JPET Fast Forward. Published on July 21, 2010 as DOI: 10.1124/jpet.110.169748

JPET#169748

TITLE PAGE

Selexipag: a selective prostacyclin receptor agonist that does not affect rat gastric function

Keith Morrison, Roland Ernst, Patrick Hess, Rolf Studer, Martine Clozel

Drug Discovery Department, Actelion Pharmaceuticals, Ltd
Allschwil, Switzerland

RUNNING TITLE PAGE

Running Title: Selexipag and gastric function

Corresponding Author: Dr. Martine Clozel,
Drug Discovery Department,
Actelion Pharmaceuticals Ltd.,
Gewerbestrasse 16,
Allschwil CH4123
Switzerland
E-mail: martine.clozel@actelion.com

Text Pages: 26

Number of tables: 1

Number of Figures: 6

Number of References: 33

Abstract word count: 250

Introduction word count: 525

Discussion word count: 1498

Abbreviations: IP receptor, prostacyclin receptor; PAH, pulmonary arterial hypertension; PGI_2, prostacyclin.

Section: Gastrointestinal, Hepatic, Pulmonary, and Renal

ABSTRACT

Selexipag is an orally available prostacyclin receptor (IP receptor) agonist that is chemically distinct from prostacyclin and is in clinical development for the treatment of pulmonary arterial hypertension. Selexipag is highly selective for the human IP receptor *in vitro*, whereas analogs of prostacyclin can activate prostanoid receptors other than the IP receptor. The goal of this study was to determine the impact of selectivity for the IP receptor on gastric function by measuring 1) contraction of rat gastric fundus *ex vivo*, and 2) the rates of gastric emptying and intestinal transport in response to selexipag in comparison to other prostacyclin analogs. The rat gastric fundus expresses mRNA encoding multiple prostanoid receptors to different levels: EP_1 > EP_3, IP > DP_1, TP. Selexipag and metabolite ACT-333679 did not contract gastric fundus at concentrations up to 10^{-3} M. In contrast, the prostacyclin analogs iloprost and beraprost evoked concentration-dependent contraction of gastric fundus. Contraction to treprostinil was observed at high concentration (10^{-4} M). Contraction to all prostacyclin analogs was mediated via activation of EP_3 receptors, although EP_1 receptors also contributed to contraction of gastric fundus to iloprost and beraprost Antagonism of IP receptors did not affect responses. Oral selexipag did not significantly alter gastric function *in vivo*, as measured by rates of stomach emptying and intestinal transport, whereas beraprost slowed gastro-intestinal transport. The high functional selectivity of selexipag and ACT-333679 for the IP receptor precludes a stimulatory action on gastric smooth muscle, and may help minimize gastric side-effects such as nausea and vomiting.

INTRODUCTION

An imbalance in activities between vasodilator (nitric oxide [NO], prostacyclin [PGI_2]) and vasoconstrictor (thromboxane, endothelin) pathways contributes to the pathogenesis of pulmonary arterial hypertension (PAH) (Tuder et al, 1999). Restoration of PGI_2 function is one strategy in the effective management of the disease, and analogs of PGI_2 provide therapeutic benefits in PAH. Activation of the PGI_2 receptor (IP receptor) by PGI_2 analogs leads to vasodilation and reduced proliferation of vascular smooth muscle cells (Gomberg-Maitland and Olschewski, 2008). Administration of these drugs, however, can be painful and inconvenient for the patient (Humbert et al, 1999, Sitbon et al, 2002). In addition, gastric side-effects including abdominal cramping, gastric pain, nausea, vomiting and diarrhea are common in patients treated with PGI_2 analogs (Widlitz et al, 2007). These compounds are not selective for the IP receptor (Kiriyama et al, 1997; Narumiya et al, 1999; Abramovitz et al, 2000) and gastric irritation may result from activation of other prostanoid receptors that are expressed in the gastro-intestinal tract (Dey et al, 2006). Binding assays demonstrate that both iloprost and beraprost have high affinity for EP_1 and EP_3 receptors (Abramovitz et al, 2000; Kiriyama et al, 1997). These receptor subtypes mediate contraction of rat gastric fundus as evidenced by contraction to the EP_3/EP_1 receptor agonist sulprostone (Bennett et al, 1980). Further, contraction of rat fundal strips (Dong et al, 1986) and guinea pig ileum (Lawrence et al, 1992) to PGI_2 analogs has been reported.

Structural derivatives of 4,5-diphenyloxazole are potent agonists at IP receptors and were developed as potential anti-thrombotic agents (Meanwell et al, 1994). Selexipag (recommended INN; NS-304, ACT-293987) is a diphenyl pyrazine derivative

(Kuwano et al, 2007) and is an orally available IP receptor agonist that is in clinical development for the treatment of PAH. Selexipag is chemically distinct from analogs of PGI_2. *In vitro*, selexipag has high selectivity for the human IP receptor over other prostanoid receptors (Kuwano et al, 2007). Selexipag is readily hydrolysed to the active metabolite ACT-333679 (MRE-269), which is also a potent and selective agonist at IP receptors. ACT-333679 has at least 130-fold higher affinity for the IP receptor over other prostanoid receptors. The receptor selectivity profile of selexipag and ACT-333679 was derived from radioligand displacement studies using recombinant cells engineered to express human prostanoid receptors. Selectivity of selexipag and ACT-333679 for the IP receptor using functional *ex vivo* and whole body systems has not been systematically evaluated.

This study sought to determine the impact of selectivity of selexipag and ACT-333679 for the IP receptor on gastric function by measuring 1) contraction of rat gastric fundus *ex vivo*, and 2) gastric emptying and intestinal transport *in vivo* in response to selexipag and PGI_2 analogs. Reactivity of rat gastric fundus was studied since perturbation of the gastro-intestinal system by PGI_2 analogs is commonly observed in the clinic (Gomberg-Maitland and Olschewski, 2008) and prostaglandins regulate gastric function (Wallace, 2008). The PGI_2 analogs iloprost, beraprost and treprostinil were studied in comparison with selexipag and metabolite ACT-333679.

Data presented here demonstrate that selexipag and ACT-333679, unlike analogs of PGI_2, do not affect gastric function, which may help minimize off-target side-effects in the gastro-intestinal system such as nausea and vomiting.

METHODS

Animals. Male Wistar rats (12 wks) were obtained from the Biotechnology and Animal Breeding Division (Harlan, Füllinsdorf, Switzerland). All rats were housed in climate-controlled conditions with a 12-h light/dark cycle and had free access to normal pelleted rat chow and drinking water in accordance with local guidelines (Basel-Landschaft cantonal veterinary office).

Measurement of receptor mRNA expression.

Expression levels of mRNA encoding prostanoid receptors in the rat gastric fundus were first measured in order to establish that this tissue expressed multiple prostanoid receptor subtypes. Rats were euthanized by CO_2 asphyxiation and the stomach removed. Total RNA was isolated from gastric fundus without mucosa (30 mg samples) using the RNeasy fibrous tissue kit according to the manufacturer's protocol (Qiagen, Germany). Remaining genomic DNA was digested using the DNAse free kit (Ambion, USA). The quantity of RNA was analyzed using a Nanodrop spectrophotometer (Thermo Fisher, USA) and RNA quality was assessed using an Agilent Bioanalyzer 2100. Total RNA was reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems, USA). QPCR was performed on the ABI 7500 machine using TaqMan probes. The following TaqMan primers and probes from Applied Biosystems and Roche Diagnostics were used for QPCR: DP-Rn00824628_m1, EP1-Rn01432713_s1, EP3-Rn0121735_m1, IP-Rn01764022_m1 and 18s rRNA assay number 4130983E from Applied Biosystems. TBXAR2 forward ggtggagatgatggttcagc, reverse gcaaggtctgcaggatgaag and probe 77 ggtggtgg of the Universal Probe Library system from Roche Diagnostics. Results were

calculated using a modified delta delta cT method. This method allows comparison between gene expression values for different genes based on an identical linear scale. A value of 1 is defined as no expression.

Rat isolated gastric fundal strips. Following euthanasia, fundal strips were prepared from rats according to the method described by Vane (Vane, 1957). Briefly, the stomach was excised and the fundus separated from the pyloric antrum. The fundus was opened by cutting along the lesser curvature and the mucosa was carefully removed. Longitudinal incisions were performed along the length of the fundus to produce strips (13 mm) that were then suspended in tissue baths (10ml) containing Krebs-Henseleit buffer of the following composition (mM): NaCl 115; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.5; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 10. Bathing solution was maintained at 37°C and aerated with 95% O_2 / 5% CO_2 (pH 7.4). A resting force of 2 g (19.6 mN) was applied to the strips which were then allowed to stabilize for 45 minutes prior to the start of the experiment. Changes in force generation were measured using an isometric force recorder (EMKA Technologies Inc, Paris, France). Viability of each strip was determined by exposure to carbachol (10^{-5} M) and subsequent responses expressed as a percentage of this reference contraction. Fundal strips were exposed to either drug vehicle or receptor antagonists for 30 minutes prior to obtaining cumulative concentration-response curves to agonists. The choice and concentrations of receptor antagonists were based on published data. The following receptor antagonists were used: DBTSA (EP_3 receptor; Gallant et al, 2002, Kuwano et al, 2008); SC19220 (EP_1 receptor; Bennett et al, 1980); SC51322 (EP_1 receptor; McCormick et al, 2010); GR32191 (TP

receptor; Lumley et al 1989); CAY10441 (IP receptor; Jones et al, 2006); AL8810 (FP receptor; Behm et al, 2009) and BW A868C (DP_1 receptor; Giles et al, 1989).

Rat isolated pulmonary artery. Relaxation of rat pulmonary artery was measured to confirm the pharmacological activity of selexipag, ACT-333679 and iloprost. The main pulmonary artery and two side branches were isolated, and rings prepared. Care was taken to avoid damage to the endothelium. Rings of pulmonary artery were suspended in tissue baths (10ml) containing Krebs-Henseleit buffer. Bathing solution was maintained at 37°C and aerated with 95% O_2 / 5% CO_2 (pH 7.4). A resting force of 0.5 g (4.9 mN) was applied to the vessels. Endothelial function was tested by measuring the ability of acetylcholine (10^{-5} M) to relax arterial rings contracted with phenylephrine (10^{-6} M). Rings of pulmonary artery were then contracted with prostaglandin $F_{2\alpha}$ (10^{-5} M). When the developed force had stabilized, cumulative concentration-relaxation curves to selexipag, ACT-333679 and iloprost were obtained. The interval between additions of higher concentrations of compounds to the baths was determined by the time required for the response to reach plateau. Arterial rings were exposed to CAY10441 (10^{-6} M) for 15 minutes prior to contraction with $PGF_{2\alpha}$ in experiments that measured the effect of IP receptor antagonism on relaxation to iloprost.

Measurement of gastric emptying and intestinal transport.

The effects of selexipag and beraprost on gastro-intestinal function *in vivo* were tested. Rats were fasted overnight. Selexipag, beraprost or drug vehicle were administered by oral gavage and a charcoal meal (1 ml/ rat, 5% carbon powder suspended in 10% arabic

gum) was given one hour later. Rats were sacrificed after 30 minutes and the abdomen was opened and the stomach and intestine were isolated. The effects of the compounds on intestinal transport were determined by measuring the distance that the charcoal meal had traveled along the gastro-intestinal tract and was expressed as a percentage of the total distance from the pylorus to the caecum. Stomach weights were measured in the same animals as an index of gastric emptying. Validation of the gastro-intestinal transport model was confirmed using the inhibitor atropine (3 mg/kg/ml, i.p.) and the stimulant neostigmine (30 mg/kg/10ml, p.o.).

Materials. Selexipag, ACT-333679 and (2E)-3-(3',4'-dichlorobiphenyl-2-yl)-N-(2-thienylsulfonyl)acrylamide (DBTSA) were synthesized by Nippon Shinyaku Co. Ltd (Kyoto, Japan). Iloprost, beraprost, treprostinil, AL8810, CAY10441, SC19220, SC51322 and sulprostone were obtained from Cayman Chemical. Acetylcholine, atropine, BW A868C, carbamyl choline chloride (carbachol), GR32191B, neostigmine, phenylephrine, and prostaglandin $F_{2\alpha}$ were purchased from Sigma (St Louis, MO, USA).

Statistical evaluation of results. Contraction of rat fundal strips is expressed as a percentage of the reference contraction to carbachol (10^{-5} M). Results are presented as mean ± S.E.M. In some experiments, the SEM values are smaller than the data symbol. n values refer to the number of animals. pEC_{50} values are defined as the negative logarithm of the concentration of agonist that evokes half maximal response. The functional inhibitory potency (pD'_2 value) for DBTSA was calculated according to the method of van Rossum (1963) and was defined as the negative logarithm of the

JPET Fast Forward. Published on July 21, 2010 as DOI: 10.1124/jpet.110.169748
This article has not been copyedited and formatted. The final version may differ from this version.

concentration causing a 50% reduction in the maximum force generated by sulprostone: $pD'_2 = pD'_x + \log (X - 1)$, where pD'_x is the negative logarithm of the concentration of DBTSA, and X is the ratio of maximal contraction to sulprostone in the absence and presence of DBTSA. The effects of receptor antagonists on contraction of gastric fundus to analogs of PGI_2 were quantified by comparing calculated areas under the agonist concentration-response curves in the absence and presence of antagonists. Statistical comparisons between control and treated groups were performed using student's paired t test (two-tailed). Significance was accepted at $P < 0.05$.

RESULTS

Prostanoid receptor mRNA expression and function.

Initial experiments using RT-QPCR established expression of mRNA encoding multiple prostanoid receptors was expressed in rat gastric fundus. A differential expression pattern for receptor mRNA was recorded (figure 1). The highest levels of mRNA encoded the EP_1 receptor, although mRNA for both the IP and EP_3 receptors was also expressed. TP and DP receptor mRNA expression was detectable but to an almost negligible degree.

In order to test the functionality of EP_1 and EP_3 receptors, the response of isolated gastric fundal strips to the EP_3/EP_1 receptor agonist sulprostone was measured. Sulprostone contracted rat gastric fundus in a concentration-dependent manner (10^{-10} M - 10^{-5} M). Contraction to sulprostone was significantly inhibited by DBTSA (10^{-5} M) (Table 1). The calculated pD'_2 value for DBTSA was 4.7 ± 0.1 (n = 6). Also, the EP_1 receptor antagonist SC19220 (3 x 10^{-5} M) caused a significant right-ward displacement of the concentration-contraction curve to sulprostone (Table 1).

IP receptor selectivity and gastric fundus.

The impact of selectivity for the IP receptor on gastric responsiveness was first studied by measuring contraction of rat gastric fundus to selexipag and ACT-333679 in comparison to analogs of PGI_2. The IP receptor selective compounds selexipag and ACT-333679 did not contract rat fundal strips above baseline force, even at concentrations up to 10^{-3} M (figure 2). Indeed, both compounds evoked weak relaxation of baseline force both in the absence and presence of the IP receptor antagonist CAY10441 (3 x 10^{-6} M) (figure 3).

JPET Fast Forward. Published on July 21, 2010 as DOI: 10.1124/jpet.110.169748
This article has not been copyedited and formatted. The final version may differ from this version.

Selexipag and ACT-333679 were shown to be pharmacologically active in rat since both compounds evoked concentration-dependent relaxation of precontracted rat pulmonary artery (pEC_{50} values of 5.38 ± 0.03 and 5.61 ± 0.06, respectively; n = 6). Similarly, iloprost relaxed rat pulmonary artery (control pEC_{50} value of 6.6 ± 0.1, n = 4). Relaxation to iloprost was significantly inhibited by CAY10441 (10^{-6} M) (areas under curves: control vs treated $P < 0.001$, n = 4).

In contrast to the findings with selexipag and ACT-333679, the non-selective PGI_2 analogs iloprost and beraprost induced concentration-dependent contraction of rat fundal strips (figure 4). Also, treprostinil evoked a small contraction of gastric fundus at the highest concentration tested (10^{-4} M). The, contraction to iloprost and beraprost was significantly inhibited by the EP_3 receptor antagonist DBTSA (10^{-5} M) (iloprost $P < 0.05$, beraprost $P < 0.01$; figure 4A, B). Contraction to the highest concentration of treprostinil tested was also significantly inhibited by DBTSA ($P < 0.05$; figure 4C). The IP receptor CAY10441 did not significantly alter contraction of rat fundal strips to the PGI_2 analogs (figure 4D-F). The role of EP_1 receptors in the contraction to PGI_2 analogs was investigated using the antagonist SC19220. Contraction to iloprost and beraprost, but not treprostinil, was inhibited by SC19220, as indicated by significant reductions in the areas under the curves (iloprost control vs treated, $P < 0.05$; beraprost control vs treated $P < 0.05$) (figure 5A and D). The inhibitory effect of SC19220 on contraction to iloprost and beraprost was confirmed using the congener SC51322 (10^{-6} M) (areas under curves: iloprost control vs treated, $P < 0.001$, n = 5; beraprost control vs treated, $P < 0.05$, n = 5). Antagonism of TP receptors and DP_1 receptors using GR32191B and BW A868C, respectively, did not significantly inhibit contraction of rat fundal strips to PGI_2 analogs

(figure 5). In addition, the selective FP receptor antagonist AL8810 (10^{-5} M) did not significantly affect contraction to iloprost, beraprost or treprostinil (areas under curves: control vs treated, $P > 0.05$, $n = 4$).

Gastro-intestinal activity in rats

The effects of selexipag and beraprost were next compared on the rates of gastric emptying and intestinal transport. Doses of selexipag and beraprost were chosen to attain concentrations in the stomach that reflect the highest concentrations used in contractility studies *ex vivo*. Calculation of doses for *in vivo* experiments was based on a rat stomach volume of 2.5 ml. Preliminary experiments validated the charcoal assay for measurement of intestinal transport using standard reference drugs. The inhibitor of acetylcholinesterase neostigmine accelerated gastro-intestinal transport (122 ± 4 % versus control 100%; $n = 12\text{-}13$, $P < 0.01$), whereas the non-selective muscarinic receptor antagonist atropine inhibited gastro-intestinal transport (78 ± 5 % versus control 100%, $n = 10\text{-}13$, $P < 0.01$). Selexipag did not retard gastric emptying or intestinal transport at 1 or 3 mg/kg (figure 6). Also, beraprost at a dose of 0.03 mg/kg had no significant effect on emptying of the stomach or rate of intestinal transport compared to vehicle control. A higher dose of beraprost (0.1 mg/kg), however, significantly slowed gastric emptying and intestinal transport ($P < 0.05$; figure 6).

DISCUSSION

The results of this study demonstrate that selexipag can be distinguished from analogs of PGI_2 in terms of gastric reactivity. Selexipag and metabolite ACT-333679 do not contract rat gastric fundus *ex vivo*, and selexipag does not retard gastric emptying or intestinal transport at pharmacologically relevant doses. In contrast, analogs of PGI_2 contract gastric fundus via activation of EP_3 and EP_1 receptors, and beraprost slows gastric function. Contraction of rat fundus may disturb the regulated and coordinated waves of contraction in the stomach leading to dysregulation of food mixing and gastric emptying (Pal et al, 2007). The pronounced contraction of gastric smooth muscle to analogs of PGI_2 reported here may help explain the abdominal pain and cramping associated with use of these compounds in the clinic. Also, perturbation of gastric function *in vivo* observed in response to beraprost is consistent with the emetic characteristic of this class of compound. Selexipag, on the other hand, lacks contractile efficacy and does not disrupt gastric function in this model, suggesting that compounds with high selectivity for the IP receptor may offer improved gastric tolerability over non-selective PGI_2 analogs.

The rat gastric fundus was chosen as the test model in this study since prostaglandins regulate gastric function (Wallace, 2008), and perturbation of the gastro-intestinal system by analogs of PGI_2 is commonly observed in the clinic. The suitability of gastric fundus to study selectivity of IP receptor agonists over other prostanoid receptors was established in initial experiments that measured the expression of mRNA encoding multiple prostanoid receptors. Also, the predominant EP_1 and EP_3 receptors were shown to be functional as measured by contraction of gastric fundus to the EP_3/EP_1

receptor agonist sulprostone. Both DBTSA and SC19220 effectively inhibited fundal contraction to sulprostone, supporting the involvement of EP_3 and EP_1 receptors.

Displacement binding studies using recombinant cell expression systems demonstrate that the IP receptor agonists selexipag and ACT-333679 are highly selective for the human IP receptor (Kuwano et al, 2007). For example, the K_i value for ACT-333679 at the IP receptor is 130-fold lower than that at other prostanoid receptors. The affinity values for selexipag and ACT-333679 at the rat IP receptor are 2.1×10^{-6} M and 2.2×10^{-7} M, respectively, and both compounds would be expected to activate IP receptors at the concentrations used in the current study. Neither selexipag nor ACT-333679 induced contraction of the rat gastric fundus above baseline demonstrating that smooth muscle IP receptors do not play a direct role in contraction of rat fundus. The possibility that activation of the IP receptor could inhibit an excitatory effect of these compounds in the gastric fundus can be excluded given that a contractile response to selexipag or ACT-333679 was not revealed in the presence of the IP receptor antagonist CAY10441 (Jones et al, 2006). Also, it seems unlikely that conversion of selexipag to metabolite ACT-333679 occurred over the duration of the experiment, given that responses to both compounds were similar, and that the mucosa was removed, negating any mucosal hydrolytic activity (Fukuhara et al, 1996). The absence of significant responses to selexipag and ACT-333679 in rat gastric fundus did not extend to other tissues in this species. Pharmacological activity of both compounds was confirmed by measuring concentration-dependent relaxation of the rat pulmonary artery pre-contracted with $PGF_{2\alpha}$. The calculated pEC_{50} value for relaxation is in good agreement with previous findings (Kuwano et al, 2008). The lack of efficacy of selexipag and ACT-

333679 on gastric reactivity relates directly to the high degree of IP receptor selectivity of these compounds. The PGI_2 analog cicaprost, which is also more selective than other PGI_2 analogs for the IP receptor (Abramovitz et al, 2000), also fails to contract rat gastro-intestinal smooth muscle at sub-micromolar concentrations (Qian and Jones, 1995).

The analogs of PGI_2 tested in this study contracted rat gastric fundus. Iloprost and beraprost evoked robust contractile responses, whereas weak contraction to treprostinil was observed at the highest concentration tested. Maximal responses to PGI_2 analogs were inferior to that evoked by the EP_3/EP_1 receptor agonist sulprostone, supporting the previous findings of Dong et al (1986). Contraction to all PGI_2 analogs tested here was sensitive to antagonism of EP_3 receptors. These data are consistent with findings in the $EP3^{-/-}$ mouse model where contraction of fundal strips to PGI_2 is abolished (Okada et al, 2000). Iloprost and beraprost both bind EP_3 receptors with high affinity (Abramovitz et al, 2000, Kuwano et al, 2007) and might be expected to activate EP_3 receptors at concentrations used in this study. Affinity data for treprostinil at non-IP receptors is not available, but the observation that treprostinil contracted fundus at high concentration via activation of EP_3 receptors suggests that this PGI_2 analog is also not selective for the IP receptor.

A role for the EP_1 receptor subtype in the contractile response to PGI_2 analogs was also considered given that the level of EP_1 receptor mRNA was highly expressed in gastric fundus, and iloprost has high affinity for this receptor subtype (equal K_i values at EP_1 and IP receptors) (Abramovitz et al, 2000). Indeed, the EP_1 receptor antagonist SC19220 caused a statistically significant inhibition of contraction to both iloprost and beraprost. These data are consistent with the findings of Bennett et al (1980).

DBTSA may also antagonize TP and DP_1 receptors at the concentration used in this study (Gallant et al, 2002). The possibility that antagonism of TP receptors contributed to the effect of DBTSA is unlikely, however, since the selective TP receptor antagonist GR32191B did not significantly inhibit contraction. Similarly, antagonism of DP_1 receptors by DBTSA can be dismissed since the DP_1 receptor antagonist BWA868C did not influence contraction to any of the PGI_2 analogs tested. Furthermore, although contractile FP receptors may be present in gastric fundus (Dong et al, 1986), the selective FP receptor antagonist AL8810 did not significantly affect contraction to any of the PGI_2 analogs tested.

The second goal of this study was to establish the impact of selectivity for the IP receptor on gastric performance *in vivo*. To this end, the effects of selexipag and beraprost on the rates of gastric emptying and intestinal transport were compared. Doses of selexipag and beraprost were chosen to attain concentrations in the stomach that reflect the high concentrations studied using the isolated gastric fundus. Also, the doses chosen effectively lower pulmonary arterial pressure and reduce ventricular hypertrophy in a rat model of PAH (Kuwano et al, 2008). Selexipag at 1 and 3 mg/kg, doses in the stomach that are equivalent to 2×10^{-4} M and 6×10^{-4} M, respectively, had no effect on the rate of gastric emptying or intestinal transport. These data are consistent with *ex vivo* data showing that selexipag does not contract gastric fundus even at 10^{-3} M. Beraprost at 0.1 mg/kg, equivalent to a contractile concentration of 2.7×10^{-5} M, significantly reduced the rates of stomach emptying and intestinal transport. A lower dose of beraprost (0.03 mg/kg) which is equivalent to 7×10^{-6} M did not impede gastric function despite

displaying contractile efficacy *ex vivo*. Taken together, these data suggest that robust contraction of gastric smooth muscle is required to disturb gastric physiology *in vivo*.

Normal gastric emptying requires the coordinated and rhythmic contraction of the stomach (Pal et al, 2007). Disruption of gastric smooth muscle activity underlies nausea and vomiting (Holmes et al, 2009). Thus, contraction of gastro-intestinal smooth muscle, as observed in this study, has important implications for the oral tolerability of a compound. Several lines of evidence support a role for EP_3 receptors in the perturbation of gastric function. The EP_3 receptor agonist sulprostone evokes emesis and diarrhea in the ferret (Kan et al, 2002). Iloprost also induces emesis in the ferret, whereas the selective IP receptor agonist cicaprost has no effect (Kan et al, 2002). Moreover, in the present study, iloprost evoked EP_3 receptor-dependent contraction of rat fundus, which may disturb the regulated and coordinated waves of contraction in the stomach leading to dysregulation of food mixing and gastric emptying (Pal et al, 2007). Importantly, gastric muscular dysrhythmias are initiated by EP_3 receptor agonists in the mouse (Forrest et al, 2009). Studies on emesis cannot be performed in rodents (Holmes et al, 2009) but delayed gastric emptying and increased chewing and swallowing are observed in the rat in response to substances that would be expected to be emetic in other species such as the ferret (Andrews and Horn, 2006). Delayed gastric emptying in the rat is considered as a surrogate marker for vomiting (Bradner and Schurig, 1981). The finding in the present study that beraprost contracted gastric fundus via activation of EP_3 receptors and slowed gastric emptying is consistent with the emetic properties of the drug in the clinic.

In conclusion, the results of this study demonstrate that the selective IP receptor agonist selexipag can be distinguished from PGI_2 analogs in terms of gastric reactivity in

JPET Fast Forward. Published on July 21, 2010 as DOI: 10.1124/jpet.110.169748
This article has not been copyedited and formatted. The final version may differ from this version.

the rat. Selexipag does not disrupt gastric function, and offers potential for improved gastric tolerability over non-selective PGI_2 analogs currently used in the clinic for the treatment of PAH.

ACKNOWLEDGMENTS

The authors thank Franck Haag and Celine Bortolamiol for technical assistance.

REFERENCES

Abramovitz M, Adam M, Boie Y, Carrière MC, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, Belley M, Gallant M, Dufresne C, Gareau Y, Ruel R, Juteau H, Labelle M, Ouimet N, and Metters KM (2000) The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochimica et Biophysica Act* **1483:** 285-293.

Andrews PLR and Horn CC (2006) Signals for nausea and emesis: Implications for models of upper gastrointestinal diseases. *Auton Neurosci* **125:** 100-115.

Behm DJ, Ogbonna A, Wu C, Burns-Kurtis CL, and Douglas SA (2009) Epoxyeicosatrienoic acids function as selective, endogenous antagonists of native thromboxane receptors: identification of a novel mechanism of vasodilation. *J Pharmacol Exp Ther* 328: 231-239.

Bennett A, Jarosik C, Sanger GJ, and Wilson DE (1980) Antagonism of prostanoid-induced contractions of rat gastric fundus muscle by SC-19220, sodium meclofenamate, indomethacin or trimethoquinol. *Br J Pharmacol* **71:** 169-175.

Bradner WT and Schurig JE (1981) Toxicology screening in small animals. *Cancer Treat Rev* **8:** 93-102.

Dey I, Lejeune M, and Chadee K (2006) Prostaglandin E_2 receptor distribution and function in the gastrointestinal tract. *Br J Pharmacol* **149:** 611-623.

Dong YJ, Jones RL, and Wilson NH (1986) Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Br J Pharmacol* **87:** 97-107.

Forrest AS, Hennig GW, Jokela-Willis S, Park CD, and Sanders KM (2009) Prostaglandin regulation of gastric slow waves and peristalsis. *Am J Physiol Gastrointest Liver Physiol* **296:** G1180-G1190.

Fukuhara A, Imai T, and Otagiri M (1996) Stereoselective disposition of flurbiprofen from a mutual prodrug with a histamine H_2-antagonist to reduce gastrointestinal lesions in the rat. *Chirality* **8:** 494-502.

Gallant M, Carrière MC, Chateauneuf A, Denis D, Gareau Y, Godbout C, Greig G, Juteau H, Lachance N, Lacombe P, Lamontagne S, Metters KM, Rochette C, Ruel R, Slipetz D, Sawyer N, Tremblay N, and Labelle M (2002) Structure-activity relationship of biaryl acylsulfonamide analogues on the human EP_3 prostanoid receptor. *Bioorg Med Chem Letts* **12:** 2583-2586.

Giles H, Leff P, Bolofo ML, Kelly MG, and Robertson AD (1989) The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br J Pharmacol* **96:** 291-300.

Gomberg-Maitland M and Olschewski H (2008) Prostacyclin therapies for the treatment of pulmonary arterial hypertension. *Eur Respir J* **31:** 891-901.

Humbert M, Sanchez O, Fartoukh M, Jagot JL, Le Gall C, Sitbon O, Parent F, and Simonneau G (1999) Short-term and long-term epoprostenol (prostacyclin) therapy in pulmonary hypertension secondary to connective tissue diseases: results of a pilot study. *Eur Respir J* **13:** 1351-1356.

Holmes AM, Rudd JA, Tattersall FD, Aziz Q, and Andrews PLR (2009) Opportunities for the replacement of animals in the study of nausea and vomiting. *Br J Pharmacol* **157:** 865-880.

Jones RL, Wise H, Clark R, Whiting RL, and Bley KR (2006) Investigation of the prostacyclin (IP) receptor antagonist RO1138452 on isolated blood vessel and platelet preparations. *Br J Pharmacol* **149:** 110-120.

Kan KK, Jones RL, Ngan MP, and Rudd JA (2002) Actions of prostanoids to induce emesis and defecation in the ferret. *Eur J Pharmacol* **453:** 299-308.

Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, and Narumiya S (1997) Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* **122:** 217-224.

Kuwano K, Hashino A, Asaki T, Hamamoto T, Yamada T, Okubo K, and Kuwabara K (2007) 2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide (NS304), an orally available and long-acting prostacyclin receptor agonist prodrug. *J Pharmacol Exp Ther* **322:** 1181-1188.

Kuwano K, Hashino A, Noda K, Kosugi K, and Kuwabara K (2008) A long-acting and highly selective prostacyclin receptor agonist prodrug, 2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide (NS304), ameliorates rat pulmonary hypertension with unique relaxant responses of its active form, {4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy} acetic acid (MRE-269), on rat pulmonary artery. *J Pharmacol Exp Ther* **326:** 691-699.

Lawrence RA, Jones RL, and Wilson NH (1992) Characterization of receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig ileum. *Br J Pharmacol* **105:** 271-278.

Lumley P, White BP, and Humphrey PPA (1989) GR32191, a highly potent and specific thromboxane A_2 receptor blocking drug on platelets and vascular and airways smooth muscle *in vitro*. *Br J Pharmacol* **97:** 783-794.

McCormick C, Jones RL, Kennedy S, and Wadsworth RM (2010) Activation of prostanoid EP receptors by prostacyclin analogues in rabbit iliac artery: implications for anti-restenotic potential. *Eur J Pharmacol* doi:10.1016/j.ejphar.2010.04.035.

Meanwell NA, Rosenfeld MJ, Trehan AK, Wright JJK, Brassard CL, Buchanan JO, Federici ME, Fleming JS, Gamberdella M, Hartl KS, Zavoico GB, and Seiler SM (1994) Non-prostanoid prostacyclin mimetics. 6. Derivatives of 2-[3-[2-(4,5-diphenyl-2-oxazolyl)ethyl]phenoxy]acetic acid modified β-to the oxazole ring. *Drug Design Disc* **11:** 73-89.

Narumiya S, Sugimoto Y, and Ushikubi F (1999) Prostanoid receptors: structures, properties, and functions. *Physiol Rev* **79:** 1193-1226.

Okada Y, Hara A, Ma H, Xiao CY, Takahata O, Kohgo Y, Narumiya S, and Ushikubi F (2000) Characterization of prostanoid receptors mediating contraction of the gastric fundus and ileum: studies using mice deficient in prostanoid receptors. *Br J Pharmacol* **131:** 745-755.

Pal A, Brasseur JG, and Abrahamsson B (2007) A stomach road or "Magenstrasse" for gastric emptying. *J Biomechanics* **40:** 1202-1210.

Qian Y and Jones RL (1995) Inhibition of rat colon contractility by prostacyclin (IP-) receptor agonists: involvement of NANC neurotransmission. *Br J Pharmacol* **115:** 163-171.

Sitbon O, Humbert M, Nunes H, Parent F, Garcia G, Hervé P, Rainisio M, and Simonneau G (2002) Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival. *J Am Coll Cardiol* **40:** 780-788.

Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, and Voelkel NF (1999) Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med* **159:** 1925-1932.

van Rossum JM (1963) Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch Int Pharmacodyn Ther* **143:** 299-330.

Vane JR (1957) A sensitive method for the assay of 5-hydroxytryptamine. *Br J Pharmacol* **12:** 344-349.

Wallace JL (2008) Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? *Physiol Rev* **88:** 1547-1565.

Widlitz AC, McDevitt S, Ward GR, and Krichman A (2007) Practical aspects of continuous intravenous treprostinil therapy. *Crit Care Nurse* **27:** 41-50.

LEGENDS FOR FIGURES

Figure 1) Expression of mRNA encoding EP_1, EP_3, IP, TP and DP_1 receptors in rat gastric fundus. Data are shown as mean ± S.E.M.; n = 6.

Figure 2) Effects of selexipag (A) and ACT-333679 (B) on baseline force of rat gastric fundus. Data are shown as mean ± S.E.M.; n = 6.

Figure 3) Effects of the IP receptor antagonist CAY10441 (3×10^{-6} M) on response of rat gastric fundus to selexipag (A) and ACT-333679 (B). Data are shown as mean ± S.E.M.; n = 6.

Figure 4) Effects of the EP_3 receptor antagonist DBTSA (10^{-5} M) and the IP receptor antagonist CAY10441 (3×10^{-6} M) on contraction of rat gastric fundus to iloprost (A,D), beraprost (B,E) and treprostinil (C,F). Data are shown as mean ± S.E.M.; n = 5. * $P < 0.05$, ** $P < 0.01$.

Figure 5) Effects of the EP_1 receptor antagonist SC19220 (3×10^{-5} M), the TP receptor antagonist GR32191B (3×10^{-6} M) and the DP_1 receptor antagonist BW A868C (10^{-7} M) on contraction of rat gastric fundus to iloprost (A,B,C), beraprost (D,E,F) and treprostinil (G,H,I). Data are shown as mean ± S.E.M.; n = 5. * $P < 0.05$.

Figure 6) Effects of selexipag and beraprost on gastric emptying (A) and intestinal transport (B) in rats. Data are shown as mean ± S.E.M.; n = 5. * $P < 0.05$.

JPET Fast Forward. Published on July 21, 2010 as DOI: 10.1124/jpet.110.169748
This article has not been copyedited and formatted. The final version may differ from this version.

TABLES

Table 1. Inhibition of contraction of gastric fundus to sulprostone.

Receptor	Antagonist	pEC_{50} control	pEC_{50} treated	E_{max} control	E_{max} treated	Control vs Treated Difference in AUC	n
EP_3	DBTSA (10^{-5} M)	7.5±0.1	6.8±0.1**	78.0±4.1	52.0±4.3**	***	6
EP_1	SC19220 ($3x10^{-5}$ M)	7.4±0.2	6.8±0.1**	80.1±3.3	83.3±4.4	n.s.	6

** P < 0.01, *** P< 0.001 control vs treated
n.s., not significant
AUC, area under curve

Figure 1

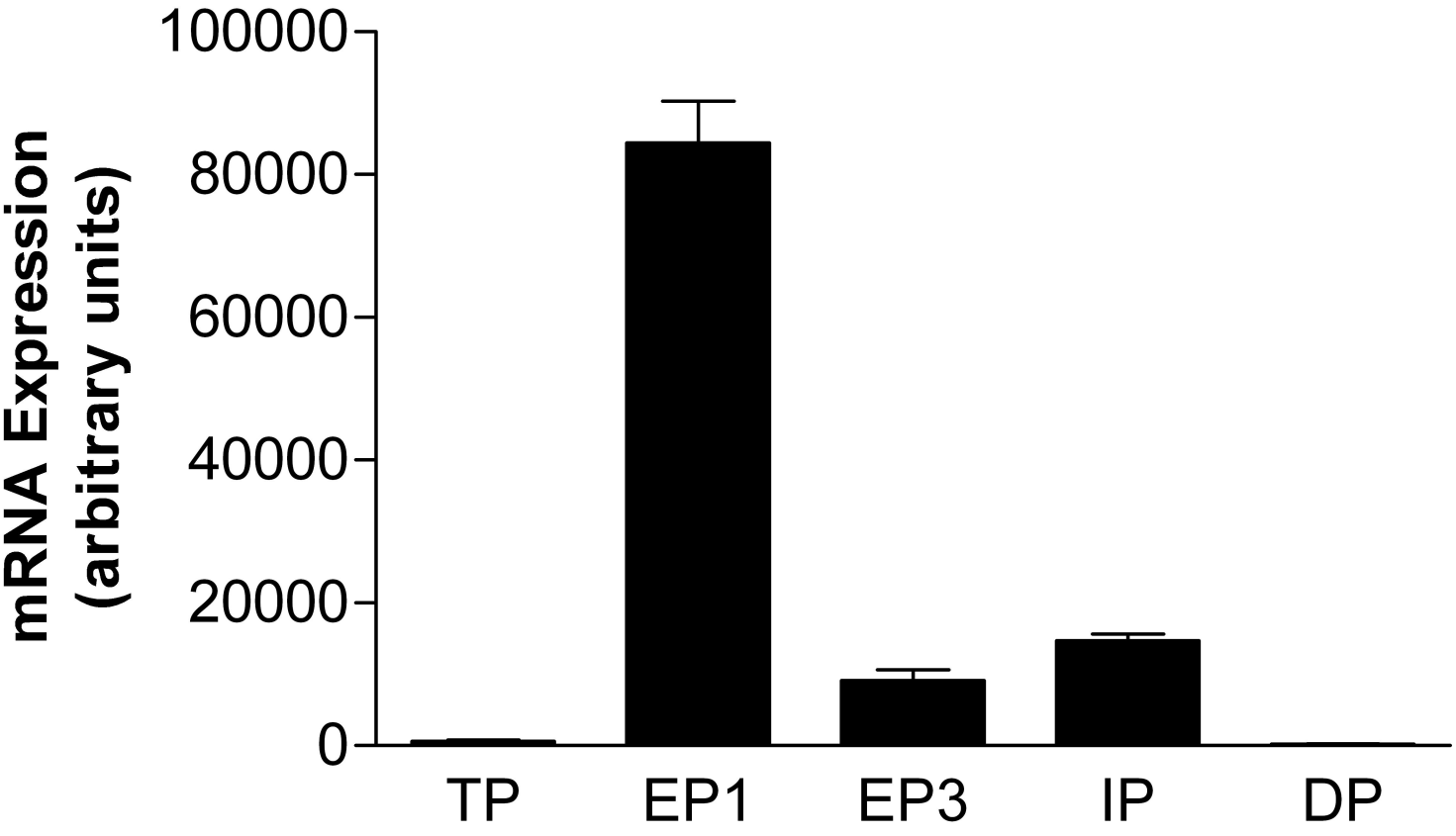
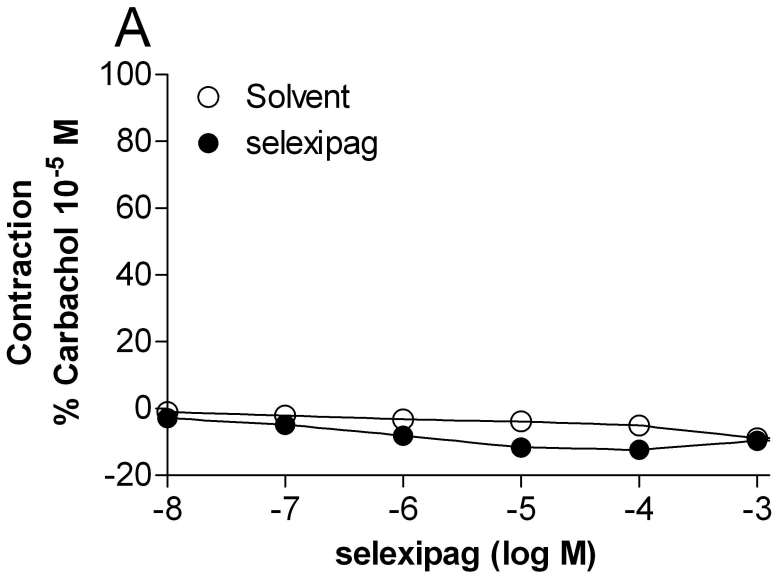


Figure 2



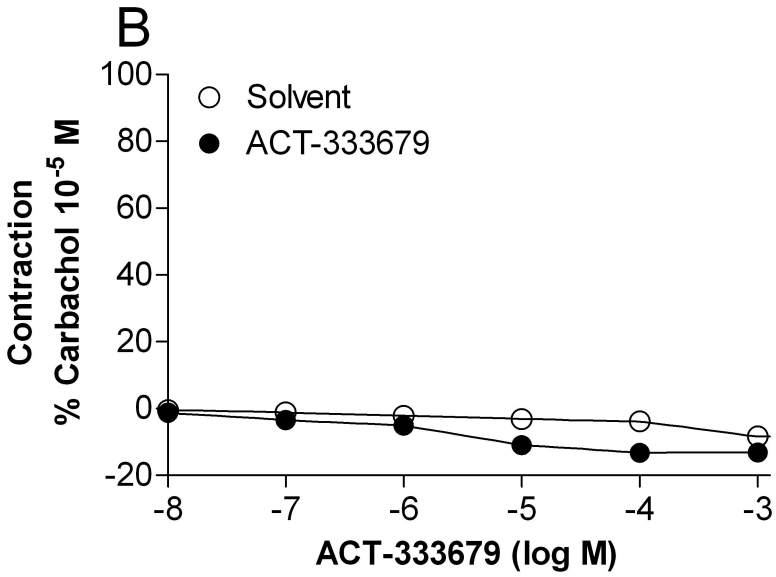
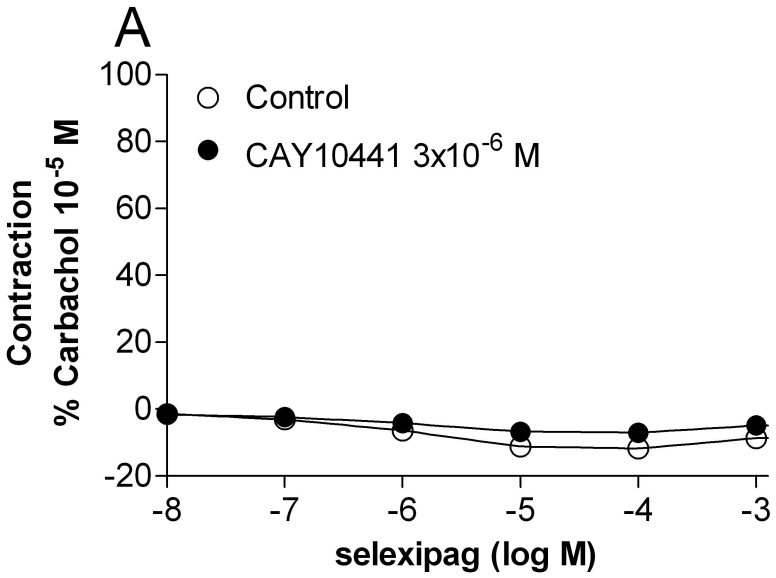


Figure 3



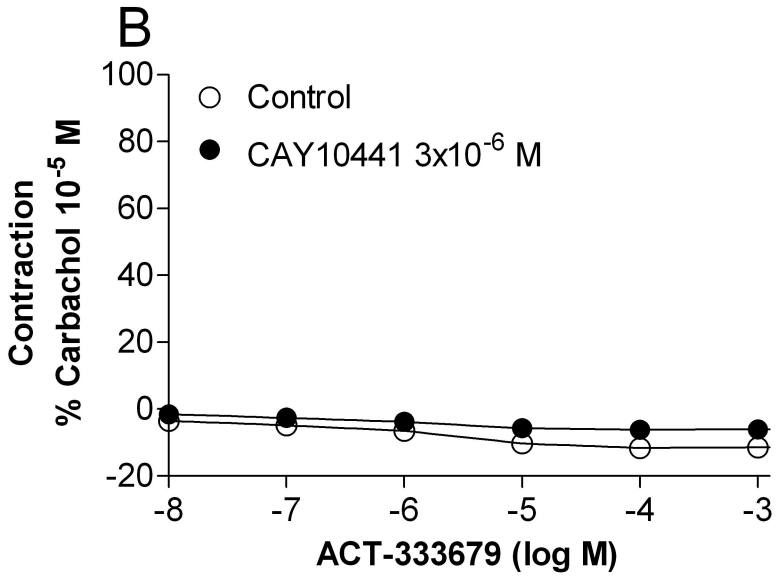
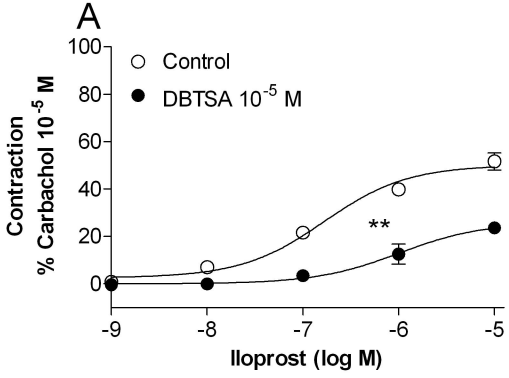
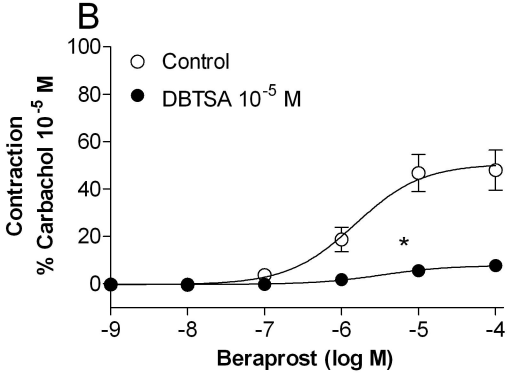
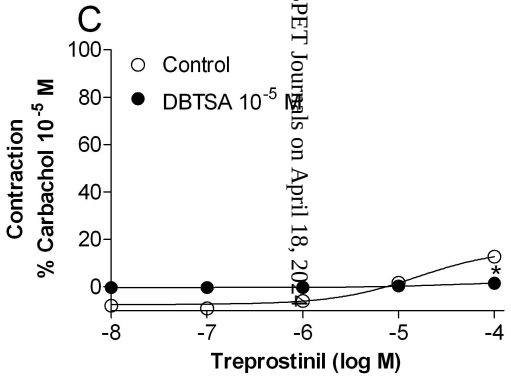
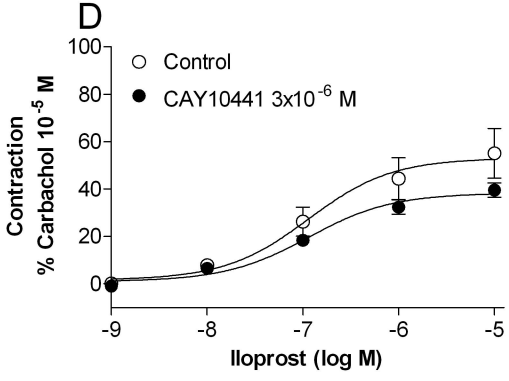


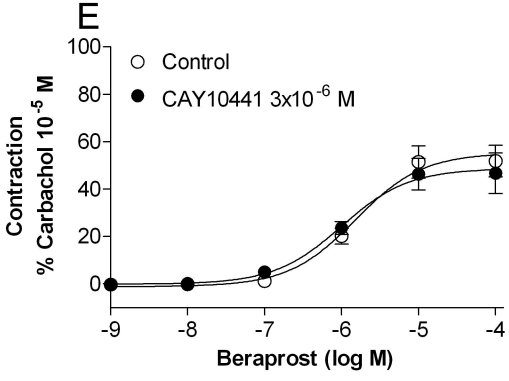
Figure 4











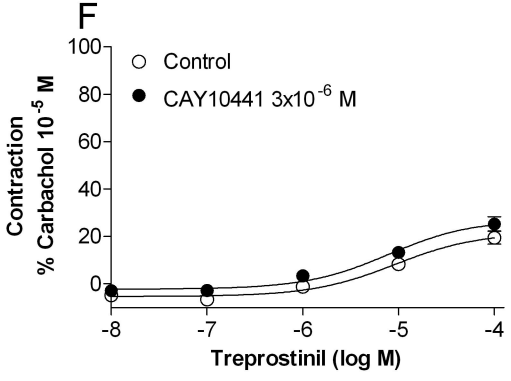


Figure 5

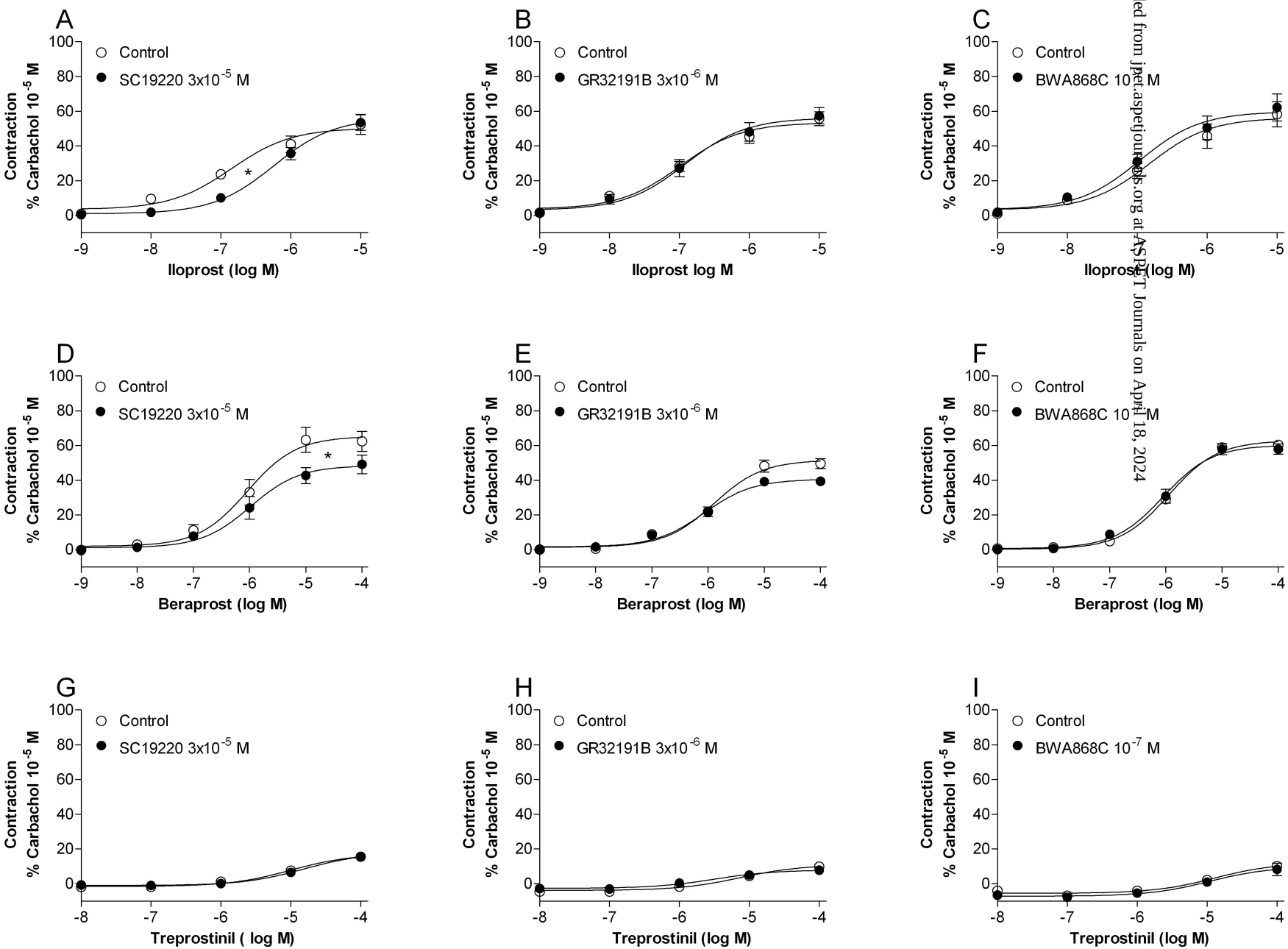


Figure 6