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RESEARCH PAPER

Bronchoprotection in conscious guinea pigs by budesonide and the NO-donating analogue, TPI 1020, alone and combined with tiotropium or formoterol

DL Turner[1], N Ferrari[2], WR Ford[1], EJ Kidd[1], B Nevin[1], L Paquet[1]*, P Renzi[2†] and KJ Broadley[1]

[1]*Division of Pharmacology, Welsh School of Pharmacy, Cardiff University, Cathays Park Campus, Cardiff, Wales, UK, and* [2]*Topigen Pharmaceuticals Inc, Montreal, QC, Canada*

Correspondence
Professor Kenneth J Broadley, Division of Pharmacology, Welsh School of Pharmacy, Cardiff University, Cathays Park Campus, King Edward VII Avenue, Cardiff, Wales CF10 3XF, UK. E-mail: broadleykj@cardiff.ac.uk

*Present address: Institut de Recherche en Pharmacologie, Université de Sherbrooke, 3001 12e Avenue Nord, Sherbrooke, Quebec J1H 5N4, Canada.
†Present address: CHUM Research Center, Notre-Dame Hospital, 2065 Alexandre-de-Sève, Room Z-8905, Montreal, Quebec H2L 2W5, Canada.

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BACKGROUND AND PURPOSE

Inhaled corticosteroids, anticholinergics and β_2-adrenoceptor agonists are frequently combined for treating chronic respiratory diseases. We examine the corticosteroid, budesonide, and novel NO-donating derivative, TPI 1020, against histamine- and methacholine-induced bronchoconstriction and whether they enhance the β_2-adrenoceptor agonist formoterol or muscarinic antagonist tiotropium in conscious guinea pigs.

EXPERIMENTAL APPROACH

Dunkin-Hartley guinea pigs received inhaled histamine (3 mM) or methacholine (1.5 mM) and specific airway conductance (sG_{aw}) was measured before and 15 or 75 min after treatment with budesonide, TPI 1020, tiotropium or formoterol alone or in combinations.

KEY RESULTS

Formoterol (0.7–10 μM) and budesonide (0.11–0.7 mM) inhibited histamine-induced bronchoconstriction and tiotropium (2–20 μM) inhibited methacholine-induced bronchoconstriction by up to 70.8 ± 16.6%, 34.9 ± 4.4% and 85.1 ± 14.3%, respectively. Formoterol (2.5 μM) or tiotropium (2 μM) alone exerted small non-significant bronchoprotection. However, when co-administered with TPI 1020 0.11 mM, which alone had no significant effect, there was significant inhibition of the bronchoconstriction (45.7 ± 12.2% and 79.7 ± 21.4%, respectively). Co-administering budesonide (0.11 mM) with tiotropium (2 μM), which alone had no effect, also significantly inhibited the methacholine bronchoconstriction (36.5 ± 13.0%), but there was no potentiation of formoterol against histamine. The NO scavenger, CPTIO, prevented the bronchoprotection by SNAPand TPI 1020.

CONCLUSIONS AND IMPLICATIONS

TPI 1020 potentiated the bronchoprotection by formoterol and tiotropium. Budesonide also enhanced the effects of tiotropium but not formoterol. Combination of TPI 1020 with a long-acting β_2-adrenoceptor agonist or muscarinic receptor antagonist may therefore be a more potent therapeutic approach for treatment of chronic respiratory diseases.

Abbreviations

BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; DMSO, dimethyl sulfoxide; sGaw, specific airways conductance; SNAP, S-nitroso-N-acetyl-DL-penicillamine; TPI 1020, budesonide 21 (4′ nitrooxymethyl)benzoate

Introduction

TPI 1020 is a novel NO-donating compound with anti-inflammatory (Nevin and Broadley, 2004) and bronchodilating activities (Turner *et al.*, 2010). Enhanced bronchoprotective activity was observed when TPI 1020 was co-administered with the short-acting β_2-adrenoceptor agonist salbutamol and it is hypothesized that similar effects may be observed when TPI 1020 is combined with the long-acting bronchodilators formoterol or tiotropium, providing a novel therapy in the treatment of asthma and chronic obstructive pulmonary disease (COPD).

COPD is the fourth leading cause of death worldwide, claiming the lives of 3 million people in 2005 and it affects more than 210 million worldwide. Vagal cholinergic tone is increased in COPD patients (Gross *et al.*, 1989) and this increased tone is the most significant reversible component of bronchoconstriction in these patients. Anticholinergics directly antagonize the muscarinic receptor-mediated bronchoconstrictor action of acetylcholine released from cholinergic nerves. Tiotropium is a long-acting anticholinergic bronchodilator agent, lasting for more than 24 h (O'Connor *et al.*, 1996; Gross, 2004). COPD exacerbations are associated with deterioration in lung function, quality of life and mortality (Seemungal *et al.*, 1998), while also accounting for the majority of costs associated with treating the disease (Hilleman *et al.*, 2000). Many patients experience between one and four exacerbations each year (Hagedorn, 1992). The use of tiotropium in COPD has consistently been correlated with a fall in the number of hospitalizations and the frequency of exacerbations (Dusser *et al.*, 2006; Tashkin *et al.*, 2008).

β_2-Adrenoceptor agonists are also used in the management of COPD as they inhibit bronchoconstriction regardless of the mechanism; however, they are most widely used in the treatment of asthma. Asthma is a chronic disease of the airways characterized by bronchoconstriction, airway hyperreactivity, inflammatory cell influx into the airways and airway remodelling. Long-acting β_2-adrenoceptor agonists combined with an inhaled corticosteroid is the recommended first-line therapy in asthma. Such combinations have been shown to be beneficial in the treatment of COPD, rapidly improving airway function, which can be maintained for 12 months or more in patients with severe COPD, while those with mild to moderate COPD see fewer benefits (Cazzola and Dahl, 2004; Cazzola and Hanania, 2006).

TPI 1020 is a novel anti-inflammatory compound with a dual mechanism of action based on a corticosteroid moiety (budesonide) and NO donation. We have shown that TPI 1020 potentiates the bronchoprotective effects of the short-acting β_2-adrenoceptor agonist salbutamol (Turner *et al.*, 2010), In the present study, we hypothesize that a similar potentiation may be observed when TPI 1020 is co-administered with other bronchodilating agents such as the long-acting β_2-adrenoceptor agonist, formoterol and the anticholinergic compound, tiotropium. This study therefore examines the effects of TPI 1020 and its parent compound, budesonide, on histamine- and methacholine-induced bronchoconstriction in conscious guinea pigs. Our study identifies for the first time a bronchoprotective action of inhaled budesonide, a non-genomic property, which would serve as an adjunct to its main action as an anti-inflammatory in the treatment of asthma. An enhanced bronchoprotection when TPI 1020 is combined with formoterol and tiotropium has relevance to the treatment of airways diseases, which would allow the doses of each to be reduced for equivalent activity, thereby reducing potential side effects.

Methods

Animals

Groups of six, male Dunkin-Hartley guinea pigs weighing 300–400 g were used. Animals received food and water *ad libitum*, and room temperature (22°C) and lighting (maintained on a 12-h cycle) were regulated. The total number of guinea pigs in used in this study was 138. This work complied with the guidelines for the care and use of laboratory animals according to the Animals (Scientific Procedures) Act, 1986. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Measurement of respiratory function

Respiratory function in conscious guinea pigs was monitored using whole-body plethysmography. Changes in airway calibre (bronchoconstriction and bronchodilatation) were recorded as specific airways conductance (sG_{aw}). The method was as described by Griffiths-Johnson *et al.* (1988), although a computerized data acquisition system replaced the original oscilloscope and angle resolver (Smith and Broadley, 2007).

The guinea pig was held in a restrainer, a face mask positioned over the snout and then placed into a sealed plethysmograph chamber. As the animal breathed, a computerized Biopac data acquisition system (Biopac systems, Santa Barbara, CA, USA) acquired and stored data (AcqKnowledge software) referring to air flow across a pneumotachograph (Model FIL, Mercury Electronics, Glasgow, UK). The resulting change in box volume (pressure) was simultaneously measured. Two UP pressure transducers (Pioden Controls LTD, Canterbury, Kent, UK) measured the changes in air flow (UP1) and box pressure (UP2). The resultant waveforms could then be analysed rapidly by comparing the gradients of the flow and the box pressure waves at a point where flow tended towards zero. At least five breaths were analysed, for each animal, at each time point. The average of two baseline determinations of airway function (sG_{aw}) was obtained. Before

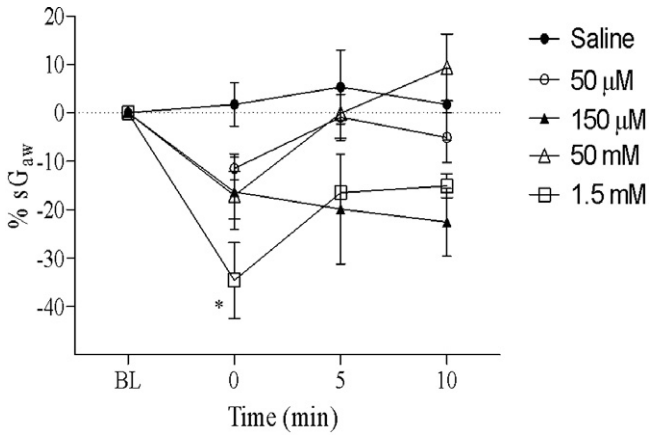

Figure 1

The dose-response effects of inhaled methacholine (50 µM to 1.5 mM). Methacholine 1.5 mM caused a significant degree of bronchoconstriction (*$P < 0.05$, Student's paired *t*-test). Each point represents the mean ± SEM change in sG_{aw} recorded at zero time and 5 and 10 min after exposure of conscious guinea pigs to methacholine expressed as % of the baseline (BL) value before methacholine exposure.

each experiment, the animals were handled and familiarized with the equipment to reduce stress.

Histamine and methacholine exposures

Bronchoconstrictor responses to nebulized histamine (3 mM) or methacholine (0.05–1.5 mM) delivered as nose-only exposures for 20 s were assessed. The dose of 1.5 mM methacholine caused significant bronchoconstriction and was selected for further experiments (Figure 1). The level of bronchoconstriction induced by this concentration of methacholine was not reproducible in the same animal when administered at 24-h intervals (Figure 2A) but was when the interval was extended to 48 h (Figure 2B). Drugs were therefore administered 48 h after the initial methacholine exposure and 24 h after the initial histamine exposure. The second histamine or methacholine exposure was made 15 or 75 min after exposure to nebulized vehicle or drug (TPI 1020, budesonide, tiotropium, formoterol, SNAP or combinations of TPI 1020 or budesonide with formoterol or tiotropium). On each occasion, sG_{aw} readings were taken before histamine or methacholine inhalation (baseline) and at 0, 5 and 10 min after their inhalation challenge.

Drug inhalation by box exposure

Guinea pigs were placed in a sealed chamber (15 × 15 × 30 cm) and drugs were delivered for 15 min via a Wright nebulizer, which supplied air at a pressure of 138 kPa (1.38 bar) and a rate of 0.3 mL·min^{-1}. Animals received either TPI 1020 (0.11, 0.33 or 0.7 mM), formoterol (0.7–25 µM), tiotropium (2–20 µM), budesonide (0.11, 0.33 or 0.7 mM), SNAP (0.2 µM), CPTIO (2 mM) or the vehicles for TPI 1020, SNAP and budesonide (30% ethanol: 30% DMSO: 40% saline), formoterol (50% DMSO: 50% saline) or tiotropium and CPTIO (saline). Animals also received combined inhalation exposures of formoterol with TPI 1020 or budesonide or tiotropium with TPI 1020 or budesonide.

Measurement of NO metabolite (total nitrate and nitrite) levels

Within 20 min of assessing airway responses to the second responses to histamine, animals were overdosed with pentobarbitone sodium (400 mg·kg^{-1} i.p. Euthatal®) and the trachea cannulated. Normal saline (1 mL·100 g^{-1}, twice) was injected through the cannula into the lungs and recovered after 3 min. This bronchoalveolar lavage fluid (BALF) sample was then centrifuged (500× *g*, 6 min), the supernatant removed and frozen (–20°C). The levels of the NO metabolites (total nitrate and nitrite) were determined after thawing by the Greiss reaction as an index of NO production by inhaled NO donors (Nevin and Broadley, 2004). The supernatant from BALF or 100 µL of sodium nitrate (0–20 µg·mL^{-1}) in normal saline were incubated (37°C) for 30 min with HEPES buffer (50 mM, pH 7.4), FAD (5 µM), NADPH (0.1 mM), distilled water (290 µL) and nitrate reductase (0.2 U·mL^{-1}) for nitrite conversion to nitrate. Any unreacted NADPH in the solution (500 µL) was then oxidized by incubating (25°C) for 10 min with potassium ferricyanide (1 mM). The samples were then incubated (25°C) with 1 mL of Greiss reagent (NED: 0.2% (w v^{-1}), sulphanilamide: 2% (w v^{-1}) solubilized in double distilled water : phosphoric acid (95:5, v/v) for 10 min. The plate was then read on a plate reader (MRX TC revelation Dynex technologies, Jencons-PLS, East Sussex, UK) at 543 nm to determine total nitrite (nitrate and reduced nitrite to nitrate) concentration (µM). Each sample was assayed in duplicate.

Calculation of responses

sG_{aw} values were determined at baseline before histamine or methacholine inhalation and at each time point after exposure. The percentage change from baseline was calculated. The peak fall in sG_{aw} in response to histamine or methacholine was then determined for each animal. In all histograms, the values for histamine or methacholine before and after box-inhaled drugs are shown. To illustrate the effects of the box-inhaled drugs, the values after treatment were also expressed as a percentage of the pretreatment peak fall in sG_{aw}, as shown in dose-response curves. All data are presented as the mean ± SEM.

Statistical analysis

Student's paired *t*-test was used to determine significance of inhibitory effects on the histamine- or methacholine-induced bronchoconstriction, from the pre- and post-drug responses to histamine or methacholine. One-way ANOVA, followed by *post hoc* Tukey's or Dunnett's test was used to determine significant differences between different treatment groups. Significance was determined as $P < 0.05$.

Drugs and solutions

The following drugs and solvents were used: aspergillus nitrate reductase (NADPH : nitrate oxido-reductase) 2.0 U·mL^{-1} was purchased from Boehringer Mannheim (Indianapolis, IN, USA) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) from Tocris (Avonmouth, Bristol, UK). Acetyl β-methylcholine chloride (methacholine),

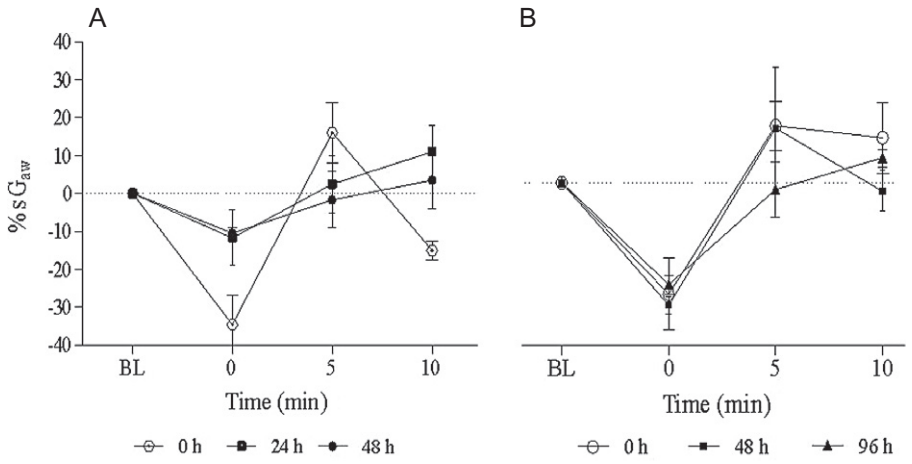

Figure 2

Time courses for the bronchoconstriction to methacholine (1.5 mM) delivered by aerosol on consecutive days (A) or at 48 h intervals (B). Each point represents the mean ± SEM (n = 6) change in sG_{aw} recorded at zero time and 5 and 10 min after exposure of conscious guinea pigs to methacholine expressed as % of the baseline (BL) value before methacholine exposure.

β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), budesonide, CPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt), flavine adenine dinucleotide disodium salt dehydrate (FAD), formoterol fumarate dehydrate, histamine diphosphate, HEPES buffer, N-1-napthylethylenediamine dihydrochloride (NED), potassium ferricyanide, phosphoric acid and sulphanilamide were supplied by Sigma-Aldrich, (St Louis, MO, USA). DMSO was supplied by Fisher Scientific (Loughborough, UK), Euthetal® (pentobarbital sodium 200 mg·mL^{-1}) by Rhone Merieux (Harlow Essex, UK) and tiotropium bromide and TPI 1020 were supplied by Topigen (Montreal, Canada).

TPI 1020 and budesonide were dissolved in a vehicle of 30% DMSO, 30% ethanol and 40% saline. Tiotropium bromide and histamine diphosphate were dissolved in saline, formoterol in a solution of 50% DMSO and 50% saline. When combinations of drugs were administered, a single solution was used, formoterol dissolved in the DMSO component of the TPI 1020/budesonide vehicle, and tiotropium dissolved in the saline component, allowing a single solution to be nebulized.

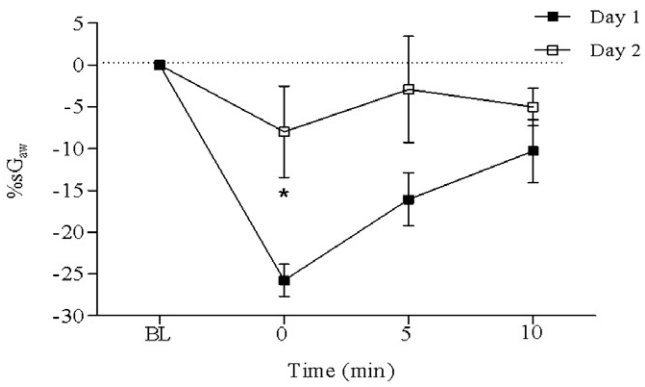

Figure 3

Responses recorded at zero time and 5 and 10 min after exposure of conscious guinea pigs to histamine. Histamine exposure was at 24 h before and 75 min after inhalation of 7 μM formoterol. Each point represents the mean ± SEM change in sG_{aw} expressed as % of the baseline (BL) value before each histamine exposure. Formoterol significantly inhibited histamine-induced bronchoconstriction at time zero, *P < 0.05, Student's paired t-test.

Results

The bronchoprotective effects of formoterol and TPI 1020 against histamine-induced bronchoconstriction

Inhalation of 3 mM histamine caused a bronchoconstriction seen as an immediate significant fall in sG_{aw}. On repeating the exposure 24 h later, at 75 min after 7 μM formoterol, the bronchoconstriction was significantly inhibited (Figure 3). The vehicles for formoterol (50% saline: 50% DMSO) and TPI 1020 (30% DMSO: 30% ethanol: 40% saline) had no significant effect on the histamine response (Figures 4 and 5).

Formoterol (0.7–10 μM) inhibited histamine-induced bronchoconstriction 75 min post-treatment, with a maximum inhibition of 70.8 ± 16.6% at 7 μM (Figures 3 and 4). Significant inhibition, however, was not observed at the highest dose of formoterol (25 μM, 9.8 ± 10.7%), (Figure 4).

TPI 1020 at 0.11 mM caused small apparent but non-significant inhibitory effects 15 and 75 min after treatment, inhibiting the histamine response by 16.8 ± 5.6% and 13.8 ± 10.9%, respectively (Figure 5). Increasing the dose of TPI 1020 to 0.33 and 0.7 mM significantly inhibited histamine-induced bronchoconstriction 15 min after inhalation by 50.5 ± 8.1 and 72.1 ± 15.3%, respectively (P < 0.05, Student's paired t-test). Unlike formoterol, TPI 1020 effects were lost by 75 min after inhalation, the response in fact increasing by 20.4 ± 24.2% (Figure 5).

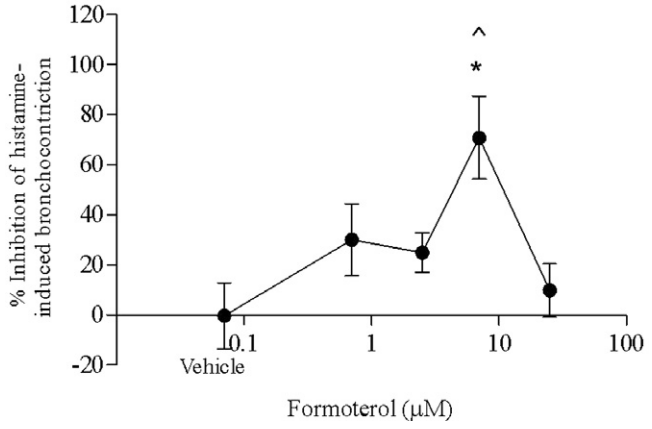

Figure 4

Effect of formoterol or vehicle (DMSO : saline 50:50) on the bronchoconstrictor response to histamine 75 min after inhalation. Histamine-induced bronchoconstriction was inhibited in a dose-dependent manner from 0.7 to 7 μM; however, this effect was reversed at a higher dose of 25 μM. Each point represents the mean peak fall in sG_{aw} post-treatment as a percentage of the pretreatment histamine response ± SEM (n = 6). *Histamine response in the presence of formoterol significantly different from the histamine response 24 h beforehand $P < 0.05$, Student's paired t-test. ^Significantly different from vehicle $P < 0.05$, ANOVA, followed by *post hoc* Dunnett's.

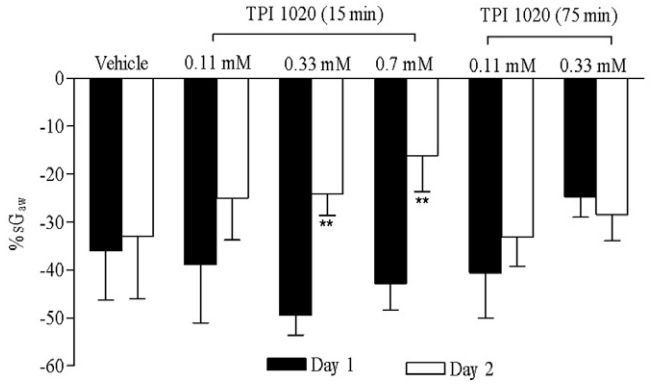

Figure 5

Effect of TPI 1020 or vehicle (30% ethanol: 30% DMSO: 40% saline) on histamine-induced bronchoconstriction. TPI 1020 (0.33 and 0.7 mM) significantly inhibited histamine-induced bronchoconstriction 15 min after administration (**$P < 0.01$, Students paired t-test) but had no effect at 75 min. Each column represents the mean peak fall in sG_{aw} ± SEM (n = 6) following histamine exposure recorded before (day 1) and after TPI 1020 (day 2).

Combined effects of TPI 1020 or budesonide with formoterol

Co-administering TPI 1020 (0.11 mM) with formoterol (2.5 μM), which by themselves had non-significant effects (24.9 ± 7.9% inhibition; Figure 6A), significantly inhibited the histamine response by 45.7 ± 12.2% 75 min after inhalation (Figure 6B; $P < 0.05$, Student's paired t-test). Increasing the dose of TPI 1020 to 0.33 mM, which again had no effect on its own, further increased the bronchoprotective effect of formoterol (2.5 μM) to 63.8 ± 14.2% 75 min after inhalation (Figure 6B; $P < 0.01$, Student's paired t-test) and was significantly superior to either drug alone ($P < 0.05$, ANOVA, *post hoc* Tukey's). In contrast, combinations of the same concentrations (0.11 and 0.33 mM) of the parent compound, budesonide, with formoterol failed to significantly inhibit the histamine-induced bronchoconstriction at 15 min (32.3 ± 13.6 and 26.5 ± 6.0% inhibitions, respectively) and 75 min (24.3 ± 29.3 and 20.9 ± 19.5%, respectively) after exposure (Figure 6B). Budesonide alone at these concentrations did not exert significant inhibitory effects on histamine-induced bronchoconstriction, but at 0.7 mM, there was significant bronchoprotection by 34.9 ± 4.4% ($P < 0.05$, Student's paired t-test; Figure 6A). The degree of potentiation of formoterol by TPI 1020 and budesonide was measured as the difference between the percentage inhibition of histamine bronchoconstriction for formoterol alone and in combination. There was potentiation by TPI 1020 but not by budesonide (Figure 6C).

Effects of TPI 1020 and tiotropium against methacholine-induced bronchoconstriction

Tiotropium (2–20 μM) dose-dependently inhibited the methacholine-induced bronchoconstriction, inhibiting it by between 23.6 ± 15.7% (non-significant) and 85.1 ± 14.3% (Figure 7); significant inhibition of methacholine-induced bronchoconstriction occurred at 6.5 and 20 μM, ($P < 0.05$, Student's paired t-test). The saline vehicle for tiotropium had no significant effect on methacholine-induced bronchoconstriction, the change in the methacholine response being 1.76 ± 5.6%.

Combined effects of TPI 1020 or budesonide with tiotropium

Low-dose TPI 1020 (0.11 mM) did not exert significant inhibitory effects 15 and 75 min after treatment (6 ± 13.9% and 13.1 ± 8.7%, respectively; Figure 8). However, a higher dose of TPI 1020 (0.33 mM) significantly inhibited methacholine-induced bronchoconstriction at 15 (81.6 ± 19.8%) and 75 min (34.7 ± 13.8%) after nebulization (Figure 8; $P < 0.01$ and $P < 0.05$, respectively, Student's paired t-test). The vehicle for TPI 1020 had no significant inhibitory effect on the methacholine response.

TPI 1020 (0.11 mM), tiotropium (2 μM) and budesonide (0.11 mM) did not exert significant bronchoprotective effects when administered alone 75 min before methacholine (Figure 9). However, when these doses of budesonide and tiotropium were given simultaneously, methacholine-induced bronchoconstriction was significantly inhibited by 36.5 ± 13.0% ($P < 0.01$, Student's paired t-test), although this was not significantly greater than either budesonide or tiotropium alone. When these ineffective doses of TPI 1020 (0.11 mM) and tiotropium (2 μM) were given simultaneously, methacholine-induced bronchoconstriction was significantly inhibited by 79.7 ± 21.4% ($P < 0.01$, Student's paired t-test). The combination of TPI 1020 and tiotropium inhibited the methacholine response to a significantly greater extent than TPI 1020 alone ($P < 0.05$, ANOVA, *post hoc* Tukey's; Figure 9).

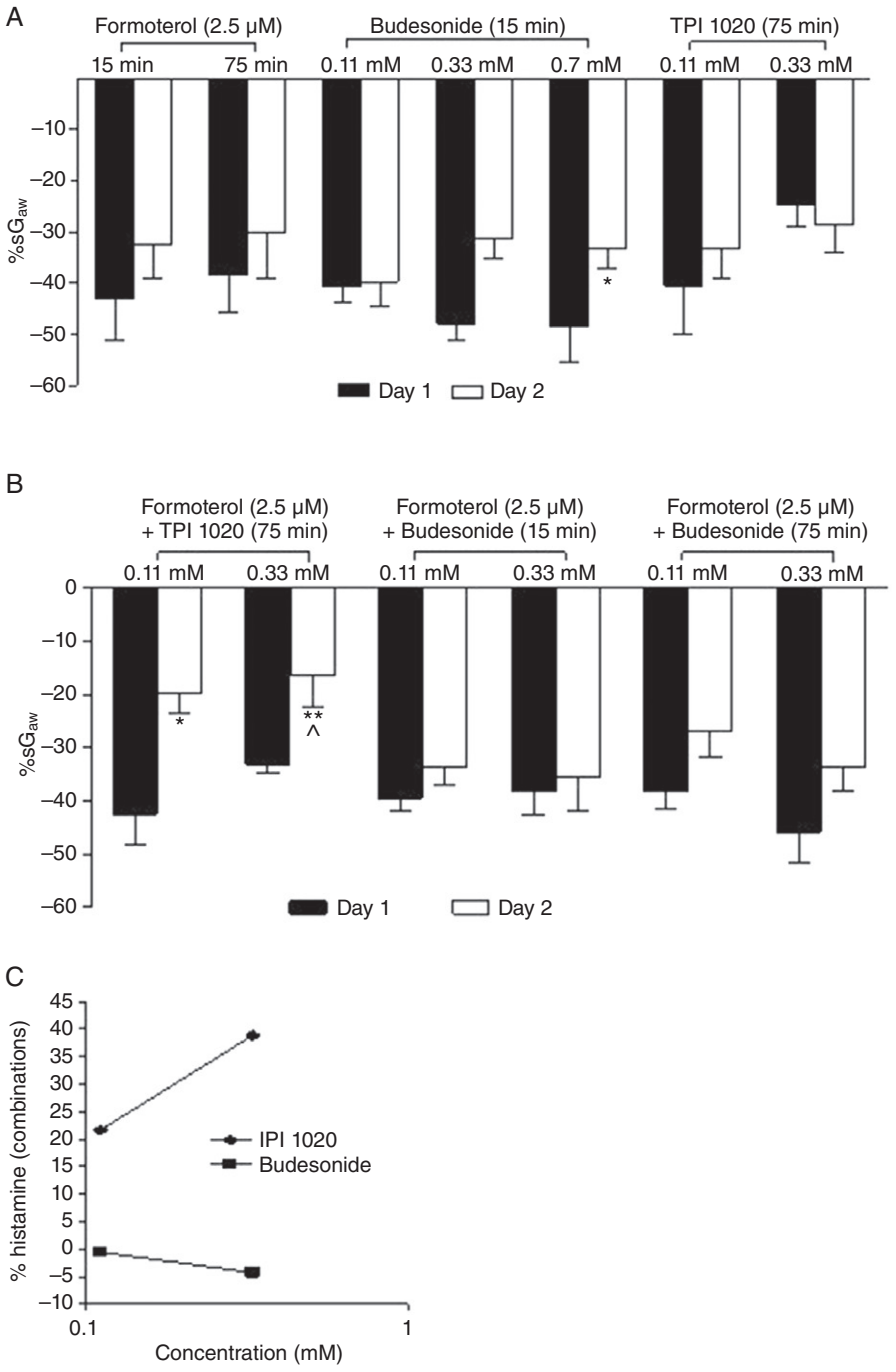

Figure 6

Individual (A) and combined (B) effects of TPI 1020 or budesonide with formoterol on histamine-induced bronchoconstriction 15 or 75 min after inhalation. The combination of TPI 1020 (0.11 and 0.33 mM) with formoterol (2.5 μM), which alone had no significant effect on histamine-induced bronchoconstriction, significantly inhibited bronchoconstriction 75 min after inhalation (*$P < 0.05$, **$P < 0.01$, Student's paired *t*-test). This inhibition was significantly greater than with formoterol or TPI 1020 alone (^$P < 0.05$, ANOVA, followed by *post hoc* Tukey's). Budesonide (0.11 or 0.33 mM) combined with formoterol (2.5 μM) did not significantly inhibit histamine-induced bronchoconstriction at either 15 or 75 min after inhalation. Each column represents the mean peak fall in sG_{aw} ± SEM ($n = 6$) following histamine exposure recorded before (day 1) and after formoterol, budesonide, TPI 1020 or their combinations (day 2). (C) Potentiation of formoterol (2.5 μM) by TPI 1020 (0.11 and 0.33 mM) expressed as the difference in mean % inhibition of histamine-induced bronchoconstriction between formoterol alone and combined with TPI 1020. There was no potentiation by budesonide (0.11 and 0.33 nM).

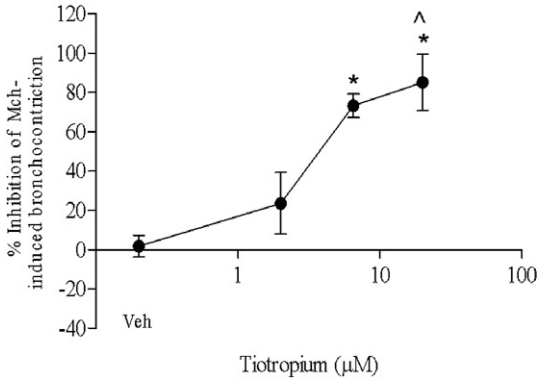

Figure 7

Effect of tiotropium or vehicle (veh, saline) on methacholine (Mch)-induced bronchoconstriction at 75 min after methacholine inhalation. A significant bronchoprotection was observed at 6.5 and 20 μM (*P < 0.05, Student's paired t-test). Each point represents the mean ± SEM (n = 6) peak fall in sG_{aw} post-treatment as a percentage of the pretreatment methacholine response. ^Significantly different from vehicle P < 0.05, ANOVA, followed by *post hoc* Dunnett's.

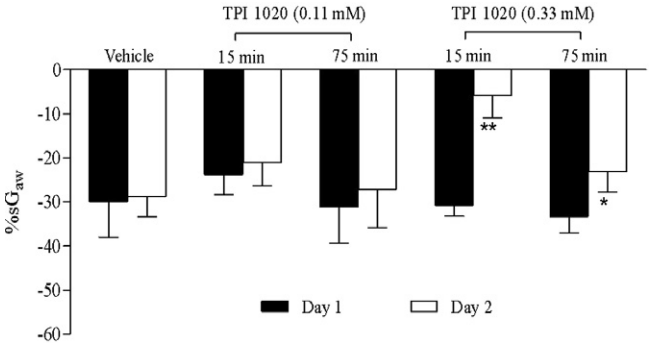

Figure 8

Effect of TPI 1020 or vehicle (on methacholine-induced bronchoconstriction. TPI 1020 (0.33 mM), but not 0.11 mM significantly inhibited methacholine-induced bronchoconstriction 15 and 75 min after inhalation (**P < 0.01, *P < 0.05, Student's paired t-test). Each column represents the mean peak fall in sG_{aw} ± SEM (n = 6) following methacholine exposure recorded before (day 1) and after TPI 1020 (day 2).

Comparison of TPI 1020 against histamine- and methacholine-induced bronchoconstriction

TPI 1020 at 0.11 mM did not distinguish between histamine and methacholine-induced bronchoconstrictions at either 15 or 75 min after inhalation. However, at 0.33 mM, TPI 1020 significantly inhibited methacholine-induced bronchoconstriction by 81.6 ± 19.8%, compared to 50.5 ± 8.1% of the histamine response at 15 min after inhalation. At 75 min after treatment with TPI 1020 (0.33 mM), the methacholine response was inhibited by 34.7 ± 13.8% while histamine-induced bronchoconstriction was not inhibited, increasing by +20.14 ± 24.17%. The inhibitory effects of TPI 1020 were

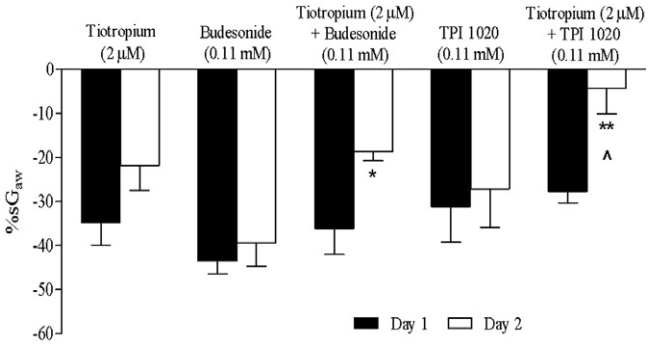

Figure 9

Combined effects of TPI 1020 or budesonide with tiotropium. Tiotropium (2 μM), budesonide (0.11 mM) and TPI 1020 (0.11 mM) did not significantly inhibit methacholine-induced bronchoconstriction 75 min after the drug exposure. Budesonide (*P < 0.05, Student's paired t-test) or TPI 1020 (**P < 0.01, Student's paired t-test) co-administered with tiotropium significantly inhibited the methacholine-induced bronchoconstriction. The combination of TPI 1020 and tiotropium was significantly more effective than TPI 1020 alone (^P < 0.05, ANOVA, *post hoc* Tukey's). Each column represents the mean peak fall in sG_{aw} ± SEM (n = 6) following methacholine exposure recorded before (day 1) and after TPI 1020 (day 2).

therefore significantly greater against bronchoconstriction induced by methacholine than by histamine (P < 0.05, Student's unpaired t-test).

Effects of formoterol, salbutamol, budesonide, TPI 1020 and tiotropium alone or in combinations on baseline sG_{aw}

Baseline sG_{aw} values were measured before the histamine or methacholine inhalations (Table 1). For comparison, we also included data on baseline values for salbutamol (120 μM) alone and salbutamol (80 μM) combined with budesonide or TPI 1020 recorded at 15 min after inhalation, taken from our previous study where these values were not presented (Turner *et al.*, 2010). Salbutamol alone inhibited histamine bronchoconstriction by 84.4 ± 17.2% while salbutamol (80 μM) combined with budesonide (0.33 mM) inhibited histamine by 62.0 ± 1.0% and combined with TPI 1020 (0.11 and 0.33 mM) inhibited by 76.7 ± 13.2% and 106.9 ± 39.5%, respectively. However, there were no significant differences between baseline sG_{aw} values before and after any exposures to formoterol, budesonide, TPI 1020 or tiotropium alone or in combination.

Effects of the NO scavenger, CPTIO and BALF NO metabolite levels

The NO scavenger, CPTIO (2 mM; Akaike *et al.*, 1993) or its vehicle (saline) were administered by box inhalation for 15 min, 30 min before inhalation exposures to the NO donor, SNAP (0.2 μM) or TPI 1020 (0.7 mM), which were in turn followed after a further 30 min by the second histamine exposure. The histamine-induced bronchoconstriction was virtually abolished by SNAP and this effect was totally reversed by CPTIO (Figure 10A). Similarly, the significant inhibition of histamine-induced bronchoconstriction by TPI 1020 (0.7 mM) was also abolished by CPTIO (Figure 10A).

Table 1

Baseline sG_{aw} values before and after inhalation of salbutamol, formoterol, tiotropium, budesonide or TPI 1020 alone or in combinations

Drug alone			Drug combinations				
				Combined with budesonide		Combined with TPI 1020	
Dose, time			Dose, time	0.11 mM	0.33 mM	0.11 mM	0.33 mM
Salbutamol 120 μM, 15 min	Before	0.470 ± 0.018	80 μM, 15 min	0.492 ± 0.021	0.455 ± 0.014	0.456 ± 0.010	0.495 ± 0.007
	After	0.498 ± 0.048*		0.502 ± 0.013	0.508 ± 0.014*	0.486 ± 0.007*	0.483 ± 0.006*
Formoterol 7 μM, 75 min	Before	0.572 ± 0.023	2.5 μM, 15 min	0.177 ± 0.011	0.303 ± 0.010		
	After	0.606 ± 0.022*		0.165 ± 0.009	0.326 ± 0.021		
	Before		2.5 μM, 75 min	0.302 ± 0.018	0.293 ± 0.021	0.564 ± 0.026	0.543 ± 0.060
	After			0.315 ± 0.054	0.300 ± 0.027	0.528 ± 0.029*	0.589 ± 0.055*
Tiotropium 20 μM, 75 min	Before	0.558 ± 0.023	2 μM, 75 min	0.313 ± 0.013		0.527 ± 0.028	
	After	0.586 ± 0.030*		0.254 ± 0.038*		0.562 ± 0.019*	
Budesonide 0.7 mM, 15 min	Before	0.527 ± 0.030					
	After	0.562 ± 0.023*					
TPI 1020 0.7 mM, 15 min	Before	0.374 ± 0.017					
	After	0.408 ± 0.013*					

sG_{aw} values are in s^{-1} cmH_2O^{-1} and are the mean ± SEM. Values were measured immediately prior to the inhalations of histamine (salbutamol, formoterol, budesonide or tiotropium) or methacholine (tiotropium) and at 15 or 75 min after the inhalation exposures of salbutamol, formoterol, tiotropium, budesonide or TPI 1020 alone or in combination. Values were compared by analysis of variance followed by *post hoc* Tukey's test and no significant differences were found between values before and after individual drugs or their combinations. sG_{aw} values are shown mainly for doses and times after inhalation exposures where there was significant inhibition of histamine- or methacholine-induced bronchoconstriction (i.e. bronchoprotection). Those producing effective inhibition of histamine or methacholine bronchoconstriction (bronchoprotection) are shown by *.

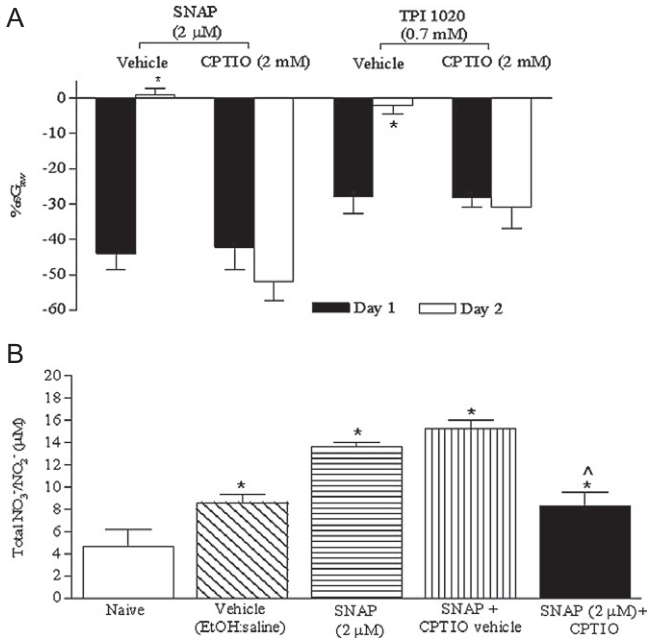

Figure 10

(A) Effects of CPTIO (2 mM) on inhibition of histamine-induced bronchoconstriction by inhaled SNAP (2 μM) and TPI 1020 (0.7 mM). SNAP and TPI 1020 significantly inhibited histamine-induced bronchoconstriction of conscious guinea pigs treated with the vehicle for CPTIO (saline; *$P < 0.05$, Student's paired *t*-test). This inhibition was abolished by CPTIO. Each column represents the mean peak fall in sG_{aw} ± SEM ($n = 6$) following histamine exposure recorded before (day 1) and after SNAP or TPI 1020 (day 2). (B) NO metabolites (total nitrate and nitrite, NO_3^-/NO_2^-) in BALF determined after inhalation of SNAP. The vehicle for SNAP (ethanol : saline) significantly increased NO metabolites compared with naïve guinea pigs, which was further increased by SNAP alone and SNAP after the CPTIO vehicle (saline; *$P < 0.05$, ANOVA, *post hoc* Tukey's). CPTIO significantly reduced NO metabolites to control levels (^$P < 0.05$, ANOVA, *post hoc* Tukey's). Each column is the mean total NO_3^-/NO_2^- level ± SEM ($n = 6$).

The combined NO_3^-/ NO_2^- levels in BALF from guinea pigs treated with the vehicle for TPI 1020 (30% DMSO, 30% ethanol, 40% saline) showed significant increases (8.7 ± 0.8 μM) compared with naïve animals (4.7 ± 1.5 μM), and this obscured any further increases after both SNAP and TPI 1020 exposures. The vehicle for SNAP was therefore changed to 30% ethanol, 70% saline which also increased the combined nitrite/nitrate levels but there was a further significant increase in their levels after SNAP (Figure 10B). This increase was not affected by the vehicle for CPTIO but was significantly inhibited to the vehicle levels by CPTIO (Figure 10B).

Discussion

This study has demonstrated enhanced bronchoprotective effects of the novel anti-inflammatory, NO-releasing compound, TPI 1020, when co-administered with the long-acting bronchodilator compounds, formoterol, a long-acting β_2-adrenoceptor agonist and tiotropium, a muscarinic receptor antagonist.

Formoterol exerted a bronchoprotective effect 75 min after inhalation in conscious guinea pigs. Formoterol has a longer duration of action than salbutamol, which is thought to arise because it forms a depot within the cell membrane that slowly leaches out and continues to interact with the β_2-adrenoceptor (Lötvall, 2002). This study confirmed the longer duration of action of formoterol, since in our previous study in conscious guinea pigs (Turner *et al.*, 2010) a dose of salbutamol that produced an equivalent response at 15 min had no bronchoprotective effect at 75 min.

The higher dose of formoterol of 25 μM, however, did not exert a bronchoprotective effect in conscious guinea pigs. Although rapid desensitization and internalization of the β_2-adrenoceptor exposed to high concentrations of formoterol (January *et al.*, 1997) could explain this observation, it is probably unlikely. In contrast to the present study, previous studies with formoterol in conscious guinea pigs have shown a progressive concentration-related increase in the bronchoprotective effects against 5-hydroxytryptamine-induced bronchoconstriction (Battram *et al.*, 2006). These authors also failed to demonstrate any desensitization with repeated dosing with formoterol. A possible reason for this discrepancy is that they administered formoterol by dry powder directly into the trachea, whereas we administered a nebulized solution by inhalation.

TPI 1020 at 0.33 mM inhibited histamine-induced bronchoconstriction 15 min after aerosol exposure but these effects were diminished 75 min after administration. This suggests that the bronchoprotective effect of TPI 1020 was of relatively short duration. Pharmacokinetics data in humans show that plasma levels of the budesonide released from TPI 1020 do not peak until 3 h after a single inhaled dose of TPI 1020 (Boulet *et al.*, 2009). Whether the NO released from TPI 1020 follows the same time course is not known, but our results suggest an immediate release which is not sustained to 75 min. When TPI 1020 was combined with the long-acting β_2-adrenoceptor agonist, formoterol, the histamine-induced bronchoconstriction was inhibited to a greater extent than with either drug administered alone. The bronchoprotection by TPI 1020 and formoterol is exerted through different pathways. TPI 1020 releases NO, which causes smooth muscle relaxation through activation of soluble guanylyl cyclase, stimulating production of cGMP. Formoterol, as a β-adrenoceptor agonist, is a functional antagonist, opposing histamine-induced bronchoconstriction by increasing cAMP levels in airway smooth muscle cells. The relaxation by both occurs independently of the bronchoconstrictive mechanism.

One of the reported benefits of long-acting β-adrenoceptor agonists and inhaled corticosteroid combinations is their apparent additive effects. A study in COPD patients showed a greater bronchodilator effect when budesonide and formoterol were co-administered compared to formoterol alone, demonstrating a 15% improvement in FEV_1 within 11 min when they were co-administered but 21 min when formoterol was given alone (Cazzola *et al.*, 2004). Moreover, our previous studies examining TPI 1020 alone or combined with salbutamol demonstrated that the bronchoprotective effects of the combined drugs exceeded that of the individual drugs (Turner *et al.*, 2010). The enhanced effect of TPI 1020 combined with formoterol could be due to at least

two separate mechanisms arising from both the NO and budesonide components of the TPI 1020 molecule. The NO-related mechanisms will be considered later. Non-genomic effects of corticosteroids have been documented, which are associated with rapid effects seen within minutes both *in vitro* and *in vivo* (Rodrigo and Rodrigo, 1998; Ketchell *et al.*, 2002; Rodrigo, 2006). Indeed, budesonide alone exerted bronchoprotective effects, which were significant at the highest dose (0.7 mM). A comparison of the dose-response curves for TPI 1020 and the parent compound, budesonide, alone showed that TPI 1020 was slightly more effective. However, in potentiating formoterol, TPI 1020 was substantially more effective. Another non-genomic property of corticosteroids is on the uptake, distribution and elimination of β-adrenoceptor agonists such as formoterol through membrane transporters such as organic cation transporters (OCTs). OCT3 is highly expressed in the airway bronchial and vascular smooth muscle (Horvath *et al.*, 2003) and is involved in the disposal and elimination of drugs in the airway. More recent studies demonstrated an inhibitory effect of corticosteroids on the uptake of β_2-adrenoceptor agonists, including formoterol, which is more pronounced on vascular than bronchial smooth muscle (Horvath *et al.*, 2007a). These inhibitory actions of corticosteroids could therefore prolong or enhance the bronchodilator effect of formoterol. When budesonide was administered in combination with formoterol in the present study, however, there was no potentiation of the bronchoprotection at either 15 or 75 min after inhalation. In our previous study, the combination of the short acting β_2-adrenoceptor agonist, salbutamol, with budesonide resulted in an enhanced bronchoprotection (Turner *et al.*, 2010).

The cholinergic antagonist, tiotropium, also exerted bronchoprotective effects in conscious guinea pigs but in this case against methacholine-induced bronchoconstriction. Tiotropium is a long-acting competitive muscarinic receptor antagonist (Haddad *et al.*, 1994) that exerts bronchodilator effects through the antagonism of acetylcholine released from vagal nerves at muscarinic M_3 receptors on airway smooth muscle. This contrasts with formoterol, which acts as a functional antagonist, opposing bronchoconstriction by increasing cAMP levels and resulting in bronchoprotection regardless of the mechanism of the bronchoconstriction. TPI 1020 also inhibited the methacholine-induced bronchoconstriction but to a greater extent than it inhibited histamine-induced bronchoconstriction. This may, in part, be due to different distributions of muscarinic M_3 and histamine H_1 receptors, the former being more densely distributed in the larger, central airways (Coulson and Fryer, 2003) while H_1 receptors are widely distributed in the smaller, peripheral airways.

When TPI 1020 and tiotropium were combined, they inhibited the methacholine response to a greater extent than either drug alone. The mechanism of the potentiation of tiotropium with TPI 1020 could be attributed to either the corticosteroid or NO components of TPI 1020, or both. In a study of 300 COPD patients, a combination of budesonide and tiotropium was found to be superior to tiotropium alone as measured by an improved quality of life, although no significant differences were found in FEV_1 or the use of rescue medication with salbutamol (Um *et al.*, 2007). When budesonide and tiotropium were combined, there was also an enhanced bronchoprotection, suggesting that the corticosteroid component of TPI 1020 may be responsible, in part, for the potentiation of tiotropium. The mechanism for this, however, remains to be established.

NO would appear to contribute to the potentiation of both tiotropium and formoterol by TPI 1020. We have previously demonstrated (Turner *et al.*, 2010) that the inhibitory effects of TPI 1020 on histamine-induced bronchoconstriction are almost abolished by the inhibitor of NO-sensitive guanylyl cyclase, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Garthwaite *et al.*, 1995). Further evidence that NO release was responsible for the bronchoprotective effect of TPI 1020 was provided in the present study, since the NO scavenger, CPTIO (Akaike *et al.*, 1993) inhibited its bronchoprotective action. We were unable to determine the release of NO into lavage fluid by TPI 1020 since the vehicle masked any increases in total nitrate and nitrite. However, inhalation of the reference NO donor, SNAP, produced increases in the NO metabolites in BALF and SNAP exerted a bronchoprotective action against histamine-induced bronchoconstriction; these increases in NO metabolites and the bronchoprotection by SNAP were abolished by the NO scavenger, CPTIO. Therefore, it is suggested that the bronchoprotective effects of TPI 1020 against histamine- or methacholine-induced bronchoconstriction are predominantly mediated by the cGMP-signalling pathway in airway smooth muscle cells. The increased production of cGMP results from the activation of soluble guanylyl cyclase by NO, which causes bronchodilatation by activation of protein kinase G. The NO is presumed to be derived from a NO donor cleaved from the TPI 1020 molecule by esterases once deposited within the airways to yield an NO donor and budesonide (Tallet *et al.*, 2002). We could not compare the non-genomic corticosteroid activity of TPI 1020 with that of budesonide as it was always linked to the NO-donating moiety. A further mechanism whereby inhaled corticosteroids may enhance the bronchodilator activity of tiotropium and formoterol is through vasoconstriction in the airways and reduced mucosal blood flow, which could reduce the rate of clearance of tiotropium and formoterol from the airways (Mendes *et al.*, 2003).

The mechanisms behind the enhanced activity of TPI 1020 in combination with tiotropium or formoterol remain unclear; however, the potent bronchoprotective activity of TPI 1020 in combination with a long-acting β_2-adrenoceptor agonist, or an anticholinergic such as tiotropium may provide a novel therapy for asthma, COPD or other airway diseases associated with bronchoconstriction and inflammation. We did not demonstrate any anti-inflammatory activity of TPI 1020 in the present study. Other studies have demonstrated *in vivo* that TPI 1020 is superior to budesonide in inhibiting airway hyper-reactivity and neutrophil infiltration in an animal model of COPD (Nevin and Broadley, 2004) and studies in man have shown that it is as effective as budesonide in improving FEV_1 and superior to budesonide in reducing sputum neutrophils in asthmatic smokers (Boulet *et al.*, 2009).

Combinations of long-acting β_2-adrenoceptor agonists and inhaled corticosteroids are the first-line therapy for persistent asthma; individually, these drugs target the two major

components of asthma, bronchoconstriction and airway inflammation. Our data suggest that interactions between formoterol and TPI 1020 produce a greater inhibitory effect against histamine-induced bronchoconstriction. However, budesonide itself did not enhance the bronchoprotective effect of formoterol. The reason for this could not be deduced from the present study, but this observation may help explain the mechanism for the potentiation by budesonide of salbutamol (Turner *et al.*, 2010) and tiotropium in this study. Since not all bronchodilators were potentiated, it can be assumed that it was not simply due to bronchodilatation by budesonide opening up the airways to allow more drugs to reach the lower airways. Indeed, neither budesonide nor any of the other drugs or combinations in doses that were bronchoprotective exerted any bronchodilator action, as measured by the baseline sG_{aw} values before and after their inhalation. Thus, they exerted bronchoprotection against histamine/methacholine but no bronchodilatation. Could the potentiation by budesonide be due to inhibition of uptake of the drugs allowing more to be available for receptor activation? Formoterol is transported via OCT3, which is blocked by budesonide (Horvath *et al.*, 2007a) but no information was found on salbutamol, which was potentiated in our previous study. This suggests that inhibition of OCT3 by budesonide is not responsible for the potentiation of salbutamol. Tiotropium (Nakamura *et al.*, 2010), salbutamol and formoterol but not salmeterol (Horvath *et al.*, 2007b) are transported via pH-dependent organic cation/carnitine transporters (OCTN1 and OCTN2) in bronchial epithelial cells. These transporters are not inhibited by corticosteroids (Horvath *et al.*, 2007b) and are not therefore the site of the potentiation of salbutamol or tiotropium by budesonide. Further experiments with a wider range of β_2-adrenoceptor agonists may help explain the site of potentiation by budesonide.

Although the use of inhaled corticosteroids in COPD is controversial, up to 50% of patients with stable COPD receive them (Van Andel *et al.*, 1999; Highland, 2004). Inhaled corticosteroids appear to be beneficial in COPD, particularly in patients in the more severe stages of the disease, when they have been shown to reduce the frequency and duration of exacerbations (Sin and Tu, 2001) and reduce mortality rates (Soriano *et al.*, 2002). The enhanced bronchoprotective effects of TPI 1020 when co-administered with formoterol or tiotropium suggests that the control of airway function in airways diseases may be improved by such combinations, assuming that these results apply to human subjects.

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Conflict of interest

The authors declare that no conflict of interest exists.

References

Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K *et al.* (1993). Antagonistic action of imidazolineoxyl N oxides against endothelium-derived relaxing factor/.NO through radical reaction. Biochemistry 32: 827–832.

Battram C, Charlton SJ, Cuenoud B, Dowling MR, Fairhurst RA, Farr D *et al.* (2006). *In vitro* and *in vivo* pharmacological characterization of 5-[(*R*)-2-(5,6-diethyl-indan-2-ylamino)-1-hydroxy-ethyl]-8-hydroxy-1*H*-quinolin-2-one (indacaterol), a novel inhaled β_2 adrenoceptor agonist with a 24-h duration of action. J Pharmacol Exp Ther 317: 762–770.

Boulet LP, Lemiere C, Gauvreau G, Boulet LP, Lemière C, Gauvreau G *et al.* (2009). Safety, pharmacodynamics and pharmacokinetics of TPI 1020 in smokers with asthma. Respir Med 103: 1159–1166.

Cazzola M, Dahl R (2004). Inhaled combination therapy with long-acting beta 2-agonists and corticosteroids in stable COPD. Chest 126: 220–237.

Cazzola M, Hanania NA (2006). The role of combination therapy with corticosteroids and long-acting beta2-agonists in the prevention of exacerbations in COPD. Int J Chron Obstruct Pulmon Dis 1: 345–354.

Cazzola M, Santus P, Di Marco F, Carlucci P, Mondoni M, Matera MG *et al.* (2004). Onset of action of formoterol/budesonide in single inhaler vs. formoterol in patients with COPD. Pulm Pharmacol Ther 17: 121–125.

Coulson FR, Fryer AD (2003). Muscarinic acetylcholine receptors and airway diseases. Pharmacol Ther 98: 59–69.

Dusser D, Bravo ML, Iacono P (2006). The effect of tiotropium on exacerbations and airflow in patients with COPD. Eur Respir J 27: 547–555.

Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. Mol Pharmacol 48: 184–188.

Griffiths-Johnson DA, Nicholls PJ, McDermott M (1988). Measurement of specific airway conductance in guinea pigs. A noninvasive method. J Pharmacol Methods 19: 233–242.

Gross NJ (2004). Tiotropium bromide. Chest 126: 1946–1953.

Gross NJ, Co E, Skorodin MS (1989). Cholinergic bronchomotor tone in COPD. Estimates of its amount in comparison with that in normal subjects. Chest 96: 984–987.

Haddad EB, Mak JC, Barnes PJ (1994). Characterization of [3H]Ba 679 BR, a slowly dissociating muscarinic antagonist, in human lung: radioligand binding and autoradiographic mapping. Mol Pharmacol 45: 899–907.

Hagedorn SD (1992). Acute exacerbations of COPD. How to evaluate severity and treat the underlying cause. Postgrad Med 91: 105–107. 110–112.

Highland KB (2004). Inhaled corticosteroids in chronic obstructive pulmonary disease: is there a long-term benefit? Curr Opin Pulm Med 10: 113–119.

Hilleman DE, Dewan N, Malesker M, Friedman M (2000). Pharmacoeconomic evaluation of COPD. Chest 118: 1278–1285.

Horvath G, Sutto Z, Torbati A, Conner GE, Salathe M, Wanner A (2003). Norepinephrine transport by the extraneuronal monoamine transporter in human bronchial arterial smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 285: L829–L837.

Horvath G, Mendes ES, Schmid N, Schmid A, Conner GE, Salathe M *et al*. (2007a). The effect of corticosteroids on the disposal of long-acting beta2-agonists by airway smooth muscle cells. J Allergy Clin Immunol 120: 1103–1109.

Horvath G, Schmid N, Fragoso MA, Schmid A, Conner GE, Salathe M *et al*. (2007b). Epithelial organic cation transporters ensure pH-dependent drug absorption in the airways. Am J Repir. Cell Mol Biol 36: 53–60.

January B, Seibold A, Whaley B, Hipkin RW, Lin D, Schonbrunn A *et al*. (1997). beta2-adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. J Biol Chem 272: 23871–23879.

Ketchell RI, Jensen MW, Lumley P, Wright AM, Allenby MI, O'Connor BJ (2002). Rapid effect of inhaled fluticasone propionate on airway responsiveness to adenosine 5′-monophosphate in mild asthma. J Allergy Clin Immunol 110: 603–606.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010) NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160:1577–1579.

Lötvall J (2002). The long and short of β_2-agonists. Pulm Pharmacol Ther 15: 497–501.

McGrath J, Drummond G, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Mendes ES, Pereira A, Danta I, Duncan RC, Wanner A (2003). Comparative bronchial vasoconstrictive efficacy of inhaled glucocorticosteroids. Eur Respir J 21: 989–993.

Nakamura T, Nakanishi T, Haruta T, Shirasaka Y, Keogh JP, Tamai I (2010). Transport of ipratropium, an anti-chronic obstructive pulmonary disease drug, is mediated by organic cation/carnitine transporters in human bronchial epithelial cells: implications for carrier-mediated pulmonary absorption. Mol Pharm 7: 187–195.

Nevin BJ, Broadley KJ (2004). Comparative effects of inhaled budesonide and the NO-donating budesonide derivative, NCX 1020, against leukocyte influx and airway hyperreactivity following lipopolysaccharide challenge. Pulm Pharmacol Ther 17: 219–232.

O'Connor BJ, Towse LJ, Barnes PJ (1996). Prolonged effect of tiotropium bromide on methacholine-induced bronchoconstriction in asthma. Am J Respir Crit Care Med 154: 876–880.

Rodrigo G, Rodrigo C (1998). Inhaled flunisolide for acute severe asthma. Am J Respir Crit Care Med 157: 698–703.

Rodrigo GJ (2006). Rapid effects of inhaled corticosteroids in acute asthma: an evidence-based evaluation. Chest 130: 1301–1311.

Seemungal TA, Donaldson GC, Paul EA, Bestall JC, Jeffries DJ, Wedzicha JA (1998). Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 157: 1418–1422.

Sin DD, Tu JV (2001). Inhaled corticosteroids and the risk of mortality and readmission in elderly patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 164: 580–584.

Smith N, Broadley KJ (2007). Optimisation of the sensitisation conditions for an ovalbumin challenge model of asthma. Int Immunopharmacol 7: 183–190.

Soriano JB, Vestbo J, Pride NB, Kiri V, Maden C, Maier WC (2002). Survival in COPD patients after regular use of fluticasone propionate and salmeterol in general practice. Eur Respir J 20: 819–825.

Tallet D, Del Soldato P, Oudart N, Burgaud JL (2002). NO-steroids: potent anti-inflammatory drugs with bronchodilating activity *in vitro*. Biochem Biophys Res Commun 290: 125–130.

Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S *et al*. (2008). A 4-year trial of tiotropium in chronic obstructive pulmonary disease. N Engl J Med 359: 1543–1554.

Turner DL, Ferrari N, Ford WR, Kidd EJ, Paquet L, Renzi P *et al*. (2010). TPI 1020, a novel anti-inflammatory, nitric oxide donating compound, potentiates the bronchodilator effects of salbutamol in conscious guinea-pigs. Eur J Pharmacol 641: 213–219.

Um SW, Yoo CG, Kim YW, Han SK, Shim YS (2007). The combination of tiotropium and budesonide in the treatment of chronic obstructive pulmonary disease. J Korean Med Sci 22: 839–845.

Van Andel AE, Reisner C, Menjoge SS, Witek TJ (1999). Analysis of inhaled corticosteroid and oral theophylline use among patients with stable COPD from 1987 to 1995. Chest 115: 703–707.