation causes bronchodilation.1) High selectivity to $\beta_2$ adrenoceptor is important for a $\beta_2$ adrenoceptor agonist, because low selectivity always induces side effects like tachycardia, caused by stimulation of the $\beta_1$ adrenoceptor; currently available drugs such as salbutamol and terbutaline are not as satisfactory in this sense.2)

We recently synthesized 2-(4-amino-3-chloro-5-trifluoromethyl-phenyl)-2-tert-butylamino-ethanol chloride (SPFF) (Fig. 1) as a novel bronchodilator which possesses $\beta_2$ adrenoceptor stimulation activity. In this paper, we reported some preliminary results showing the potency, selectivity, and duration of action of SPFF in guinea pigs and rabbits for the first time; and the effects of SPFF are compared with those of other $\beta_2$ adrenoceptor agonists, isoprenaline and salbutamol. SPFF exhibited both a potent trachea relaxing activity and high $\beta_2$ selectivity.

MATERIAL AND METHODS

Drugs and Chemicals The composition of the Krebs–Henselte solution was as follows (in g·l$^{-1}$): NaCl (6.92), KCl (0.35), CaCl$_2$ (0.28), MgSO$_4$·7H$_2$O (0.29), NaHCO$_3$ (2.1), KH$_2$PO$_4$ (0.16) and glucose (2.0). The Krebs solution used had the following composition (g·l$^{-1}$): NaCl (6.92), KCl (0.35), MgSO$_4$·7H$_2$O (0.15), NaHCO$_3$ (2.1), KH$_2$PO$_4$ (0.16), glucose (2.0) and CaCl$_2$ (0.24), isoprenaline hydrochloride was obtained from Hefeng Pharmaceutical Co., Ltd. (Shanghai, China). Salbutamol sulphate was from YanCheng Pharmaceutical Co. (Jiangsu, China). ICI-118551 [(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)]-
male Hartley guinea pigs (Bristol, U.K.). Histamine phosphate and acetylcholine chloride were from Sigma (St. Louis MO, U.S.A.). [3H]Dihydroalprenolol (1480 GBq/mmol) was from the National Institute of Atomic Energy (Beijing, China). SPFF was synthesized by Dr. M. S. Cheng and L. Pan (The Department of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang, China).

**Animals** Guinea pigs (Hartley) and rabbits were provided by the Experimental Animal Center of Shenyang Pharmaceutical University. Animals for in vitro experiments were sacrificed by decapitation under light ether anesthesia.

**Bronchorelaxation Effect on Resting Isolated Guinea Pig Trachea Strips** Hartley guinea pigs of either sex weighing 355±35 g were sacrificed and the trachea strips were prepared; the preparation was then mounted under a resting tension of 2 g in an organ bath containing 10 ml of Krebs–Heinsleit solution (KH, pH 7.4) at 37 °C and superfused with a gas mixture (O2 95%, CO2 5%).3) The trachea strips were left for 1.5 h and KH solution was changed at 15 min intervals before SPFF or isoprenaline was accumulatively added to the bath. The maximum relaxation produced by isoprenaline (10⁻⁷ M) was taken as 100% and dose–response curves of each drug were made.

**Bronchorelaxation Effect on Precontracted Isolated Guinea Pig Trachea Strips** The isolated guinea pig trachea strips were precontracted with acetylcholine (3 μM) or histamine (5 μM). The maximum contraction induced by histamine or acetylcholine was taken as 100% and the cumulative concentration–response curves for isoprenaline (10⁻⁹—3×10⁻⁴ M) or SPFF (10⁻⁹—10⁻⁴ M) were established. The concentration of acetylcholine and histamine used in this experiment produced 50—60% maximum response of each contractor (tested in preliminary experiments). The contact time was 20 min, enough to produce a steady level of contraction.4) EC₅₀, the concentration needed to decrease the tone of trachea strips by 50%, was obtained from the dose–response curves to express the relaxant effects of SPFF and isoprenaline.

**Competitive Effect of ICI-118551 on SPFF** The isolated guinea pig trachea strips were precontracted with histamine (5 μM). Following the addition of a specific β₂ adrenoceptor antagonist, ICI-118551 (10⁻⁸, 10⁻⁷ M), SPFF was added into the organ bath accumulatively, and the maximum relaxation produced by SPFF taken as 100%; the dose–response curves of SPFF in the presence of ICI-118551 were established.

**Positive Chronotropic Effect of SPFF on Isolated Guinea Pig Left Atria** Left atria were isolated from freshly excised hearts of male guinea pigs (250—350 g). The preparations were suspended in an organ bath containing 20 ml Krebs solution at 37 °C and superfused with 95% O₂ and 5% CO₂ under a resting tension of 0.2 g.9) The spontaneous beating rate was measured with a heart rate counter triggered by atrial contraction. After an equilibration of 30 min, SPFF or isoprenaline was added to the organ bath accumulatively. Increases in beating rate were expressed as percentage of maximum increase caused by isoprenaline and the concentration–response curves were made.

**[3H]Dihydroalprenolol Binding in Guinea Pig Lung and Ventricular Membranes** Male Hartley guinea pigs (300—350 g) were killed and the cardiac ventricle and lung were removed and homogenized in 20 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.5) using a Polytron (setting 7—8, 30 s×2). The homogenate was centrifuged at 1500×g for 10 min, and the supernatant was recentrifuged at 45000×g for 30 min. The pellet was homogenized using a Potter type glass Teflon homogenizer with 7—8 passes. The homogenate was centrifuged again at 45000×g for 20 min. The final pellet was resuspended in the above buffer and stored in liquid nitrogen until use. The above procedure was conducted at 2—4 °C.7)

The saturation experiments were performed in duplicate by incubating an aliquot of the membrane preparations (ventricle, 500 μg, lung 100 μg) with various concentrations of [3H]Dihydroalprenolol (0.1—5 nM) for 20 min at 37 °C (final incubation volume of 250 μl). Incubation was terminated by the addition of 6 ml of ice-cold 50 mM Tris–HCl buffer. Membrane-bound [3H]Dihydroalprenolol was separated from free radioligand by filtration using a cell harvester (ZT-2, Weixin, China) over glass fiber filters (Whatman, GF/B). The filters were washed with an addition of 6 ml of ice-cold Tris–HCl buffer. The radioactivity of the membrane-bound [3H]Dihydroalprenolol was determined with a liquid scintillation counter (LS-6500, Beckman, U.S.A.). Specific binding was defined as the difference between the amount of [3H]Dihydroalprenolol bound in the absence and presence of 400 nM propranolol.

In the competition experiment, an aliquot of the membrane suspension was incubated with [3H]Dihydroalprenolol (2 nM) and various concentrations of competing drug, SPFF (ventricle: 1 nM—1 mM; lung: 0.1 nM—0.1 mM) or isoprenaline (ventricle: 1 nM—1 mM; lung: 0.1 nM—10 μM). The specific binding was obtained as described above.

Protein concentration was determined using the Coomassie brilliant blue assay reagent (Jiancheng, Nanjing, China).

**The Protection Effects on the Bronchospasm Induced by Histamine–Acetylcholine Aerosol in Guinea Pigs** Male Hartley guinea pigs weighing 150±30 g were put under a bell cover (4 l) and exposed to the aerosol (mixed with 2% acetylcholine and 0.1% histamine) produced by a nebulizer at a constant flow-rate of 2 ml·min⁻¹ for 5 s.8) SPFF (0.0625, 0.125, 0.25, 0.5, 1.0 mg·kg⁻¹) and salbutamol (1, 3, 9 mg·kg⁻¹) were dissolved in 1% CMC-Na, then administered i.e.g. 1 h before the aerosol exposure. The asphyxia response to the aerosol mixture was measured as latency time before collapse was developed. When asphyxia collapse did not develop within 6 min, the animals were considered “completely protected” and the latency time was taken as 6 min. Protection rates, the percentage of completely protected animals, were obtained and the ED₅₀ values of each compound were estimated using the Bliss method.

**Konzett and Rössler Experiment in Anesthetized Rabbits** Male rabbits weighing 3.2±0.6 kg were anesthetized with pentobarbital (30 mg·kg⁻¹, i.v.). Body temperature was maintained at 36—37 °C with a heating pad and the Konzett and Rössler experiment8) was performed. The trachea was cannulated and ventilated for 40 min⁻¹ with a tidal air volume of 15 ml·kg⁻¹ using a respirator. The overflow of air was measured by a flow transducer and recorded by a bio-signal recording system (Ms2000, Guangdong College of Pharmacy, Guangzhou, China). Acetylcholine (30 μg·kg⁻¹)
was given i.v. 30, 45, 60, 90, 120, 180, 240 min after intraduodenal administration of normal saline (N.S), SPFF (80, 8, 1 μg·kg⁻¹) or salbutamol (80 μg·kg⁻¹). The overflow induced by acetylcholine 1 min before N.S or bronchodilator or administration was taken as 100% and the inhibition rate of each bronchodilator was calculated at different time points.

**Statistical Analysis**

The results are expressed as means ± S.E. of n (number of experiments). Agonistic potency was expressed as pD₂ values, and antagonistic potency was expressed as pA₂ values, obtained from Schild plotting with a confidence limit of 95%. Statistical significance of differences between groups was verified with unpaired Student’s t test. The equilibrium dissolution constant (K_d) and maximum number of binding sites (B_max) for [³H]Dihydroalprenolol binding to both membrane preparations were determined by Scatchard analysis. IC₅₀ values of the radioligand competition curves were calculated by Hill plots.

**RESULTS**

**Bronchorelaxation Effects on Resting Isolated Guinea Pig Trachea Strip**

SPFF decreased the tone of the trachea muscle at concentrations higher than 10⁻¹⁰ M and reached the maximum effect at 10⁻⁶ M (Fig. 2). The pD₂ values of SPFF and isoprenaline were 7.66±0.68 and 8.79±0.19, respectively (95% confidence limits), which meant that SPFF was about 10 times less potent than isoprenaline.

**Effect on Precontracted Isolated Guinea Pig Trachea Strips**

In guinea pigs trachea strips precontracted with acetylcholine (3 μM), SPFF (10⁻⁷—10⁻⁴ M) and isoprenaline (10⁻⁵—10⁻⁶ M) induced a concentration-dependent relaxation (Fig. 3). Both drugs produced less than 100% relaxation of acetylcholine-induced contraction. The EC₅₀ values of SPFF and isoprenaline were 10.2±0.7 and 3.8±0.6 μM (n=8), respectively. Meanwhile, SPFF (10⁻⁷—10⁻⁴ M) and isoprenaline (10⁻⁹—10⁻⁶ M) also produced a concentration-dependent relaxation on histamine (5 μM) precontracted trachea strips (Fig. 4). The maximum relaxation produced by both drugs was more than 100% of the histamine induced contraction, and the EC₅₀ values were shown to be 550±38.2 and 67.8±40.0 nm, respectively.

**Competitive Effect of ICI-118551 on SPFF**

ICI-118551 was shown as a competitive antagonist to SPFF by the slope of this plot was 1.02±0.05.
shifting right the dose–response curve of SPFF at low concentrations of 3×10⁻⁹ and 3×10⁻¹¹ M in isolated guinea pig trachea strips (Figs. 5, 6). The pA₂ value of the antagonistic activity of ICI-118551 was 8.90±0.01.

**Positive Chronotropic Effect on Isolated Guinea Pig Left Atria** As shown in Fig. 7, SPFF (3×10⁻⁸—10⁻⁴ M) induced concentration-related increase in the beating rate of contraction on guinea pig left atria, with a pD₂ of 5.41±0.38. The positive chronotropic effect of SPFF was about 2188 times less potent than that of isoprenaline, whose pD₂ value was 8.75±0.24.

**β₁/β₂ Selectivity** The β₁/β₂ adrenoceptor selectivity ratio (selectivity index) was obtained from the difference between the mean pD₂ values obtained from the isolated right atria and trachea strips in guinea pigs. The selectivity index values indicated that SPFF and isoprenaline were 178 and 1.1 times more effective on the trachea than on the right atria, respectively; therefore, SPFF was considered more highly selective to β₂ adrenoceptor than isoprenaline (Table 1).

**Affinity and Selectivity for β Adrenoceptor Subtypes by Radioligand Binding** Specific binding of [³H]Dihydroalprenolol to the guinea pig ventricle was saturable, and Scatchard analysis indicated a single population of binding sites with a K_D of 8.5±3.7 nM, and B_max of 25.3±0.3 fmol/mg protein (4 experiments). In the lung membrane, specific binding was also saturable and the K_D and B_max were 8.3±0.2 nM and 351.2±6.2 fmol/mg protein (4 experiments), respectively. These data were similar to those previously reported.11,12) The Scatchard plots were linear and the apparent Hill coefficients were unity in both membrane preparations, indicating the absence of cooperative interactions.

Displacement curves for SPFF and isoprenaline of [³H]Dihydroalprenolol binding to guinea pig ventricle and lung membranes were depicted in Fig. 8. The IC₅₀ values and overall β₂ selectivity ratios are shown in Table 2.

**Protective Effect on the Bronchospasm Induced by Histamine–Acetylcholine Aerosol** As shown in Table 3, both SPFF and salbutamol significantly prolonged the latency time of histamine and acetylcholine-induced collapse. The ED₅₀ of each drug was 0.32±0.05 and 2.37±0.22 mg·kg⁻¹, respectively, meaning that SPFF was about 7 times more potent than salbutamol in inhibiting the bronchospasm induced by bronchoconstrictor aerosol in conscious guinea pigs (Table 3).
Bronchodilation Effect on Anaesthetised Rabbits

As shown in Table 4, intraduodenally administered SPFF and salbutamol significantly inhibited the increase of lung overflow induced intravenously by acetylcholine, but the effect of salbutamol diminished quickly 120 min after drug administration; the effect of SPFF, however, remained throughout the 240 min duration of all the experiments.

DISCUSSION

In isolated guinea pig trachea strips, the dose–response curves of SPFF were paralleled by the reference agonist, isoprenaline. The curves of SPFF were shifted right by the addition of ICI-118115, and the pA2 value of ICI-118551 for SPFF was 2188 times less potent than isoprenaline. The curves of SPFF were paralleled by the reference agonist, isoprenaline. Comparable results were also reported in the studies of other β2 agonists.15

In the studies of the β2 adrenoceptor selectivity of SPFF, it was found that SPFF was 2188 times less potent than isoprenaline in increasing the beating rate of isolated guinea pig left atria. As the atria positive chronotropic effect was always considered a sign of the non-selective side effect of β2 adrenoceptor agonist,16 we assume that SPFF had higher selectivity to β2 adrenoceptor than isoprenaline. The β2 selectivity indexes showed that the selectivity of SPFF to β2 adrenoceptor was 178, about 162 times greater than that of isoprenaline (1.1), and even greater than two widely used β2 agonists, salbutamol and terbutaline, whose selectivity index values were reported to be 17 and 18, respectively, less than that of formoterol (204).17

To confirm the β2 adrenoceptor selectivity of SPFF demonstrated by the functional experiments mentioned above, we also examined the affinity and selectivity of the compound for β-adrenoceptor subtypes by radioligand binding in comparison with isoprenaline. Results had showed that the specific binding in both membrane preparations was saturable, and the Scatchard analysis and hill plots indicated the validity of our experimental conditions. SPFF exhibited high affinity (IC50 = 27.3 nm) in the lung membrane. Selectivity of SPFF for β2 adrenoceptor assessed by the IC50 (ventricle)/IC50 (lung) was 4.6, which was 12.8 times higher than that of isoprenaline (0.36). But SPFF exhibited relatively lower selectivity in the binding experiment than in the functional experiment. Although the reason for this disagreement is not clear, it is possible that 20% of the adrenoceptors are of β1 subtype in lung membranes18,19; the non-selective binding of [3H]Dihydroalprenolol to the β1 adrenoceptor resulted in an undesirable increase in the IC50 values of SPFF in lung membrane preparation.

The bronchodilator effect of SPFF was further validated in vivo by bronchoconstrictor-induced bronchospasm and Konzett and Rössler experiment, and SPFF was found to be more potent than salbutamol in both experiments. Since the

### Table 3. Inhibition Effects of SPFF and Salbutamol on Bronchoconstrictor-Induced Bronchospasm in Guinea Pig

<table>
<thead>
<tr>
<th>Group (mg·kg⁻¹)</th>
<th>Latent period/s</th>
<th>Protection rate (%)</th>
<th>ED50 (95% confidence limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59.6±5.53</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SPFF 0.0625</td>
<td>118.3±32.6</td>
<td>12.5</td>
<td>—</td>
</tr>
<tr>
<td>0.125</td>
<td>160.5±40.5*</td>
<td>25</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>0.25</td>
<td>222.6±48.1*</td>
<td>50</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>266.4±42.6**</td>
<td>62.5</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>322.5±23.3***</td>
<td>75</td>
<td>—</td>
</tr>
<tr>
<td>Salbutamol 1</td>
<td>158.0±46.0</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>193.4±48.6*</td>
<td>37.5</td>
<td>1.82±0.19</td>
</tr>
<tr>
<td>3</td>
<td>323.9±35.77****</td>
<td>87.5</td>
<td>—</td>
</tr>
</tbody>
</table>

*a p<0.05, ** p<0.01, *** p<0.001 vs. control group, n=8, Student’s t-test.

### Table 4. The Effect of Intraduodenal Administration of SPFF and Salbutamol in Rabbits, (Konzett and Rössler Experiment)

<table>
<thead>
<tr>
<th>Group/µg·kg⁻¹</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>7.1±9.3</td>
</tr>
<tr>
<td>SPFF group</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>43.8±8.8*</td>
</tr>
<tr>
<td>8</td>
<td>39.3±10.4*</td>
</tr>
<tr>
<td>1</td>
<td>15.5±6.8</td>
</tr>
<tr>
<td>Sab 80</td>
<td>45.0±4.8**</td>
</tr>
</tbody>
</table>

*a p<0.05, ** p<0.01, *** p<0.001 vs. control group, † p<0.05 vs. Sab group n=5, Student’s t-test.
intrinsic activity values of SPFF and salbutamol obtained in isolated trachea strips were similar (0.98, 0.91, respectively), we presumed that the higher $\beta_2$ selectivity of SPFF could account for the stronger bronchodilator effect of SPFF in vivo.

SPFF also showed a longer duration of activity than that of salbutamol in rabbits. This seemed to be due to its low sensitivity to catechol-O-methyltransferase (COMT) because the chemical structure of SPFF lacks a ring hydroxyl group on the catechol nucleus which will result in a longer effective plasma drug level. There may be some other possibilities to explain the longer duration of action of SPFF such as the appearance of some active metabolites, but all no other hypothesis has been validated so far.

In conclusion, SPFF was a potent and highly selective $\beta_2$-adrenergic receptor agonist with longer duration of action.

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**REFERENCES**