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RESEARCH PAPER

Selectivity profiling of the novel EP_2 receptor antagonist, PF-04418948, in functional bioassay systems: atypical affinity at the guinea pig EP_2 receptor

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BACKGROUND AND PURPOSE
Understanding the role of the EP_2 receptor has been hampered by the lack of a selective antagonist. Recently, a selective EP_2 receptor antagonist, PF-04418948, has been discovered. The aim of this study was to demonstrate the selectivity profile of PF-04418948 for the EP_2 receptor over other EP receptors using a range of isolated tissue systems.

EXPERIMENTAL APPROACH
PF-04418948 was profiled on a range of isolated tissues to assess its EP receptor potency and selectivity: ONO-DI-004-induced contraction of guinea pig trachea (EP_1); ONO-AE1-259 and PGE_2- induced relaxation of mouse and guinea pig trachea (EP_2); PGE_2-induced depolarization of guinea pig isolated vagus (EP_3); PGE_2-induced relaxation of human and rat trachea (EP_4). PF-04418948 was also profiled in functional murine TP, IP, DP and FP receptor assays.

KEY RESULTS
In bioassay systems, where assessment of potency/selectivity is made against the 'native' receptor, PF-04418948 only acted as an antagonist of EP_2 receptor-mediated events. PF-04418948 competitively inhibited relaxations of murine and guinea pig trachea induced by ONO-AE1-259 and PGE_2 respectively. However, the affinity of PF-04418948 was not equal in the two preparations.

CONCLUSIONS AND IMPLICATIONS
Using a wide range of bioassay systems, we have demonstrated that PF-04418948 is a selective EP_2-receptor antagonist. Interestingly, an atypically low affinity was found on the guinea pig trachea, questioning its utility as an EP_2 receptor assay system. Nevertheless, this compound should be an invaluable tool for investigating the biological activity of PGE_2 and the role of EP_2 receptors in health and disease.

Abbreviations
CCh, carbachol; KH, Krebs–Henseleit

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Introduction

Prostaglandins are potent biologically active lipid molecules that are produced by most human cell types and act as autocrine, paracrine and endocrine mediators through an interaction with specific receptors (Alexander *et al.*, 2011; Woodward *et al.,* 2011). The human lung is a rich source of prostaglandins, particularly PGE_2 (Karim *et al.*, 1967). Although the functional effects of PGE_2 in the lung and other organs have been the focus of intense research for several decades, we still have a lot to learn about the complex biology associated with this interesting molecule. This complexity associated with PGE_2 is due to the fact that it can activate nine different prostanoid receptors, preferentially activating the EP receptors (EP_{1-4}) (Coleman *et al.*, 1984; Narumiya *et al.*, 1999).

Recently, there have been major advances in our understanding of the role of EP receptors. This has been driven primarily through the development of selective pharmacological tools and the use of genetically modified animals (Woodward *et al.,* 2011). However, investigating the role of the EP_2 receptor has remained challenging because of the lack of a selective antagonist. Until recently, investigators have relied on gene deletion studies and the use of AH6809, which, in addition to blocking EP_2 receptors, also acts as an EP_1 and DP_1 receptor antagonist (Jones *et al.*, 2009; Woodward *et al.*, 2011). Clearly, both approaches have their limitations, and as such, the recent publications describing a novel and selective EP_2 receptor antagonist, PF-04418948, have sparked interest in the scientific community (Forselles *et al.*, 2011). Using receptor-binding assays and a range of functional cell-based assays where the human recombinant receptor was expressed in CHO cells, PF-04418948 was shown to be potent and selective for the EP_2 receptor over the other prostanoid receptors [EP_1, EP_3, EP_4, DP_1, DP_2 ($CRTH_2$), IP, FP and TP]. The authors of those studies also explored the functional profile of PF-04418948 on native EP_2 receptors in human myometrium, dog bronchiole and mouse trachea. However, data were not presented examining the selectivity of the compound in tissues that express multiple prostanoid receptor subtypes. While selectivity data in cell-based systems against recombinant receptors are extremely useful, we feel that an assessment of the compound selectivity in bioassays against the native EP_2 receptor is needed.

Thus, the aim of this study was to use a range of tissue-based assays to confirm the selectivity of PF-04418948 for the native EP_2 receptor relative to other PG receptors. Using these assay systems, we demonstrated here that PF-04418948 antagonizes EP_2 receptor-mediated responses in a surmountable, competitive manner, although differences in affinity were noted between species. These data complement those from a previous study by Forselles *et al.* (2011) and confirm that PF-04418948 is the first, truly selective EP_2-receptor antagonist and should be of value to the scientific community as a whole in helping to delineate the biological role of PGE_2.

Methods

Animals

Male Dunkin-Hartley guinea pigs (300–500 g), Sprague Dawley rats (200–250 g) and C57BL/6 mice (18–22 g) were purchased from Harlan (Bicester, Oxon, UK). A total of 42 mice, 26 guinea pigs and 6 rats were used. Animals were housed in a temperature-controlled (18–21°C) room on a 12-h light/dark cycle (07:00–19:00 h) with food and water freely available. All experiments were conducted in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986. Human airway samples (trachea, major bronchus) surplus to clinical requirement were obtained from lung transplants performed at The Royal Brompton and Harefield Hospital. Approval was obtained from the Royal Brompton and Harefield ethics committee after receiving the relevant consent from patients and relatives. 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).

General organ bath methodology

Rats, mice and guinea pigs were killed by an overdose of sodium pentobarbital (200 $mg \cdot kg^{-1}$, i.p.). The trachea from all species was carefully dissected and placed in Krebs-Henseleit (KH) solution (composition in mM: NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5, glucose 5.6) containing 10 μM indomethacin (to prevent the formation of endogenous prostanoids), maintained at room temperature and continuously bubbled with 95% O_2/5% CO_2. The trachea (except for mouse tissue) was opened longitudinally by cutting through the cartilage directly opposite to the smooth muscle layer. Murine trachea was left as rings and divided into two equal segments. The epithelium was left intact and tissue suspended from steel hook transducers in 10 mL baths containing KH solution warmed to 37°C. Tissues were left to equilibrate under a resting tension of 1 g (guinea pig and rat) or 0.8 g (mouse) or 2 g (human) for at least 60 min with regular (every 20 min) washing before beginning the experiment. Changes in force were measured isometrically using force–displacement transducers (model FT-03c; Grass Instrument, Quincy, MA, USA) connected to a data acquisition system (MP100) operating on a Windows PC using AcqKnowledge software (BIOPAC Systems, Goleta, CA, USA) as previously described (Birrell *et al.*, 2008). Following equilibration, the contractile response was assessed in each tissue using a supra-maximal concentration of ACh (1 mM).

Experimental design

EP_2-receptor-mediated responses: relaxation of mouse and guinea pig isolated tracheal tissue. Relaxation of induced tone in mouse tracheal tissue by ONO-AE1-259 (a selective EP_2 receptor agonist; Suzawa *et al.,* 2000) and PGE_2 has previously been shown to be mediated by the EP_2 receptor (Nials *et al.*, 1993; Fortner *et al.*, 2001; Buckley *et al.,* 2011) and was therefore used as an assay system to assess functionality at this receptor subtype. In these experiments, carbachol (CCh; 1 μM) was used to induce a stable contraction of the tissues. The tissues were then incubated with the vehicle or antagonist for 30 min before cumulative concentration–response curves were performed (with ONO-AE1-259, PGE_2 or the non-selective β-adrenoceptor agonist, isoprenaline). At the end of the experiment, the non-specific PDE inhibitor, papaverine (100 μM), was added to assess the maximum capacity for relaxation of each tissue.

EP_1-receptor-mediated responses: contraction of guinea pig isolated tracheal tissue. Contraction of guinea pig tracheal tissue under basal tone has previously been demonstrated to be due to activation of the EP_1 receptor (Coleman and Kennedy, 1985; McKenniff *et al.*, 1988; Ndukwu *et al.*, 1997; Buckley *et al.*, 2011). This provided an ideal test bed for assessing functionality at the EP_1 receptor. Once a stable basal tone was established, the tissue was incubated for 30 min with vehicle or antagonist before a cumulative concentration–response curve to a selective EP_1 agonist, ONO-D1-004 was performed (Suzawa *et al.*, 2000). The selective EP_1-receptor antagonist, GW848687X (1 μM), was included to confirm involvement of the EP_1 subtype (Giblin *et al.*, 2007).

EP_3 receptor-mediated responses: depolarization of guinea pig isolated vagal tissue. PGE_2-induced depolarization of guinea pig vagus nerve has previously been shown to be mediated by the EP_3 receptor (Maher *et al.*, 2009) and was therefore used as an assay system to assess functionality at this receptor subtype. Guinea pigs were killed as described earlier and the neck was opened by mid-line incision to expose the trachea and thorax. Segments of vagus nerve, 10–15 mm long caudal to the nodose ganglion, were removed with fine forceps and placed in KH solution and bubbled with 95% O_2/5% CO_2. The nerve was then cleared of connective tissue and carefully desheathed under a dissecting microscope. Care was taken throughout to ensure that the nerve trunks remained in oxygenated KH solution and that they were not stretched or damaged. Immediately after dissection, the desheathed nerve trunk was mounted in a 'grease-gap' recording chamber. The nerve was drawn longitudinally through a narrow channel (2 mm in diameter, 10 mm in length) in a Perspex block. The centre of the channel was filled with petroleum jelly, injected through a side-arm when the nerve was in place, onto the middle of the vagus, creating an area of high resistance and electrically isolating the extracellular space between the two ends of the nerve. One end of the nerve emerged into a wider channel and was constantly superfused with KH solution with a flow rate of approximately 2 $mL \cdot min^{-1}$. The other nerve ending remained throughout the study in a second, smaller chamber containing oxygenated KH solution. Ag/AgCl electrodes (Mere 2 Flexible reference electrodes, World Precision Instruments [WPI], Stevenage, UK) filled with KH solution, made contact at either end of the nerve trunk and recorded direct current (DC) potential via a DAM50 differential amplifier (WPI). DC voltages were amplified 10 times, filtered at 1000 Hz and sampled at 5 Hz. The temperature of the perfusate was maintained at 37°C by a water bath.

Prostanoids were applied at sub-maximal concentrations into the perfusing solution onto the mouse vagal nerves for 2 min and depolarizing responses recorded onto a chart recorder (Lectromed Multi-Trace 2, Lectromed, Welwyn Garden City, UK). Two reproducible responses were obtained for PGE_2 (10 μM). The antagonist was then perfused for 10 min, followed by PGE_2 in the presence of antagonist for a further 2 min. The nerve was then washed and PGE_2 was again applied (2 min incubation) to confirm nerve viability. The selective EP_3-receptor antagonist, ONO-AE3-240 (10 μM), was included to confirm involvement of the EP_3 subtype (Amano *et al.*, 2003).

EP_4 receptor-mediated responses: contraction of rat and human isolated tracheal tissue. PGE_2-induced relaxation of induced tone in rat and human tracheal tissue has previously been shown to be mediated by the EP_4 receptor (Lyford and McKechnie, 1994; Buckley *et al.*, 2011; Benyahia *et al.*, 2012) and, therefore, used as assay systems to assess functionality at this receptor subtype. In these experiments, CCh (1 μM) was used to induce a stable contraction of the tissues. Vehicle or antagonist was then incubated for 30 min before a cumulative concentration–response curve to PGE_2 was performed. At the end of the experiment, the non-specific PDE inhibitor, papaverine (100 μM), was added to assess the maximum capacity for relaxation of each tissue. A high concentration of the selective EP_4-receptor antagonist, ONO-AE3-208 (1 μM), was used to confirm involvement of the EP_4 subtype (Kabashima *et al.*, 2002).

DP_1, TP, IP, FP receptor-mediated responses: depolarization of mouse isolated vagal tissue. Mice were given an overdose of sodium pentobarbitone (200 $mg \cdot kg^{-1}$) and the neck was opened by mid-line incision to expose the trachea and thorax. Segments of vagus nerve, 10–15 mm long caudal to the nodose ganglion, were removed with fine forceps and placed in KH solution and bubbled with 95% O_2/5% CO_2. The nerve was then cleared of connective tissue and carefully desheathed under a dissecting microscope. Care was taken throughout to ensure that the nerve trunks remained in oxygenated KH solution and that they were not stretched or damaged. Vagal nerve depolarization studies were performed as above and as previously described (Birrell *et al.*, 2009; Maher *et al.*, 2009). The stimuli were applied at known submaximal concentrations into the perfusing solution onto the mouse vagal nerves for 2 min and depolarizing responses recorded onto a chart recorder (Lectromed Multi-Trace 2). Two reproducible responses were obtained for the prostanoid agonists: $PGF_{2\alpha}$ (FP), PGD_2 (DP), U46619 (TP) and iloprost (IP) (10 μM). The antagonists PF-04418948 (1 μM; EP_2), AL8810 (10 μM; FP), AH6809 (10 μM; DP), SQ29548 (1 μM; TP), RO3244794 (1 μM; IP) or 0.1% DMSO (vehicle) were then perfused for 10 min, followed by the prostanoid agonist in the presence of antagonist for a further 2 min. The nerve was then washed and the agonist was again applied (2 min incubation) to confirm nerve viability. Antagonists were used at concentrations 100 times higher than their reported pK_B values, as is common practice (Jones *et al.*, 2009).

Data analysis and statistical procedures

Airway smooth muscle tone experiments. Monophasic agonist concentration–effect (E/[A]) curves were fitted by least squares, non-linear iterative regression to the following form of the Hill equation (Prism 4®, GraphPad Software Inc., San Diego, CA, USA):

$$E = E_{min} + \frac{(E_{max} - E_{min})}{1 + 10^{(p[A]_{50} - p[A])n}} \quad (1)$$

where E is the effect, E_{min} and E_{max} are the lower and upper asymptotes (i.e. the basal response and maximum agonist-induced response, respectively), p[A] is the log molar concentration of agonist, $p[A]_{50}$ is a location parameter equal to the

log molar concentration of agonist producing $E_{max}/2$ and n is the gradient of the E/[A] curve at the p[A]$_{50}$ level.

Antagonist affinity was determined by least squares, non-linear regression using a modification of the Hill and Gaddum/Schild equations derived by Waud *et al.* (1978). Each family of E/[A] curves (i.e. the control agonist E/[A] curve and agonist E/[A] curves constructed in the presence of increasing concentrations of antagonist) were fitted simultaneously to Equation (2). Thus,

$$E = E_{min} + \left(\frac{(E_{max} - E_{min})}{1 + \left(\frac{10^{(p[A]_{50})}\left[1 + ([B]/10^{(-p[A]_2)})^S\right]}{[A]} \right)^n} \right) \quad (2)$$

where [A] and [B] are the molar concentrations of agonist and antagonist, respectively, S is the Schild slope factor and pA_2 is the affinity of the antagonist when $S = 1$, which is equivalent to the pK_B. To determine whether S deviated significantly from unity, the entire family of E/[A] curves that made up an individual experiment was fitted globally to Equation (2) under two conditions: one where S was constrained to a constant equal to 1 and the other where it was a shared value for all data sets. The F-test was applied to determine which equation gave the best fit, which was used for the final analysis.

Estimates of antagonist affinity were also made by performing a classic Schild analysis (Schild, 1949) and are expressed with the 95% confidence intervals (CIs). In addition, in some experiments, estimates of antagonist affinity were calculated using the equation pK_B = log(CR-1) – log[B] as described by Gaddum (1957), where CR is the concentration ratio calculated from the [A]$_{50}$ of agonist in the presence of the antagonist divided by the [A]$_{50}$ of the agonist alone, K_B is the equilibrium dissociation constant and [B] is the concentration of antagonist. In those experiments involving only one concentration of antagonist, the term pA_2 was used as this term precludes assumptions being made about the nature of the antagonism.

Vagal depolarization experiments. Data are presented as mean ± SEM of n independent observations. Inhibition of agonist responses in the isolated vagus nerve preparation were analysed by two-tailed paired t-test, comparing responses to agonist in the absence and presence of antagonist in the same piece of nerve.

Drugs, chemicals reagents and other materials

The EP$_1$-receptor antagonist, GW848687X, was a gift from GSK (Stevenage, UK). The EP$_2$-receptor antagonist, PF-04418948, was a gift from Pfizer (Kent, UK). The EP$_3$-receptor antagonist (ONO-AE-240), the EP$_4$-receptor antagonist (ONO-AE3-208), the EP$_1$-receptor agonist (ONO-D1-004) and the EP$_2$-receptor agonist (ONO-AE1-259) were gifts from Ono Pharmaceuticals (Osaka, Japan). All antagonists were reconstituted using DMSO, and 1–10 mM stocks were stored at –20°C until required. Stock solutions of the EP$_1$- and EP$_2$-receptor agonists (1 mM) were made in 10% DMSO in Krebs. PGE$_2$ was purchased from Cayman Europe (Tallinn, Estonia) and stock solutions of 10 mM were made in ethanol. Papaverine was purchased from Sigma-Aldrich (Poole, UK) and dissolved in distilled water at 100 mM. Krebs salts were obtained from BDH (Dorset, UK) and all other chemicals and reagents were purchased from Sigma-Aldrich. ACh and CCh were purchased from Sigma-Aldrich and dissolved in KH solution at 100 and 1 mM respectively.

Results

EP$_2$-receptor-mediated responses: relaxation of mouse isolated tracheal tissue

When tension was induced in the mouse trachea using CCh (1 μM) (induced tone), increasing concentrations of the EP$_2$ receptor agonist, ONO-AE1-259, produced a concentration-dependent relaxation with p[A]$_{50}$ of ~–7.8 (95% CI: –7.96 to –7.66) (approximately 90% of the maximum relaxation to 100 μM papaverine). PF-04418948 (30 min pretreatment), compared with its vehicle control, produced a graded, parallel dextral displacement of the ONO-AE1-259 concentration–response curves (Figure 1A). Enumeration of the Schild slope factor, S (0.97; 95% CI: 0.8–1.14), by simultaneously fitting each agonist concentration–response curve in the absence and presence of PF-04418948 to Equation (2), indicated that this parameter did not deviate significantly from unity ($P = 0.73$). Thus, PF-04418948 behaved in a manner that was consistent with surmountable competitive antagonism (Neubig *et al.*, 2003). Accordingly, S was constrained to a value of 1, from which a mean pK_B value of 8.3 (95% CI: 8.12–8.46) was derived (Figure 1B), which is similar to previously published values (5.4, 2.4 and 1.3 nM for human myometrium, dog bronchiole and mouse trachea, respectively; Forselles *et al.*, 2011).

In other experiments, a single concentration of PF-04418948 (30 nM), compared with its vehicle control, antagonized PGE$_2$-induced relaxations (data not shown), producing a significant rightward parallel shift of the E/[A] curve, from which a pA_2 of 8.32 (95% CI: 7.79–8.85) was derived (Gaddum, 1957). This value is identical to the pK_B obtained using ONO-AE1-259 as the agonist. PF-04418948 at a higher concentration of 1 μM did not have any direct effect on tissue tone (data not shown) and was without effect on relaxations induced by the β-adrenoceptor agonist, isoprenaline (data not shown).

EP$_2$ receptor-mediated responses: relaxation of guinea pig isolated tracheal tissue

Due to the contractile actions of PGE$_2$ acting via the EP$_1$ receptor (Coleman and Kennedy, 1985; Ndukwu *et al.*, 1997; Buckley *et al.*, 2011), PGE$_2$-induced relaxation of guinea pig trachea was performed in the presence of the EP$_1$ receptor antagonist, GW848687X (1 μM) (Giblin *et al.*, 2007). Under conditions of EP$_1$ receptor blockade, PGE$_2$ relaxed guinea pig trachea in a concentration-dependent manner with a potency (p[A]$_{50}$ = –6.5; 95% CI: –6.71 to –6.32) lower than that found in mouse tracheal tissue (cf. Figures 1A and 2A). PF-04418948, compared with its vehicle control, produced a

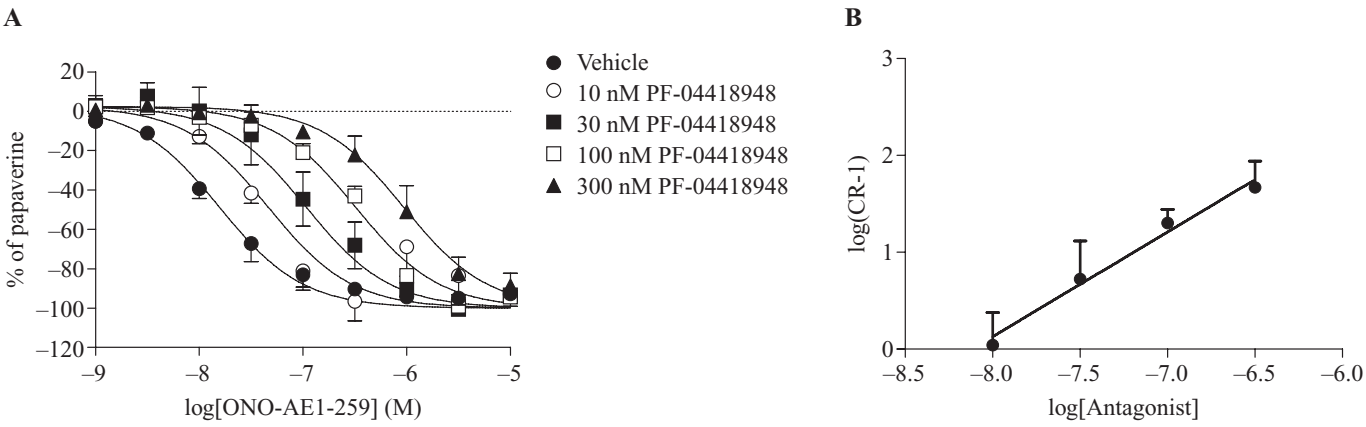

Figure 1

EP_2 receptor-driven responses: relaxation of isolated mouse tracheal tissue. (A) Effect of vehicle (DMSO, 0.1% v v^{-1}) or PF-04418948 (10, 30, 100 and 300 nM) on ONO AE1-259-induced relaxation of carbachol pre-contracted mouse tracheal tissue. (B) Schild plot analysis of the antagonism with PF-04418948. Data represent mean ± SEM (n = 4–6).

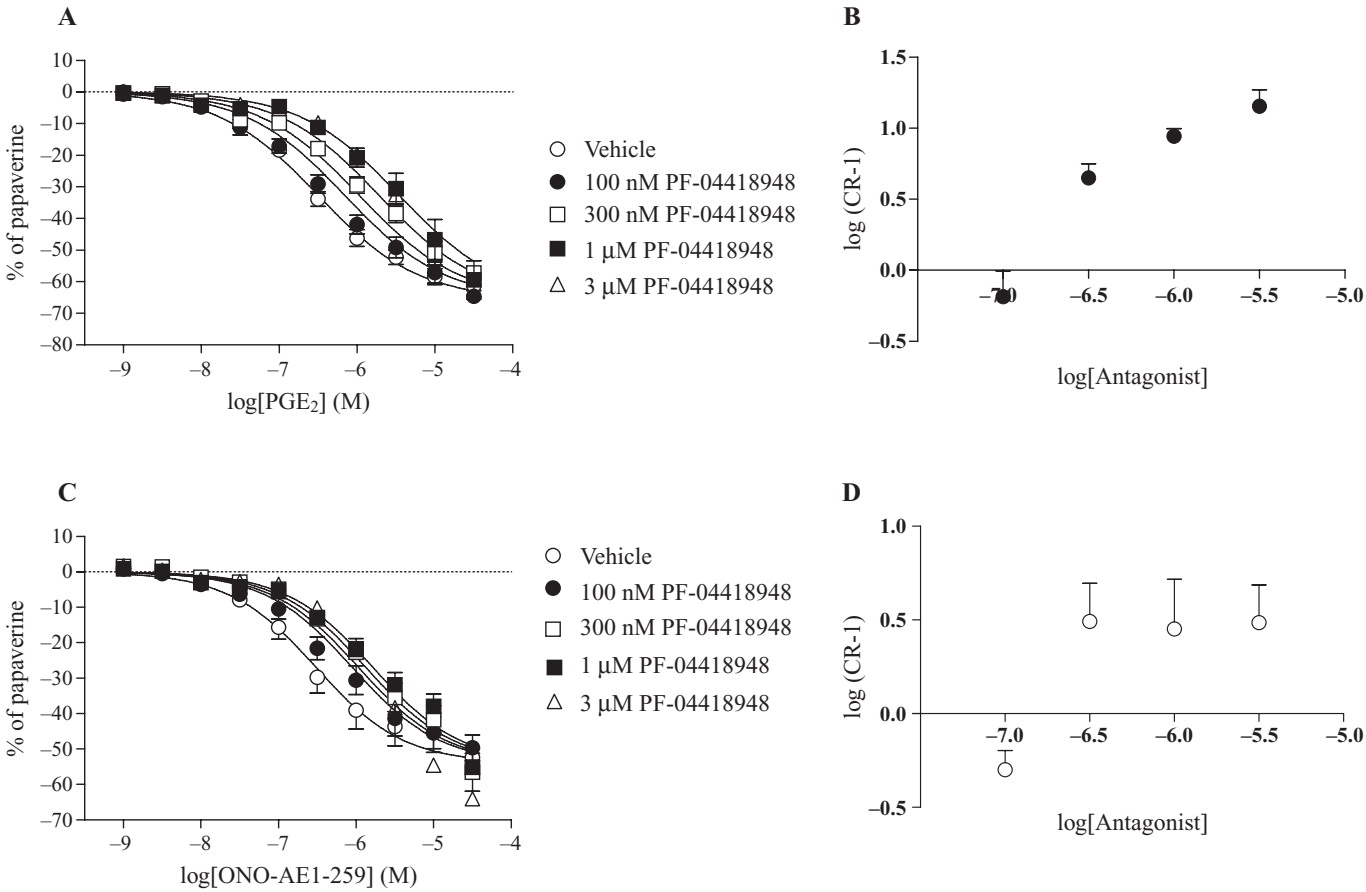

Figure 2

EP_2 receptor-driven responses: relaxation of guinea pig isolated tracheal tissue. (A) Effect of vehicle (DMSO, 0.1% v v^{-1}) or PF-04418948 (100 nM–3 µM) on PGE_2-induced relaxation of carbachol pre-contracted guinea pig tracheal tissue. (B) Schild plot analysis of the antagonism of PGE_2 with PF-04418948. Data represent mean ± SEM (n = 4–6). (C) Effect of vehicle (DMSO, 0.1% v v^{-1}) or PF-04418948 (100 nM–3 µM) on ONO-AE1-259-induced relaxation of carbachol pre-contracted guinea pig tracheal tissue. (D) Schild plot analysis of the antagonism of ONO-AE1-259 with PF-04418948. Data represent mean ± SEM (n = 4–6).

graded, parallel dextral displacement of PGE_2 *E*/[A] curves (Figure 2A,C). At concentrations of PF-04418948 lower than 3 μM, enumeration of the Schild slope factor, *S* (0.97; 95% CI: 0.8–1.14), by simultaneously fitting the agonist *E*/[A] curves in the absence and presence of PF-04418948 to Equation (2), indicated that this parameter did not deviate significantly from unity ($P = 0.38$). Thus, PF-04418948 behaved in a manner that was consistent with surmountable competitive antagonism (Neubig *et al.*, 2003). Accordingly, *S* was constrained to a value of 1, from which a mean pK_B value of 6.95 (95% CI: 6.8–7.1) was derived (Figure 2B).

When the concentration of PF-04418948 was increased to 3 μM, global, non-linear analysis using Equation (2) indicated that the antagonism of PGE_2-induced relaxation deviated from surmountable, competitive behaviour. Indeed, the Schild slope factor, *S*, was now significantly ($P < 0.001$) less than unity (PGE_2: 0.63; 95% CI: 0.48–0.77), suggesting that PGE_2 (at high concentrations) was producing relaxation by interacting with a receptor in addition to the EP_2 subtype at which PF 04418948 has weak or no affinity. This was shown in Figure 2B, where the Schild plot has become curvilinear.

The same trend was seen when ONO-AE1-259 was used as the agonist, but the deviation from surmountable competitive behaviour was much more pronounced (Figure 2D). Indeed, global curve fitting of the data shown in Figure 2C, where PF 04418948 was used at concentrations of 100 nM, 300 nM, 1 μM and 3 μM, returned a Schild slope factor, *S*, of 0.24, which was significantly different from 1 (95% CI: 0.04–0.44; $P < 0.001$). Inspection of the ONO-AE1-259 concentration–response curves shown in Figure 2C clearly shows that concentrations of PF-04418948 above 100 nM failed to antagonize relaxations, suggesting that, similar to PGE_2, ONO-AE1-259 (at high concentrations) was producing relaxation by interacting with a receptor in addition to the EP_2 subtype at which PF-04418948 has weak or no affinity. In these experiments, a single concentration of PF-04418948 (100 nM), compared with its vehicle control, antagonized PGE_2- and ONO-AE1-259-induced relaxations (data not shown), producing significant rightward, parallel shifts of the *E*/[A] curve, from which a $pA_2 = 6.81$ (95% CI: 5.95–7.67) and 6.89 (95% CI: 6.36–7.4) were derived, respectively (Gaddum, 1957). The result using PGE_2 as an agonist was similar to the pK_B obtained by global curve fitting using PF-04418948 at concentrations from 100 nM to 1 μM. PF-04418948 (1 μM) did not have any direct effect on tissue tone (data not shown) and was without effect on relaxations induced by the β-adrenoceptor agonist, isoprenaline (data not shown).

EP_1-receptor-mediated responses: contraction of guinea pig isolated tracheal tissue

The selective EP_1-receptor agonist, ONO-D1-004 (Suzawa *et al.*, 2000), caused a concentration-dependent contraction of guinea pig tracheal tissue with a $p[A]_{50}$ value of 7.10 ± 0.30 and a maximal contraction of 59.4 ± 8.5% of the response to ACh (Figure 3). The selective EP_1-receptor antagonist, GW848687X (1 μM), evoked a rightward shift of the responses to ONO-D1-004, whereas PF-04418948 (1 μM) was inactive ($p[A]_{50} = 6.99 \pm 0.29$; maximal contraction = 62.6 ± 8.7%; Figure 3).

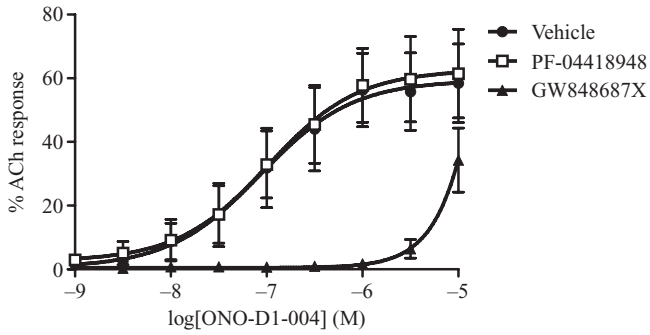

Figure 3

EP_1 receptor-mediated responses: contraction of guinea pig isolated tracheal tissue. Effect of vehicle (DMSO, 0.1% v v^{-1}), PF-04418948 (1 μM) or GW848687X (1 μM) on ONO-D1-004-induced contractions of guinea pig tracheal tissue. Data represent mean ± SEM ($n = 4$–6).

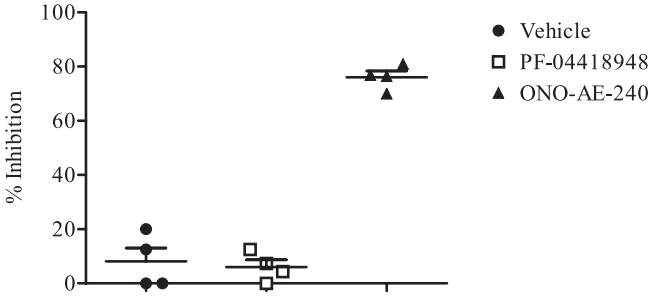

Figure 4

EP_3 receptor-mediated responses: depolarization of guinea pig isolated vagal tissue. Effect of vehicle (DMSO, 0.1% v v^{-1}), PF-04418948 (1 μM) or ONO-AE3-240 (10 μM) on PGE_2-induced depolarization of guinea pig vagal tissue. Data represent mean ± SEM ($n = 4$).

EP_3-receptor-mediated responses: depolarization of guinea pig isolated vagal tissue

PGE_2 caused depolarization of the guinea pig vagus nerve (mean change in mV was 0.17 ± 0.012), as previously reported (Maher *et al.*, 2009). While the EP_3-receptor antagonist, ONO-AE-240, evoked a statistically significant inhibition of this response, PF-04418948 (1 μM) had no effect (Figure 4).

EP_4-receptor-mediated responses: contraction of rat isolated tracheal tissue

PGE_2 caused a concentration-dependent relaxation of pre-contracted rat trachea (Figure 5A), which was inhibited by the EP_4-receptor antagonist, ONO-AE3-208 (as previously reported by Lyford and McKechnie, 1994; Buckley *et al.*, 2011). PF-04418948 (1 μM) had no effect on PGE_2-induced relaxations (Figure 5 and Table 1). Similarly, PGE_2 caused a concentration-dependent relaxation of pre-contracted human trachea (Figure 5B), which was inhibited by the EP_4-receptor antagonist, ONO-AE3-208, as previously reported

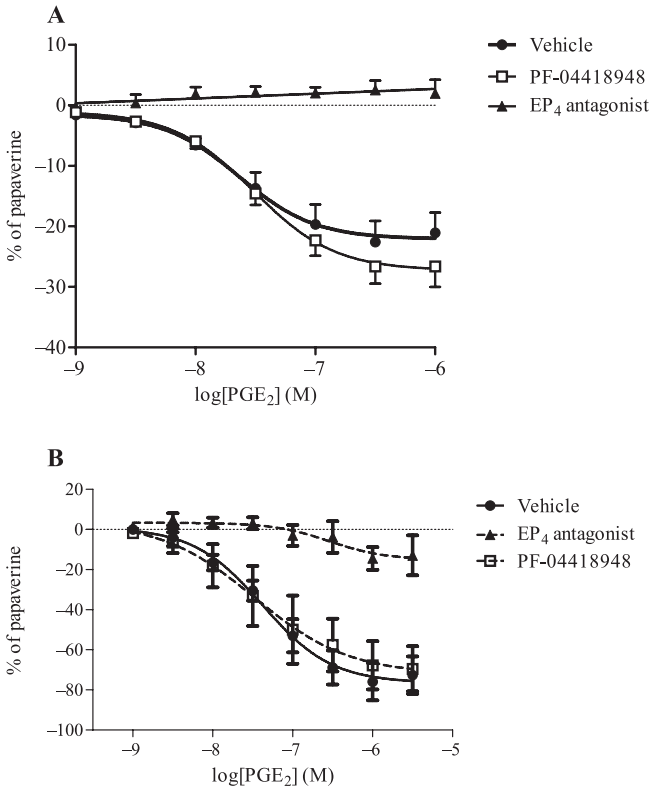

Figure 5

EP_4 receptor-mediated responses: relaxation of rat and human isolated tracheal tissue. Effect of vehicle (DMSO, 0.1% v v^{-1}), PF-04418948 (1 μM) or ONO AE3 208 (1 μM) on PGE_2-induced relaxation of carbachol pre-contracted rat (A) and human (B) tracheal tissue. Data represent mean ± SEM ($n = 4$ in both cases).

Table 1

Parameter estimates for EP_4 receptor-mediated responses: relaxation of rat and human isolated tracheal tissue

Sigmoidal dose response – Best fit values	Vehicle	PF-04418948
Rat		
Bottom (% of papaverine)	22.09 ± 1.96	27.29 ± 1.96
$p[A]_{50}$	7.64 ± 0.16	7.52 ± 0.12
Human		
Bottom (% of papaverine)	76.6 ± 6.14	72.23 ± 15.04
$p[A]_{50}$	7.38 ± 0.16	7.47 ± 0.43

Concentration–response curves were analysed by non-linear regression using the 'PRISM' curve-fitting program (Graph-Pad software) to produce curves of best fit. A four-parameter logistic equation was used to construct sigmoidal dose–response curves with a variable slope.

(Buckley *et al.*, 2011; Benyahia *et al.*, 2012). PF-04418948 (1 μM) had no effect on PGE_2-induced relaxations (Figure 5 and Table 1).

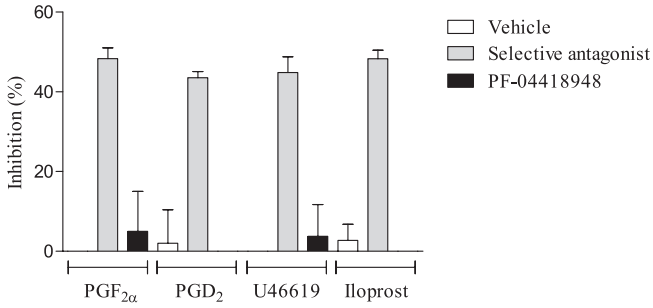

Figure 6

DP_1, TP, IP and FP receptor-mediated responses: depolarization of mouse isolated vagal tissue. The effect of the EP_2 receptor antagonist PF-04418948 (1 μM) on depolarization of the mouse vagus evoked by activation of the other prostanoid receptors (FP, DP, IP and TP); $PGF_{2\alpha}$ (FP), PGD_2 (DP), U46619 (TP) and iloprost (IP) all caused depolarization of the isolated vagus nerves. The vehicle (DMSO, 0.1% v v^{-1}) had no effect on depolarization, whereas the selective antagonists (AL8810, 10 μM; AH6809, 10 μM; SQ29548, 1 μM; RO3244794, 1 μM) inhibited responses mediated by their respective agonists. The EP_2-receptor antagonist had no effect on any of the prostanoid agonists. Data represent mean ± SEM ($n = 4$–6).

DP_1, TP, IP and FP receptor-mediated responses: depolarization of mouse isolated vagal tissue

We also assessed whether the EP_2 receptor antagonist, PF 04418948, had an effect on other prostanoid receptors (FP, IP, DP and TP) in an isolated mouse vagus nerve preparation. $PGF_{2\alpha}$ (FP), PGD_2 (DP), U46619 (TP) and iloprost (IP) induced depolarizations that were significantly inhibited in each case by selective antagonists that target these receptor subtypes (AL8810, 10 μM; AH6809, 10 μM; SQ29548, 1 μM and RO3244794 1 μM, respectively). In contrast, PF 04418948 (1 μM) did not have any effect on depolarization induced by any of these prostanoid receptor ligands, suggesting that it did not exhibit activity at the FP, DP, TP or IP receptors (Figure 6).

Discussion

Investigating the role that the EP_2 receptor plays in prostaglandin biology has been hampered by the lack of a selective antagonist. The absence of such a pharmacological tool has forced investigators to use weak EP_2 receptor agonists such as AH13205 (pK_i of 6 against the human receptor; Nials *et al.*, 1993) as well as butaprost FA, which, although useful, has affinity for EP_3 and EP_4 receptors (pK_i of 5.9–7, 5.8, 4.7 for the human EP_2, EP_3 and EP_4 receptor, respectively) (Abramovitz *et al.*, 2000; Buckley *et al.*, 2011). Although the situation improved with the development of the potent and selective EP_2 receptor agonist, ONO-AE1-259 (pK_i of 8.5 on the mouse EP_2 receptor; Suzawa *et al.*, 2000), this compound is not commercially available. Moreover, it is still generally believed that receptor characterization is achieved with greater certainty using selective antagonists. One commercially available receptor antagonist, AH6809, has been utilized extensively

given the absence of a high affinity and selective EP_2 receptor antagonist, but it is not very potent and also blocks the EP_1 and DP_1 receptors (similar affinity at human EP_2, EP_1 and DP_1 receptors with pK_is of 5.9, 5.9–6.0 and 5.8 respectively) (Abramovitz *et al.*, 2000). Another approach has been to utilize genetically modified mice, where the gene for the EP_2 receptor (*ptger2*) has been deleted. However, these studies do not address the possible developmental consequences of gene deletion. Therefore, the recent discovery of the EP_2 receptor antagonist, PF-04418948, is welcome (Forselles *et al.*, 2011).

The aim of this study was to use a range of tissue-based assays to confirm the activity of PF-04418948 against the native EP_2 receptor and to determine its selectivity for this receptor over other prostanoid receptors. PGE_2 is thought to induce relaxation of mouse and guinea pig pre-contracted trachea via activation of the EP_2 receptor. Therefore, these preparations were selected as bioassay systems to assess the functional effects of PF-04418948 on this receptor (Nials *et al.*, 1993; Sheller *et al.*, 2000; Tilley *et al.*, 2000; Fortner *et al.*, 2001; Buckley *et al.*, 2011). PF-04418948 behaved as a surmountable competitive antagonist, producing rightward parallel shifts of the PGE_2 and ONO-AE1-259 *E*/[A] curves that described relaxation of mouse trachea with an identical pK_B value of 8.32. These antagonist affinity values were very similar to those published by Forselles *et al.* (2011), differing maximally by less than fourfold (Forselles *et al.*, 2011). In addition, we showed that the inhibition of relaxation by PF-04418948 appeared to be specific to the PGE_2-driven response because the compound had no effect on isoprenaline-induced relaxations in the mouse trachea, which is mediated through β_1 adrenoceptors (Henry and Goldie, 1990).

PF-04418948 also caused a concentration-related parallel, rightward shift of the *E*/[A] curves that described the relaxation of guinea pig trachea induced by PGE_2 without changing the maximum response. Interestingly, although global analysis indicated that PF-04418948 at concentrations up to 1 μM behaved as a competitive surmountable antagonist ($S = 1.1$; not different from unity), the pK_B (~7) was 23-fold lower than that calculated for the mouse trachea. Stated differently, PF-04418948 interacted with the EP_2 receptor on guinea pig trachea with significantly lower affinity. Clearly, if these findings are applicable to other EP_2 receptor antagonists, then the utility of this preparation as a native EP_2 receptor bioassay is questionable. Indeed, PGE_2 (and ONO-AE1-259) were also relatively weak agonists on guinea pig trachea when compared with the mouse.

The low potency of PGE_2 and ONO-AE1-259 on guinea pig trachea may explain why global analysis of the antagonism produced by PF-04418948 returned Schild slopes that deviated significantly from unity. A reasonable interpretation of these data is that high concentrations (>1 μM) of PGE_2 and ONO-AE1-259 promote relaxation by activating a second receptor or receptors that are relatively insensitive to PF-04418948. If this is true, then this issue is more problematic with ONO-AE1-259, which cross-activates a second relaxant receptor(s) at lower concentrations than PGE_2. Indeed, inspection of Figure 2D shows that PF-04418948 is active as an antagonist at a concentration of 100 nM only; a higher concentration produce negligible further dextral displacements of the ONO-AE1-259 *E*/[A] curves. Collectively, these data suggest that the guinea pig EP_2 receptor on tracheal smooth muscle displays a pharmacology that is distinct from the EP_2 subtype in human and mouse airways. This difference may be antagonist-specific (i.e. peculiar to PF-04418948) or due to some structural dissimilarity of the EP_2 receptor between species, which influences ligand recognition.

Having shown that PF-04418948 had antagonist activity at the native mouse EP_2 receptor, we went on to assess whether it had activity at the other native EP receptors. Each of the assay systems was validated by using a selective antagonist for the respective receptor driving the response. PF-04418948 appeared to be selective for the EP_2 receptor, as it did not inhibit contraction of guinea pig trachea elicited by an EP_1-receptor agonist (previously shown to be mediated by an EP_1 receptor) (McKenniff *et al.*, 1988; Ndukwu *et al.*, 1997; Buckley *et al.*, 2011) and PGE_2-induced relaxation of rat and human pre-contracted trachea (previously shown to be mediated by the EP_4 receptor; Lyford and McKechnie, 1994; Buckley *et al.*, 2011; Benyahia *et al.*, 2012). An assessment of the selectivity of PF-04418948 against EP_3 receptors was made in the isolated vagus nerve assay. We have previously demonstrated that PGE_2 depolarizes the guinea pig vagus nerve via activation of the EP_3 subtype (Maher *et al.*, 2009). In the present study, PF-04418948 did not affect PGE_2-induced depolarization of the guinea pig vagus, indicating no functional activity at the native EP_3 receptor. Collectively, therefore, the data in the EP_1, EP_3 and EP_4 receptor tissue-based assays clearly show that PF-04418948 is a selective EP_2 receptor antagonist, despite the species differences in affinity.

As there is a certain amount of sequence homology and several conserved regions identified between the EP, TP, IP, DP_1 and FP receptors (Woodward *et al.*, 2011), we assessed whether PF-04418948 could act as an antagonist at these receptors in functional bioassay experiments. We have previously shown in guinea pig vagal tissue that $PGF_{2\alpha}$, PGD_2, iloprost and U46619 produce depolarization via activation of the receptors at which they exhibit the greatest affinity, that is, FP, DP_1, IP and TP, respectively (Maher *et al.*, 2009). In these experiments, the same profile of agonist activity was seen on the mouse vagus and in each case the depolarization was inhibited by the FP, DP, IP and TP selective receptor antagonists; in contrast, PF-04418948 was inactive. These data suggest that PF-04418948 does not behave as an antagonist at these receptors. In addition, in none of the assay systems employed did PF-04418948 display any agonist activity.

In conclusion, these data confirm the EP_2-selectivity profile of PF-04418948 previously found using human recombinant receptor assays. However, the present study also suggests that the guinea pig EP_2 receptor has characteristics different from the EP_2 receptor on mouse trachea, human myometrium and dog bronchiole, indicating that it may not be an ideal EP_2 receptor assay system. Nevertheless, the discovery of PF-04418948 should be of great utility in dissecting the role of PGE_2 in a variety of physiological and pathophysiological settings.

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Conflicts of interest

NP is employed by Pfizer. There are no other perceived conflicts of interest.

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