Effect of the Mixed Phosphodiesterase 3/4 Inhibitor RPL554 on Human Isolated Bronchial Smooth Muscle Tone

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ABSTRACT

The phosphodiesterase (PDE) enzyme family hydrolyzes cAMP and cGMP, second messengers that regulate a variety of cellular processes, including airway smooth muscle (ASM) relaxation and the inhibition of inflammatory cells. We investigated the activity of RPL554 [9,10-dimethoxy-2(2,4,6-trimethylphenylimino)-3-(n-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2H-pyrimido[6,1-α]isoquinolin-4-one], a dual PDE3/PDE4 inhibitor that exhibits bifunctional activity for its effects on the tone of human isolated ASM and any potential synergistic interactions with muscarinic receptor antagonists or a β2-agonist. We evaluated the influence of RPL554 on the contractile response induced by electrical field stimulation (EFS), acetylcholine (ACh), or histamine on human isolated bronchi. We also analyzed the potential synergistic effect of RPL554 on the contractile response induced by electrical field stimulation (EFS) [maximal effectiveness (Eₘₐₓ) 91.33 ± 3.37%, P < 0.001], relaxed bronchi precontracted with ACh (Eₘₐₓ 94.62 ± 2.63%, pD₂ 4.84 ± 0.12, P < 0.001), and abolished the contraction induced by histamine. Analysis of interactions indicated a weak synergism between RPL554 and salbutamol (interaction index: 0.25 ± 0.06; BI Δeffect: 0.29 ± 0.11; dose equivalence: no synergism) but significant synergism between RPL554 and atropine (interaction index: 0.09 ± 0.07; BI Δeffect: 0.54 ± 0.09; dose equivalence: synergism for low concentrations) or glycopyrrolate (ACh: BI Δeffect 0.46 ± 0.03, Berenbaum Δeffect 0.42 ± 0.02; histamine: BI Δeffect 0.46 ± 0.03, Berenbaum Δeffect 0.42 ± 0.03). This study demonstrates that RPL554 relaxes human bronchi and that it can interact with a muscarinic receptor antagonist to produce a synergistic inhibition of ASM tone. These results suggest that RPL554 may provide a novel treatment of airway diseases, either alone or in combination with antiguscarinic drugs.

Introduction

Both cAMP and cGMP are second messengers that regulate a number of critical cellular processes, such as metabolism, cell proliferation and differentiation, secretion, vascular and airway smooth muscle (ASM) relaxation, and the release of inflammatory mediators. The phosphodiesterase (PDE) enzyme family hydrolyzes cAMP and/or cGMP to inactive 5′-AMP and 5′-GMP, respectively, and thus, inhibition of PDEs represents a potential mechanism by which cellular processes can be modulated. Eleven major PDE gene families have been identified, denoted PDE1–11, which differ in primary structures, affinities for cAMP and cGMP, responses to specific effectors, sensitivities to specific inhibitors, and biochemical regulation. Each family contains at least one isoenzyme, and in some cases, the isoenzymes are splice variants of more than one gene (Beavo and Brunton, 2002; Conti et al., 2003; Bingham et al., 2006; Banner and Press, 2009).

PDE3 hydrolyzes both cAMP and cGMP with relatively high affinities; however, hydrolysis for cAMP is nearly 10-fold higher than for cGMP. PDE3A is expressed in platelets, vascular smooth muscle, cardiac myocytes, oocytes, and B-lymphocytes. PDE3B is relatively highly expressed in adipocytes, hepatocytes, and spermatocytes, but it can also be detected in vascular and ASM cells, the pancreas, T-lymphocytes, and macrophages (Gantner et al., 1998; Shakur et al., 2001; Banner and Press, 2009). PDE3 is considered the main PDE in human ASM, and this enzyme is known to be altered in ASM from subjects with asthma (Banner and Press, 2009; Cazzola et al., 2012b; Yick et al., 2013).

PDE4 has a low affinity for cAMP and only a weak affinity for cGMP. The PDE4 family comprises four genes (A, B, C, and D) that are broadly distributed in brain, gastrointestinal tract, spleen, lung, heart, testis, and kidney. In addition, PDE4 is...
expressed in almost all inflammatory cell types, except mast cells and platelets (Banner and Press, 2009; Matera et al., 2012; Cazzola et al., 2012a).

Whereas PDE4 inhibitors are efficacious inhibitors of proinflammatory mediator release from certain cell types, there is evidence to suggest that dual inhibition of PDE3 and PDE4 is additive or synergistic at suppressing the activation/functions of other cell types (e.g., macrophages, dendritic cells, epithelial cells, lymphocytes, and endothelial cells) (Banner et al., 1996; Giembycz et al., 1996; Blease et al., 1998; Wright et al., 1998; Gantner et al., 1999; Hatzelmann and Schudt, 2001; Banner and Press, 2009) but are not effective at relaxing ASM in vitro and do not cause acute bronchodilation experimentally (Boswell-Smith et al., 2006a) or clinically (Grootendorst et al., 2003). In contrast, PDE3 inhibitors are able to relax human ASM (Matera et al., 2011b) and can elicit bronchodilation in humans (Myou et al., 1999, 2003). Furthermore, PDE4 inhibitors can act synergistically with PDE3 inhibitors in a number of cell types (Schmidt et al., 2000; Banner and Press, 2009; Milara et al., 2011). Thus, it has been suggested that administration of a dual PDE3/4 inhibitor by the inhaled route may offer increased efficacy with a reduced side effect potential compared with an orally administered PDE4 inhibitor or a PDE3 inhibitor (Banner and Press, 2009), and such a drug would have bifunctional activity combining both bronchodilator and anti-inflammatory activity in a single molecule (Boswell-Smith et al., 2006b; Matera et al., 2012; Cazzola et al., 2012a).

PDE3 and PDE4B and D are expressed in human ASM cells. Some studies have demonstrated that PDE4 inhibitors can relax inherent tone in isolated human bronchial muscle, whereas other studies have found that PDE3 or PDE4 inhibitors alone are ineffective but in combination effectively relax inherent tone (Rabe et al., 1993; Naline et al., 1996; Schmidt et al., 2000; Le Jeune et al., 2002). However, to date, selective PDE4 inhibitors have not shown acute bronchodilator activity in a variety of clinical trials carried out in humans (Matera et al., 2011b), although several clinical trials with selective PDE3 inhibitors have shown clear bronchodilator activity in patients with asthma (Myou et al., 1999, 2003). Furthermore, PDE3 or PDE4 inhibition alone had no effect on allergen- or leukotriene C4 (LTC4)-induced contraction of human ASM but in combination acted synergistically to inhibit contraction. It is noteworthy that it has been demonstrated that PDE4D was the key physiologic regulator of β2-adrenoceptor-induced cAMP turnover within human ASM (Schmidt et al., 2000; Le Jeune et al., 2002; Banner and Press, 2009), suggesting that PDE inhibitors may also have the capacity to potentiate the bronchodilator actions of β2-agonists. In addition, the relaxation of ASM was associated with a reduced sensitivity to muscarinic cholinergic agonists; thus, the modulation of the parasympathetic neural control of ASM may represent another mechanism by which PDE3 and PDE4 inhibitors can influence airways function (Mehats et al., 2003; Banner and Press, 2009).

Therefore, the aim of the present study was to investigate the role of RPL554 (Fig. 1) (Boswell-Smith et al., 2006b), a novel PDE3/PDE4 inhibitor, on sensitized and nonsensitized human ASM and to evaluate any potential synergistic effects when administered with the muscarinic receptor antagonists atropine or glycopyrrolate or the β2-agonist salbutamol.

Material and Methods

Ethical Approval and Informed Consent

Ethical approval and informed consent were obtained from the Istituto Regina Elena–Istituto San Gallicano (IRE-ISG, Rome, Italy), and they were consistent with the 2009 National Committee of Bioethics, National Committee of Bio-safety, Biotechnology and Sciences, Italy, concerning the collection of biologic samples for research purposes and the Italian ethical and legal recommendations of 2010 concerning the biobank and the research biorepository (Istituto Nazionale dei Tumori—Independent Ethics Committee, 2010) and the Comitato Nazionale per la Biosicurezza, le Biotecnologie e le Scienze per la Vita (2009). Raccolta di campioni biologici a fini di ricerca: consenso informato; http://www.governo.it/bioetica/gruppo_misto/Consenso_Informato_allegato_Petrini_2009.pdf.

Preparation of Tissues

Regions of macroscopically normal lungs were taken from uninvolved areas resected from 24 patients (11 men, and 13 women, aged 60.1 ± 1.6 years) undergoing lobectomy surgery for lung cancer but without a history of chronic airway disease.

Airways were immediately placed into oxygenated Krebs-Henseleit buffer solution (KH) (119.0 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25.0 mM NaHCO3, 1.7 mM glucose, pH 7.4) containing the cyclooxygenase inhibitor indomethacin (5.0 μM) and transported at 4°C from the Regina Elena National Cancer Institute or the Sant’Andrea Hospital (Rome, Italy) to the Respiratory Research Laboratory in the Medical School of the University of Rome Tor Vergata (Rome, Italy). None of the patients had been chronically treated with theophylline, β2-agonists, or glucocorticosteroids. Serum IgE levels determined on the day of surgery were in the normal range. Preoperative lung function parameters were generally normal, and no patients had signs of respiratory infections.

In the laboratory, airways were dissected from connective and alveolar tissues. Segmental bronchi were then isolated and stored overnight in KH buffer solution at refrigeration temperature. The next morning, bronchi were cut into rings (N = 120; thickness: 1–2 mm; diameter: 5–7 mm) and transferred into 4400 four-chamber 10-ml Isolated Organ Baths (Ugo Basile Instruments, Verona, Italy) containing KH buffer (37°C) and continuously aerated with a 95.5% mixture of O2/CO2.

Preparation of Drugs

The following drugs were used: acetylcholine (ACh), histamine, salbutamol, atropine, glycopyrrolate, papaverine, and indomethacin. All substances were obtained from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in distilled water except for indomethacin and quinine, which were dissolved in ethanol and then diluted in a KH buffer. The maximal amount of ethanol (0.02%) did not influence isolated tissue responses (Freas et al., 1989; Hatake and Wakabayashi, 2000). RPL554 was kindly provided by Verona Pharma PLC, (London, UK). Compounds were stored in small aliquots at –80°C until use.

![Chemical structure of RPL554](image)
Tension Measurement

Human bronchi were placed in organ baths containing KH buffer solution (37°C), medicated with indomethacin (5.0 μM), bubbled with 95%O2/5%CO2, and suspended under passive tension (0.5–1.0 g). Bronchial rings were mounted on hooks in the organ baths, where one hook was attached with thread to a stationary rod and the other hook was tied with thread to an isometric force displacement transducer. Airways were allowed to equilibrate for 90 minutes with repeated changes of the medicated KH buffer solution every 10 minutes. Changes in isometric tension were measured with a transducer (Fort 10 WPI, Ugo Basile Instruments), and the tissue responsiveness was assessed by measuring the ASM response to ACh (100 μM); when the contractile response reached a plateau, rings were washed three times and were allowed to equilibrate for 45 minutes.

Study Design

**Influence of RPL544 on Electrical Field Stimulation.** Each organ bath was fitted with two platinum plate electrodes (1 cm²) placed alongside the tissue (10 mm apart) for electrical field stimulation (EFS). Experiments were performed using trains of 10 Hz EFS (biphasic pulse with a constant current of 10 V, 0.5 milliseconds, 10 seconds), one pulse every 5 minutes for the first hour and then at 30-minute intervals for the next 5 hours by a 3185 multiplexing pulse booster (Ugo Basile Instruments) (Binks et al., 2001). After the start of the EFS trains, tissues were incubated with RPL544 (10 or 100 μM) until maximum inhibition of the contractile response to EFS was achieved. Incubation with drug was then terminated, and the tissues were repeatedly washed over a 30-minute period and then every 30 minutes for up to 5 hours after drug administration.

**Relaxant Effect of RPL544 on Passively Sensitized Bronchi.** Human isolated bronchial rings were rotated overnight at room temperature in tubes containing KH buffer solution in the absence (nonsensitized control rings) or in the presence of 10% vol−1 sensitizing serum (sensitized rings) as described elsewhere (Watson et al., 1997; Rabe, 1998). Patients suffering from atopic asthma (total IgE defined as specific against common aeroallergens) during exacerbation provided informed consent for serum donation. Sera were prepared by centrifugation of whole blood, and sera samples were frozen at −80°C in 200-μl aliquots until required.

The next morning, after removing the adherent alveolar and connective tissues, we transferred the bronchial rings into an organ bath containing KH buffer solution (37°C) and continuously gassed with a 95%O2/5%CO2. Tissues were preincubated for 30 minutes with RPL544 (1, 10, and 100 μM) and then followed (without washing) by the construction of concentration responses curve to histamine (10 nM–1 mM) in the presence of RPL544.

**Synergistic Effect of RPL544 Plus Atropine, Glycopyrrolate, or Salbutamol.** To test the possible synergistic relaxation induced by atropine, glycopyrrolate, or salbutamol with RPL544, the bronchial tissues were contracted with ACh at the concentration required to cause the plateau, tissues were allowed a 15-minute stabilization period. Concentration response curves then were constructed to test compounds RPL544, atropine, glycopyrrolate, or salbutamol alone or RPL544 administered in combination with atropine or salbutamol (atropine-RPL544 and salbutamol-RPL544 ranging from 10:1 to 1:100) as described elsewhere (Greco et al., 1995; Tallarida, 2001; Goldoni and Johansson, 2007; Boik et al., 2008, Lee, 2010). RPL544 or glycopyrrolate was tested alone or in combination at low concentrations, inducing an EC50 of the submaximal bronchial contractile tone produced by acetylcholine or histamine.

Intervals of 20 minutes between successive concentrations were used to reach a stable level of relaxation before the administration of the next concentration. At completion of the experiment, papaverine (500 μM) was added to relax the tissues completely and provide a standard to which the relaxation of each tissue could be compared.

Analysis of Results

**Analysis of EFS Studies.** Bronchial contractile tension induced by EFS was measured as a percentage of control bronchi, and polynomial curves were constructed by fitting models of biologic data using nonlinear regression as described elsewhere (Motulsky and Christopoulos, 2004). The maximal effect (Emax) was identified as the lowest contractile force induced by EFS stimulation, and the offset (half-life, minute) indicates the time to evoke a half of maximal relaxation. For every three bronchial rings mounted in the isolated organ bath system, one was used as a time control as described elsewhere (Mercier et al., 2002).

**Analysis of Concentration Response Studies.** Appropriate curve-fitting to a sigmoidal model was used to calculate the effect (E), the Emax, and the concentration required to cause a 50% maximal effect (EC50). The equation used was log(agonist) versus response, variable slope, expressed as Y = bottom + (top − bottom)/(1 + 10^[(LogEC50 − Y0)/HillSlope]) (Motulsky and Christopoulos, 2004; Goodman et al., 2008). E/Emax was expressed as the percentage of Emax elicited by the contractile agents; EC50 values were converted to pD2 for statistical analysis (Goodman et al., 2008), and the relaxant responses were expressed as a percentage of papaverine- (500 μM) induced relaxation.

**Analysis of Synergism Studies.** Analysis of the potential synergism between RPL544 plus salbutamol, RPL544 plus atropine, or RPL544 plus glycopyrrolate was measured by applying the Berenbaum method, the Bliss Independence (BI) criterion, or the concept of dose equivalence (Berenbaum, 1977, 1989; Greco et al., 1995; Grabovsky and Tallarida, 2004; Tallarida, 2006; Goldoni and Johansson, 2007; Tallarida and Raffa, 2010).

To apply the Berenbaum method, we evaluated the interaction index for the EC50 values. If the interaction index was <1, the effect was considered synergistic; if the interaction index was >1, the effect was antagonistic; and if the interaction index was =0, the effect was considered additive (Goldoni and Johansson, 2007; Lee, 2010). The BI theory for understanding the action of two agents is expressed by the following equation: E_x = E_x = E_y = (E_x + E_y) / 2, where E is the fractional effect and x and y are the concentrations of two compounds in a combination experiment. If the combination effect is higher than the expected value from the preceding equation, the interaction is considered synergistic, whereas if this effect is lower, the interaction is antagonistic. Otherwise, the effect is additive and there is no interaction (Greco et al., 1995; Meletiadis et al., 2003; Boucher and Tam, 2006; Goldoni and Johansson, 2007; Boik et al., 2008, Lee, 2010). In this study, the BI equation was characterized by X = RPL544 and Y = salbutamol, Y = atropine, or Y = glycopyrrolate.

In further analysis performed to test for a synergistic interaction, control concentration response curves for salbutamol, atropine, and RPL544 from bronchi from each lung were fitted to a four-parameter logistic equation to calculate parameter estimates of Emax slope (n_Hill), and potency (EC50). The following parameter estimates Emax and n_Hill (mean ± S.E.M.) and EC50 (geometric, 95% confidence interval (CI)) for salbutamol (78.54 ± 4.78, 1.572 ± 0.216, and 0.283 (0.064–1.239) μM, n = 5, respectively), atropine (65.98 ± 6.912 ± 0.218, and 1.181 (0.134–10.4) μM, n = 5, respectively) and RPL544 (100 ± 0, 2.271 ± 0.318, and 21.2 (11.5–39.1) μM, n = 5, respectively) were then used to calculate the additive response for each drug pair combination to evaluate synergism using the approach based on the concept of the dose equivalence (Grabovsky and Tallarida, 2004; Lee, 2010; Tallarida and Raffa, 2010). By use of the concept of dose equivalence, the relationship a/A + b/B = 1 was reformulated as b + beq (a) = B, where beq is the dose equivalent of a and solving for beq (a) by equating the two individual concentration response curves EA = f(A) and EB = f(B). The additive response (Eab) for each dose combination with respect to B was then calculated by insertion of B into EB = f(B).
RPL554 caused a concentration-dependent relaxation of human isolated bronchial tissues precontracted with acetylcholine. RPL554 was less potent ($P < 0.05$) than atropine, glycopyrrolate, or salbutamol in bronchial relaxation; but, in contrast to the other drugs studied, RPL554 completely relaxed tissues ($P < 0.001$).

Control concentration response curves for salbutamol, glycopyrrolate, atropine, and RPL554 from bronchi from each lung were fitted to a four-parameter logistic equation to calculate parameter estimates of $E_{\text{max}}$, $n_H$, and potency ($EC_{50}$). The following parameter estimates $E_{\text{max}}, n_H$, and $EC_{50}$ (geomean, 95% CI) were as follows: for salbutamol (precontraction by ACh) $78.54 \pm 4.78, 1.572 \pm 0.216$, and $0.283 (0.064–1.239)$ $\mu$M, $n = 5$, respectively; atropine (precontraction by ACh): $65.98 \pm 6.08, 0.912 \pm 0.218$, and $1.181 (0.134–10.4) \mu$M, $n = 5$, respectively; RPL554 (precontraction by ACh: $100 \pm 0, 2.271 \pm 0.318$, and $21.2 (11.5–39.1) \mu$M; precontraction by histamine: $100 \pm 0, 0.88 \pm 0.157$, and $12.9 (8.1–20.5) \mu$M, $n = 5$, respectively; and glycopyrrolate (precontraction by ACh: $98.86 \pm 6.95, 1.946 \pm 0.796, 1.76 (1.0–3.088)$ nM; precontraction by histamine: $69.07 \pm 3.35, 0.86 \pm 0.105$, and $3.96 (2.68–5.62) \mu$M, $n = 5$, respectively) (Fig. 3, A and B).

The passive sensitization of bronchi enhanced the contractile effect of histamine compared with nonsensitized tissues. In passively sensitized bronchi, RPL554 1 and 10 $\mu$M significantly ($P < 0.001$) shifted leftward the concentration response curve to histamine compared with untreated tissues, and RPL554 100 $\mu$M completely abolished the contraction induced by histamine (Fig. 4; Table 1).

**Synergistic Relaxant Effect of RPL554 Plus Salbutamol, Atropine, or Glycopyrrolate on Human Bronchial Tone.** The BI study indicated that the interaction of RPL554 plus salbutamol induced a significant synergistic relaxant effect for RPL554 at 1 and 10 $\mu$M (both $P < 0.05$) on the human bronchial tone precontracted with acetylcholine, and the maximal synergism was detected for RPL554 at 1 $\mu$M plus salbutamol at 100 nM (BI $\Delta$effect: 0.29 $\pm$ 0.11, although there was no significant synergistic interaction between salbutamol and RPL554 at lower concentrations (10 nM and 100 nM, $P > 0.05$) (Fig. 5A). In fact, the BI analysis of the isomolar association (1:1) of RPL554 plus salbutamol only showed a signal for synergism ($P = 0.08$) based on the enhancement of the relaxant potency (Table 2). Nevertheless, the Berenbaum analysis demonstrated that RPL554 plus salbutamol elicited a synergistic interaction for RPL554 over the concentration range of 10 nM to 10 $\mu$M (interaction index: 0.25 $\pm$ 0.06) and that RPL554 significantly caused a leftward shift of the relaxant concentration response curves to salbutamol of 0.89 $\pm$ 0.14 logarithms ($P < 0.05$). However, the analysis based on the concept of dose equivalence did not reveal any statistical difference between the observed and additive relaxation response for the 1:1 dose combinations of salbutamol and RPL554, indicating no evidence of synergy (Fig. 6).

The interaction of RPL554 plus atropine produced a significant synergistic effect of RPL554 over the concentration range of 1 nM to 10 $\mu$M (1 nM: $P < 0.05$; 10 nM and 1 $\mu$M: $P < 0.01$; 100 nM and 10 $\mu$M: $P < 0.001$), and the maximal synergism was detected for the crossing concentrations of RPL554 1 $\mu$M and atropine 10 nM (BI $\Delta$effect: 0.54 $\pm$ 0.09). Lower concentrations of RPL554 and atropine interacted synergistically by significantly increasing the relaxation of bronchial rings at both 10 nM ($P < 0.05$) and 100 nM.
compared with the expected effect theoretically induced by these drugs (Fig. 5B). The results of the isomolar association (1:1) of RPL554 plus atropine on human bronchial tone precontracted with ACh indicated a statistically significant BI interaction (P < 0.01), based on the enhancement of the relaxant potency (Table 2).

Furthermore, the Berenbaum analysis demonstrated that RPL554 plus atropine elicited a synergistic interaction for RPL554 over the concentration range of 1 nM to 10 μM (interaction index: 0.09 ± 0.07) and that RPL554 significantly caused a leftward shift of the relaxant concentration response curves to atropine of 2.91 ± 0.44 logarithms (P < 0.05). In addition, the analysis based on the concept of dose equivalence also demonstrated a statistically significant difference between the observed and additive relaxation response for the 1:1 dose combinations of atropine and RPL554, indicating evidence of synergy (Fig. 6).

The surface analysis demonstrated that atropine induced a significantly higher and wider synergistic interaction extended across all the concentrations compared with salbutamol, when administered in association with RPL554 (average of atropine/salbutamol synergism ratio by three-dimensional surface analysis: 3.23 ± 0.48, P < 0.001) (Fig. 7).

Finally, the concomitant administration of low concentrations of RPL554 plus glycopyrrolate, both at isoeffective concentrations inducing EC_{20} (RPL554: 6.0 ± 1.5 μM on acetylcholine contraction and 1.7 ± 0.8 μM on histamine contraction; glycopyrrolate: 0.7 ± 0.4 nM on acetylcholine contraction and 1.4 ± 0.5 μM on histamine contraction), produced a significant synergistic interaction on isolated bronchial rings precontracted with acetylcholine (BI Δeffect: 0.46 ± 0.03, Berenbaum Δeffect: 0.42 ± 0.02) and histamine (BI Δeffect: 0.46 ± 0.03, Berenbaum Δeffect: 0.42 ± 0.03) (Fig. 8).

Discussion

Inhibition of PDE3/4 has previously been reported to induce relaxation of canine airways, guinea pig trachea, and human ASM preparations (de Boer et al., 1992; Naline et al., 1996; Torphy, 1998; Boswell-Smith et al., 2006b). We have demonstrated that the selective inhibition of PDE3/4 by RPL554 elicited relaxation of bronchial tone in human isolated airways, which extends and supports observations previously reported in guinea pig isolated trachea with this drug.
The use of human isolated bronchial rings to investigate the actions of bronchodilator drugs is well established and considered predictive of the effectiveness of such drugs clinically, and we, as well as a number of other laboratories, have previously demonstrated a range of studies with different drug classes in this model (Matera et al., 2009, 2011a, 2013; Tannu et al., 2010; Calzetta et al., 2011; Cazzola et al., 2011; Hewson et al., 2012; Rogliani et al., 2013). The inhibitory effect of RPL554 was maintained for up to 5 hours after termination of drug exposure, confirming the long duration of action of this compound in human airways, which we have subsequently confirmed in patients with asthma or chronic obstructive pulmonary disease (COPD) when this drug is nebulized to patients, confirming the predictability of our model (Cazzola et al., 2013; Franciosi et al., 2013). Furthermore, RPL554 acted to relax airways contracted with either histamine or acetylcholine. Moreover, prior incubation of tissues with RPL554 resulted in significant protection of the tissues against the contractile action of exogenously administered histamine in passively sensitized bronchi. In addition, the inhibition of RPL554 in combination with a muscarinic receptor antagonist (either atropine or glycopyrrolate), and to a lesser extent with a β2-adrenergic receptor agonist (salbutamol), demonstrated a synergistic effect on relaxation of ASM. These results show that RPL554 is a good functional antagonist against contractile agents in human ASM and, when combined with a muscarinic receptor antagonist, may have the ability to provide further bronchodilation; however, the interaction between RPL554 and salbutamol is less clear.

RPL554 caused a concentration- and time-dependent inhibition of contractile responses elicited by EFS, which had a considerably longer duration of action against EFS-induced contractile responses than other selective PDE3 or PDE4 inhibitors (Coleman et al., 1996; Spina et al., 1998; Boswell-Smith et al., 2006b). RPL554 was particularly effective at inhibiting the contractile response in passively sensitized human bronchi contracted with histamine, which is of interest because various selective PDE3 and PDE4 inhibitors have been reported to attenuate significantly acute bronchospasm induced by antigen in sensitized guinea pigs, which is predominantly mediated by histamine release from mast cells (Boswell-Smith et al., 2006b). Furthermore, the ability of PDE4 inhibitors to inhibit bronchospasm induced by allergen in animal models is likely due to inhibition of IgE/IgG-dependent mediator release from inflammatory cells rather than to functional antagonism of ASM shortening (Boswell-Smith et al., 2006b).

It is likely that this effect of RPL554 on human bronchi is via the ability of this drug to inhibit PDE3 rather than PDE4, as PDE4 inhibitors have been reported to not be quite effective at changing airway tone acutely, either preclinical or clinically (Schudt et al., 1995; Boswell-Smith et al., 2006a; Calverley et al., 2009). RPL554 also induced a noticeable decrease in the maximum response to histamine in passively sensitized bronchi. This profile of loss of $E_{\text{max}}$ to histamine resembles the response observed for indirectly acting substances that inhibit the release of endogenous intermediaries whose concentrations

### Table 1

Effect of RPL554 on contraction induced by histamine in passively sensitized bronchi

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RPL554 1 μM</th>
<th>RPL554 10 μM</th>
<th>RPL554 100 μM</th>
<th>ND</th>
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<tr>
<td>$E_{\text{max}}$</td>
<td>100.7 ± 1.7</td>
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<td>58.1 ± 2.2***</td>
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<td>$pD_2$</td>
<td>4.82 ± 0.03***</td>
<td>5.29 ± 0.03</td>
<td>4.97 ± 0.07***</td>
<td>4.98 ± 0.07***</td>
<td>ND</td>
</tr>
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ND, not determined.

![Fig. 5. Low-concentration interactions (10 and 100 nM) of salbutamol plus RPL554 (A) and atropine plus RPL554 (B). Bar graphs express the expected relaxant effect of adding doses of each drug by Berenbaum method (1989) and the observed relaxant effect of salbutamol plus RPL554 or atropine plus RPL554. Data are from experiments performed with samples of $n = 5$ different subjects, and they are represented as mean ± S.E.M. *$P < 0.05$ and **$P < 0.01$.](image-url)
are the limiting factor (Black et al., 1980; Kenakin et al., 2006). The inherent tone of passively sensitized human airways in vitro results from the spontaneous release of inflammatory mediators, in particular the cysteinyl leukotrienes and histamine, from resident inflammatory cells in the airway wall (Schmidt et al., 2000). Therefore, it could be assumed that cAMP-elevating drugs, such as mixed PDE inhibitors, might exhibit their effects on basal bronchial tone, at least in part through the inhibition of endogenous mediator release (Schmidt et al., 2000), in addition to any distinct effects on airways' smooth muscle tone caused by inhibition of PDE3.

Our results are consistent with other studies supporting the hypothesis that PDE3/4 inhibitors are able to relax human bronchi, as either a combination of PDE3 and PDE4 inhibitors or dual PDE3/4 inhibitors, and have been shown to produce ASM relaxation against carbachol-precontracted airway preparations (de Boer et al., 1992; Torphy, 1998).

We also investigated the potential synergism between RPL554 and salbutamol or atropine or glycopyrrolate by applying the Berenbaum method, the BI criterion, and the dose equivalence concept. Because the slope and the maximal relaxant effect of RPL554, salbutamol, atropine, and glycopyrrolate were different in our study, we applied modified equations for dose equivalence concept, as proposed by Tallarida and Grabovsky, to establish the correct dose-effect interaction (Grabovsky and Tallarida, 2004; Tallarida and Raffa, 2010).

The BI, the Berenbaum, and the dose equivalence concept are generally used to study combined effects of substances in vivo and in vitro (Berenbaum, 1977, 1989; Tallarida, 2001, 2006; Grabovsky and Tallarida, 2004; Boucher and Tam, 2006; Goldoni and Johansson, 2007; Boik et al., 2008; Tallarida and Raffa, 2010). The main assumption of the BI theory is that two or more agents act independently from one another in terms of the site of action of the drugs in the mixture (Greco et al., 1995; Goldoni and Johansson, 2007). The Berenbaum approach is based on the concept of effect addition where the expected effect of a mixture is the arithmetic sum of a measured effect of the single agents in the mixture of linear or linearizable models (Berenbaum, 1977, 1989). On the other hand, the concept of dose equivalence is the basis of the relation derived for the additive concentration of drugs so that the combination doses can be expressed as a dose of either one of them (Grabovsky and Tallarida, 2004; Tallarida and Raffa, 2010). These methods are characterized by both advantage and limitations. For example, the validity of the BI model has been questioned by Greco and colleagues, because it may overestimate the extent of any synergism and could therefore have low biologic plausibility (Greco et al., 1995; Goldoni and Johansson, 2007); the Berenbaum effect summation approach or combination effect is not accurate for nonlinear models (Berenbaum, 1977, 1989); and no particular mechanisms are derived from the dose equivalence concept proposed by Tallarida and colleagues, although the analysis of data obtained from the consequences of dose combination could represent a first step in determining if some mechanism is to be posited (Grabovsky and Tallarida, 2004; Tallarida and Raffa, 2010).

Therefore, the choice of the most appropriate model is important because at some coexposure concentrations, the

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**TABLE 2**

Relaxant synergistic effect of RPL554 plus salbutamol and RPL554 atropine (both isomolar, 1:1) on submaximal contraction induced by acetylcholine

Data shown are from experiments performed with samples of n = 5 different subjects, and they are represented as mean ± S.E.M. *P < 0.05 for zero interaction hypothesis Δeffect (observed vs. expected values).

<table>
<thead>
<tr>
<th></th>
<th>RPL554 + Salbutamol</th>
<th>RPL554 + Atropine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed</strong></td>
<td><strong>Expected</strong></td>
<td><strong>Observed</strong></td>
</tr>
<tr>
<td><strong>E_{max}</strong></td>
<td>96.31 ± 3.43</td>
<td>94.66 ± 4.01</td>
</tr>
<tr>
<td><strong>pD2</strong></td>
<td>6.78 ± 0.30</td>
<td>6.33 ± 0.15</td>
</tr>
<tr>
<td><strong>ΔPotency</strong></td>
<td>0.45 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 6.** Bar graph representing the relaxation response to salbutamol plus RPL554 (A) and atropine plus RPL554 (B), the additive response of each dose combination, and the observed relaxation response for each dose combination (combination 1:1). The concentrations of each agonist are shown on the x-axis. Each bar represents the mean ± S.E.M. (n = 5). The additive response was estimated by using the methods of Tallarida and Raffa (2010). *P < 0.05 (adjusted) of additive response using a one-sample t test. The 10 nM combination of atropine plus RPL554 was statistically different to the additive response, but the significance was lost with the adjustment for multiple comparisons.
differences in outcome might be dramatic (Greco et al., 1995; Goldoni and Johansson, 2007). Nonetheless, by analyzing different dose-response curves, the BI method permits an accurate statistical analysis, the Berenbaum approach provides results that are easy to be interpreted, and the concept of dose equivalence allows a high biologic plausible evaluation of synergism through useful graphic representation of data (Berenbaum, 1989; Greco et al., 1995; Tallarida, 2001; Goldoni and Johansson, 2007; Lee, 2010); hence, we chose to analyze our results using all three approaches.

In our study, the synergistic interaction suggested by the concept of dose equivalence was partially confirmed by the BI analysis and fully confirmed by the Berenbaum approach. Furthermore, our findings demonstrated a greater synergistic relaxant effect on human bronchial muscle precontracted with acetylcholine in the presence of low concentrations of atropine and glycopyrrolate compared with that elicited by salbutamol when coadministered with low concentrations of RPL554. These findings confirm that RPL554 is a good functional antagonist against contractile agents in human ASM and that across a range of concentrations is able to interact synergistically with muscarinic receptor antagonists.

However, RPL554 exhibited weak synergistic interaction with the $\beta_2$-agonist salbutamol, which may be explained considering that cAMP-elevating drugs, such as PDE inhibitors and $\beta_2$-adrenoceptor agonists, might exhibit part of their effects on basal bronchial tone, at least in part through the inhibition of endogenous mediator release (Schmidt et al., 2000). However, it has been demonstrated that PDE4 inhibitors can relax inherent tone in isolated human bronchial muscle and, moreover, that the PDE4D variant 5 is the key physiologic regulator of $\beta_2$-adrenoceptor-induced cAMP turnover within human ASM (Matera et al., 2011b). Thus, it remains plausible that the combination of a PDE3/4 inhibitor and $\beta_2$-adrenoceptor agonist may provide enhanced bronchodilation in the treatment of patients with either asthma or COPD, which is now under investigation.

In addition, the synergism between RPL554, atropine, or glycopyrrolate provides strong evidence for considering the use of these drugs in combination, particularly since glycopyrrolate was approved as a treatment of patients with COPD. Furthermore, our observations are of interest because it has been demonstrated that allergen-induced bronchial hyper-responsiveness induced by cigarette smoking exposure in animal models is mainly mediated through increased

![Fig. 7. Interaction surfaces obtained from response surface analysis of BI drug interaction model for the combination of RPL554 plus salbutamol (A) and RPL554 plus atropine (B). The horizontal axis indicates the concentration of compounds, and the vertical axis represents the $\Delta E$ (relaxation, %). The 0-plane indicates BI interactions, whereas the volume above the 0-plane represents synergistic (positive $\Delta E$) interactions. The magnitude of interactions is directly related to $\Delta E$, and the different tones in the three-dimensional plots represent different percentile bands of synergy (10%). Each point intersection represents the mean of experiments performed on samples from different subjects ($n = 5$).](image)

![Fig. 8. Interaction analysis of low concentrations of RPL554 plus glycopyrrolate inducing EC20 in human isolated bronchi submaximally precontracted with acetylcholine or histamine. Bar graphs express the expected relaxant effect of adding doses of each drug by Bliss or Berenbaum method and the observed relaxant effect of RPL554 plus glycopyrrolate. Data are from experiments performed with samples of $n = 5$ different subjects, and they are represented as mean ± S.E.M. *$P < 0.05$ versus Bliss Independence theory; **$P < 0.05$ and ***$P < 0.01$ vs. the Berenbaum method.](image)
expression of M1, M2, and M3 muscarinic receptors and the PDE4 isozyme PDE4D5 in the lung (Singh et al., 2009). These findings are also corroborated by the suggestion of a causal relationship between the PDE4D5 activity and muscarinic receptor expression in allergic asthma (Schmidt et al., 2000).

The PDE4 isozyme was identified as a major therapeutic target for novel anti-inflammatory drugs because it is the predominant isozyme in most inflammatory cells, including neutrophils, which are implicated in the pathogenesis of COPD. PDE4 is also present in ASM; to date, however, selective PDE4 inhibitors have not shown acute bronchodilator activity in a variety of clinical trials (Matera et al., 2011b). In contrast, there is considerable evidence that the PDE4 isozyme predominates in human ASM and that inhibition of this enzyme, rather than PDE4, leads to ASM relaxation (Boswell-Smith et al., 2006a). As a consequence, dual PDE4/3 inhibitors, such as RPL554, can combine bronchodilator with anti-inflammatory activity and thus provide superior efficacy over compounds that inhibit PDE4 or PDE4 alone (Banner and Press, 2009; Matera et al., 2011b). Given that it is current practice to combine different classes of bronchodilator treatment to obtain greater bronchodilation, the availability of a new class of bronchodilator represented by RPL554 that shows synergy of this drug with the two major classes of established bronchodilators is of considerable interest, particularly as there is no clinical evidence of synergism between β2-agonists and muscarinic antagonists in terms of changes in forced expiratory volume in 1 second; rather, it has been reported that the combination of these two classes of drugs produces only additive effects in improving spirometric parameters (Cazzola et al., 2005; Berton et al., 2010).

Our results suggest that inhibiting both PDE3 and PDE4 with RPL554 induces significant relaxation of human bronchi and that when administered with a muscarinic receptor antagonist, mixed PDE3/4 inhibitors such as RPL554 may have synergistic inhibition on ASM tone and thus lead to improved bronchodilation compared with either drug administered alone.

Authorship Contributions

Participated in research design: Calzetta, Page, Spina, Cazzola, Rogliani, Matera.
Conducted experiments: Calzetta, Facciolo.
Contributed new reagents or analytic tools: Page. Performed data analysis: Calzetta, Spina.
Wrote or contributed to the writing of the manuscript: Calzetta, Page, Spina, Cazzola, Rogliani, Matera.

References
