

**TITLE PAGE**

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

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## RUNNING TITLE PAGE

**Running Title:** Selexipag and gastric function

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**Abbreviations:** IP receptor, prostacyclin receptor; PAH, pulmonary arterial hypertension; PGI<sub>2</sub>, prostacyclin.

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## 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<sub>2</sub>]) and vasoconstrictor (thromboxane, endothelin) pathways contributes to the pathogenesis of pulmonary arterial hypertension (PAH) (Tuder et al, 1999). Restoration of PGI<sub>2</sub> function is one strategy in the effective management of the disease, and analogs of PGI<sub>2</sub> provide therapeutic benefits in PAH. Activation of the PGI<sub>2</sub> receptor (IP receptor) by PGI<sub>2</sub> 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<sub>2</sub> analogs (Widlitz et al, 2007). These compounds are not selective for the IP receptor (Kiriyaama 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<sub>1</sub> and EP<sub>3</sub> receptors (Abramovitz et al, 2000; Kiriyaama et al, 1997). These receptor subtypes mediate contraction of rat gastric fundus as evidenced by contraction to the EP<sub>3</sub>/EP<sub>1</sub> 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<sub>2</sub> 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<sub>2</sub>. *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<sub>2</sub> analogs. Reactivity of rat gastric fundus was studied since perturbation of the gastro-intestinal system by PGI<sub>2</sub> analogs is commonly observed in the clinic (Gomberg-Maitland and Olschewski, 2008) and prostaglandins regulate gastric function (Wallace, 2008). The PGI<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub> 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<sub>4</sub> 1.2; KH<sub>2</sub>PO<sub>4</sub> 1.5; CaCl<sub>2</sub> 2.5; NaHCO<sub>3</sub> 25; glucose 10. Bathing solution was maintained at 37°C and aerated with 95% O<sub>2</sub>/ 5% CO<sub>2</sub> (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<sup>-5</sup> 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<sub>3</sub> receptor; Gallant et al, 2002, Kuwano et al, 2008); SC19220 (EP<sub>1</sub> receptor; Bennett et al, 1980); SC51322 (EP<sub>1</sub> 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<sub>1</sub> 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<sub>2</sub> / 5% CO<sub>2</sub> (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<sup>-5</sup> M) to relax arterial rings contracted with phenylephrine (10<sup>-6</sup> M). Rings of pulmonary artery were then contracted with prostaglandin F<sub>2α</sub> (10<sup>-5</sup> 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<sup>-6</sup> M) for 15 minutes prior to contraction with PGF<sub>2α</sub> 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<sub>2α</sub> 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<sup>-5</sup> 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<sub>50</sub> values are defined as the negative logarithm of the concentration of agonist that evokes half maximal response. The functional inhibitory potency (pD'<sub>2</sub> value) for DBTSA was calculated according to the method of van Rossum (1963) and was defined as the negative logarithm of the

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<sub>2</sub> 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<sub>1</sub> receptor, although mRNA for both the IP and EP<sub>3</sub> 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<sub>1</sub> and EP<sub>3</sub> receptors, the response of isolated gastric fundal strips to the EP<sub>3</sub>/EP<sub>1</sub> 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'<sub>2</sub> value for DBTSA was  $4.7 \pm 0.1$  (n = 6). Also, the EP<sub>1</sub> receptor antagonist SC19220 ( $3 \times 10^{-5}$  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<sub>2</sub>. 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 ( $3 \times 10^{-6}$  M) (figure 3).

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<sub>50</sub> values of 5.38 ± 0.03 and 5.61 ± 0.06, respectively; n = 6). Similarly, iloprost relaxed rat pulmonary artery (control pEC<sub>50</sub> value of 6.6 ± 0.1, n = 4). Relaxation to iloprost was significantly inhibited by CAY10441 (10<sup>-6</sup> 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<sub>2</sub> 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<sup>-4</sup> M). The contraction to iloprost and beraprost was significantly inhibited by the EP<sub>3</sub> receptor antagonist DBTSA (10<sup>-5</sup> 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<sub>2</sub> analogs (figure 4D-F). The role of EP<sub>1</sub> receptors in the contraction to PGI<sub>2</sub> 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<sup>-6</sup> 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<sub>1</sub> receptors using GR32191B and BW A868C, respectively, did not significantly inhibit contraction of rat fundal strips to PGI<sub>2</sub> 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 ( $122 \pm 4$  % versus control 100%;  $n = 12-13$ ,  $P < 0.01$ ), whereas the non-selective muscarinic receptor antagonist atropine inhibited gastro-intestinal transport ( $78 \pm 5$  % versus control 100%,  $n = 10-13$ ,  $P < 0.01$ ). 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<sub>2</sub> 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<sub>2</sub> contract gastric fundus via activation of EP<sub>3</sub> and EP<sub>1</sub> 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<sub>2</sub> 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<sub>2</sub> 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 gastrointestinal system by analogs of PGI<sub>2</sub> 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<sub>1</sub> and EP<sub>3</sub> receptors were shown to be functional as measured by contraction of gastric fundus to the EP<sub>3</sub>/EP<sub>1</sub>

receptor agonist sulprostone. Both DBTSA and SC19220 effectively inhibited fundal contraction to sulprostone, supporting the involvement of EP<sub>3</sub> and EP<sub>1</sub> 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<sub>i</sub> 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 x 10<sup>-6</sup> M and 2.2 x 10<sup>-7</sup> 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<sub>2α</sub>. The calculated pEC<sub>50</sub> 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<sub>2</sub> analog cicaprost, which is also more selective than other PGI<sub>2</sub> analogs for the IP receptor (Abramovitz et al, 2000), also fails to contract rat gastrointestinal smooth muscle at sub-micromolar concentrations (Qian and Jones, 1995).

The analogs of PGI<sub>2</sub> 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<sub>2</sub> analogs were inferior to that evoked by the EP<sub>3</sub>/EP<sub>1</sub> receptor agonist sulprostone, supporting the previous findings of Dong et al (1986). Contraction to all PGI<sub>2</sub> analogs tested here was sensitive to antagonism of EP<sub>3</sub> receptors. These data are consistent with findings in the EP<sub>3</sub><sup>-/-</sup> mouse model where contraction of fundal strips to PGI<sub>2</sub> is abolished (Okada et al, 2000). Iloprost and beraprost both bind EP<sub>3</sub> receptors with high affinity (Abramovitz et al, 2000, Kuwano et al, 2007) and might be expected to activate EP<sub>3</sub> 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<sub>3</sub> receptors suggests that this PGI<sub>2</sub> analog is also not selective for the IP receptor.

A role for the EP<sub>1</sub> receptor subtype in the contractile response to PGI<sub>2</sub> analogs was also considered given that the level of EP<sub>1</sub> receptor mRNA was highly expressed in gastric fundus, and iloprost has high affinity for this receptor subtype (equal K<sub>i</sub> values at EP<sub>1</sub> and IP receptors) (Abramovitz et al, 2000). Indeed, the EP<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> receptors by DBTSA can be dismissed since the DP<sub>1</sub> receptor antagonist BWA868C did not influence contraction to any of the PGI<sub>2</sub> 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<sub>2</sub> 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  $2 \times 10^{-4}$  M and  $6 \times 10^{-4}$  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  $2.7 \times 10^{-5}$  M, significantly reduced the rates of stomach emptying and intestinal transport. A lower dose of beraprost (0.03 mg/kg) which is equivalent to  $7 \times 10^{-6}$  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<sub>3</sub> receptors in the perturbation of gastric function. The EP<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> 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<sub>2</sub> analogs in terms of gastric reactivity in

the rat. Selexipag does not disrupt gastric function, and offers potential for improved gastric tolerability over non-selective PGI<sub>2</sub> analogs currently used in the clinic for the treatment of PAH.

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## LEGENDS FOR FIGURES

**Figure 1)** Expression of mRNA encoding EP<sub>1</sub>, EP<sub>3</sub>, IP, TP and DP<sub>1</sub> 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 ( $3 \times 10^{-6}$  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<sub>3</sub> receptor antagonist DBTSA ( $10^{-5}$  M) and the IP receptor antagonist CAY10441 ( $3 \times 10^{-6}$  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<sub>1</sub> receptor antagonist SC19220 ( $3 \times 10^{-5}$  M), the TP receptor antagonist GR32191B ( $3 \times 10^{-6}$  M) and the DP<sub>1</sub> 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.

## TABLES

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

Receptor	Antagonist	pEC <sub>50</sub> control	pEC <sub>50</sub> treated	E <sub>max</sub> control	E <sub>max</sub> treated	Control vs Treated Difference in AUC	n
EP <sub>3</sub>	DBTSA (10 <sup>-5</sup> M)	7.5±0.1	6.8±0.1**	78.0±4.1	52.0±4.3**	***	6
EP <sub>1</sub>	SC19220 (3x10 <sup>-5</sup> 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

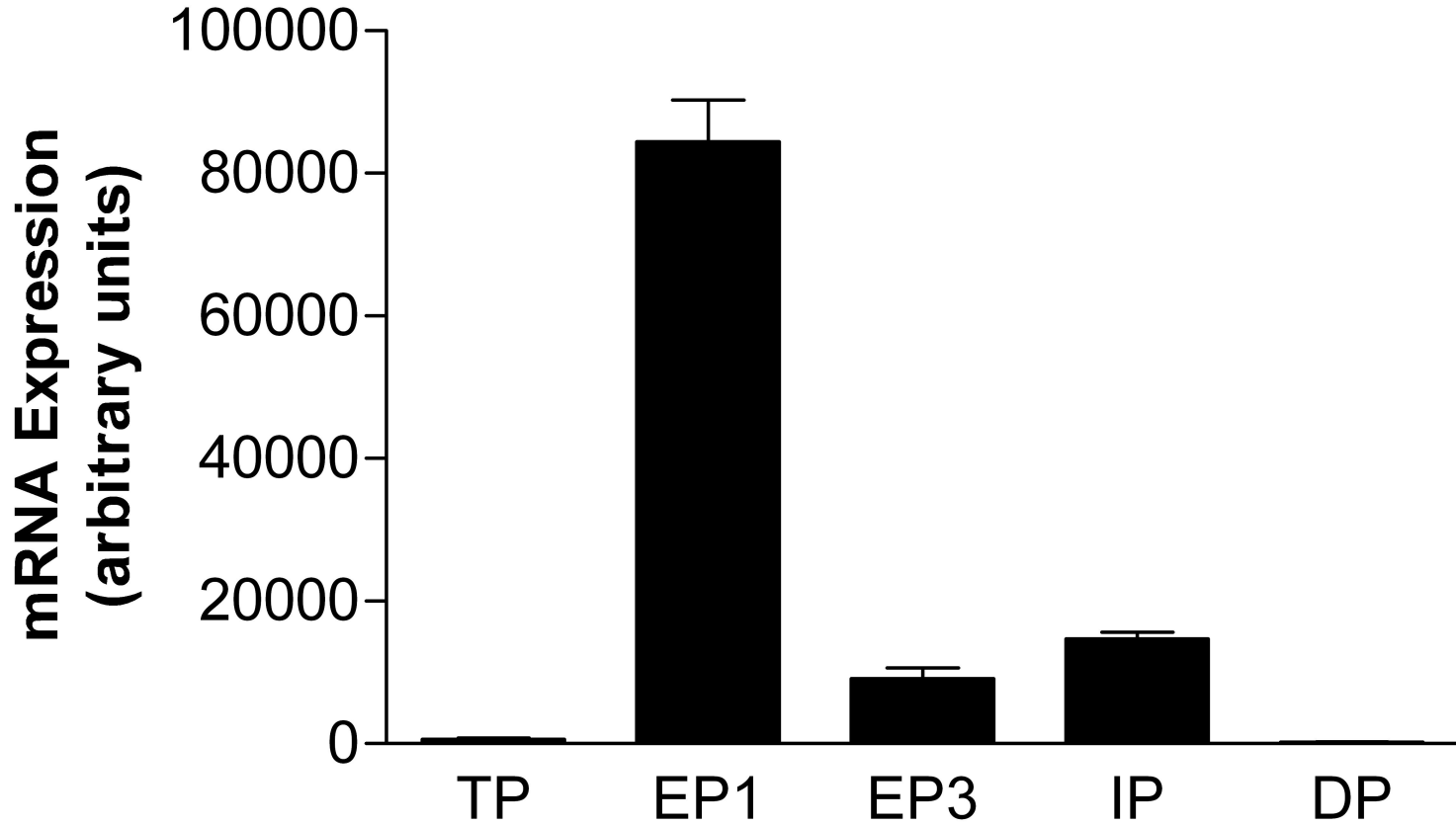


Figure 2

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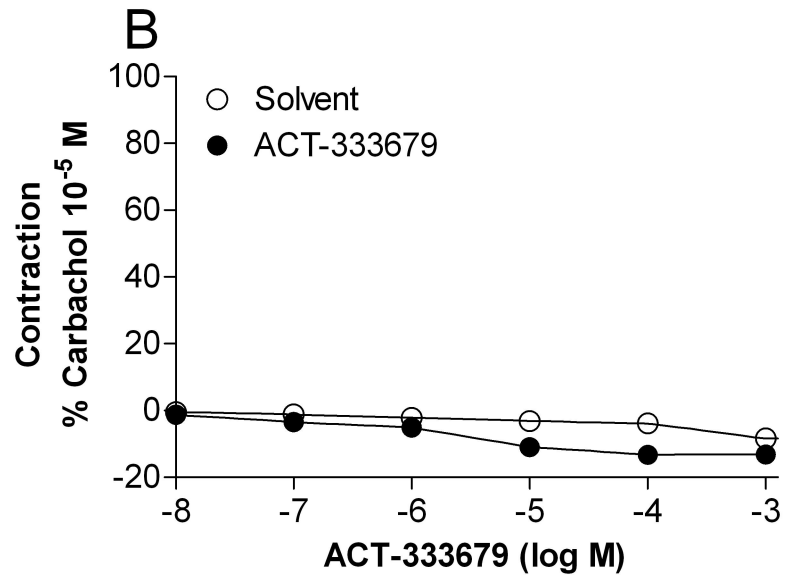
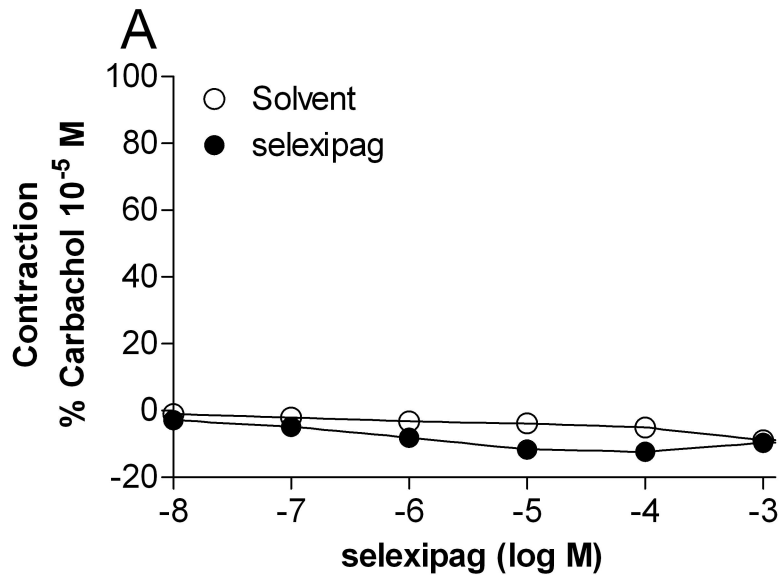
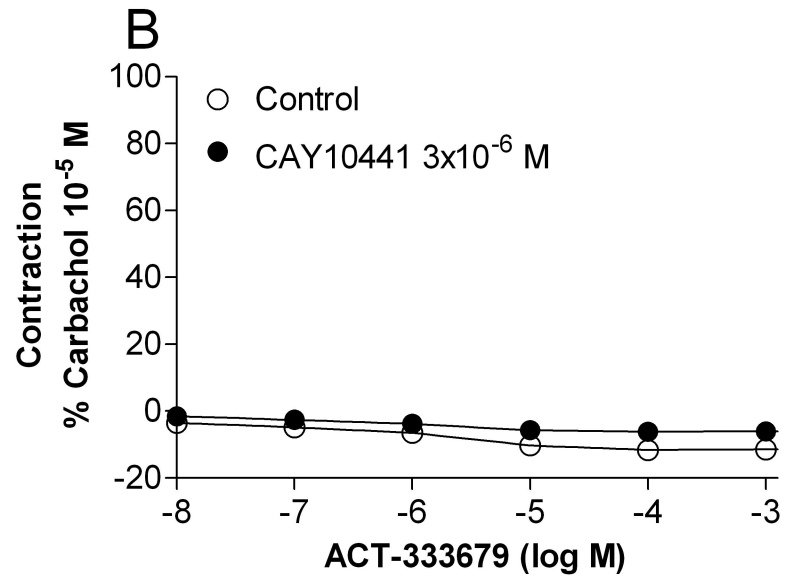
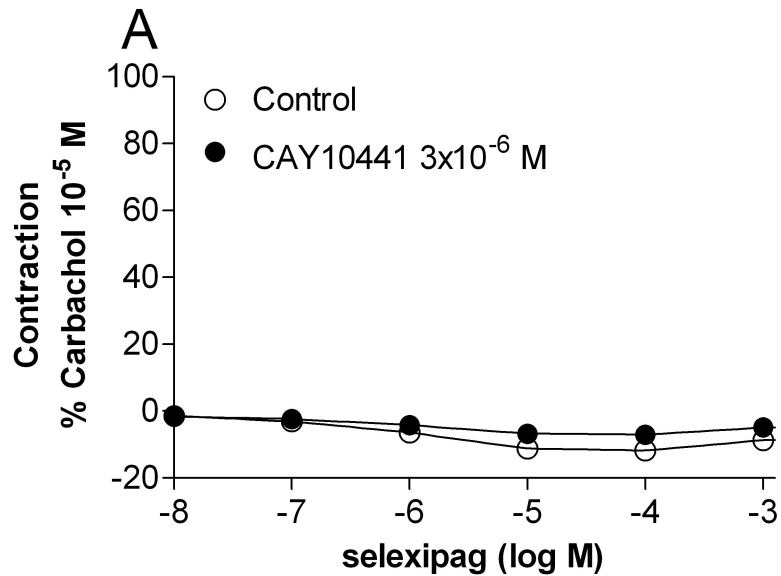
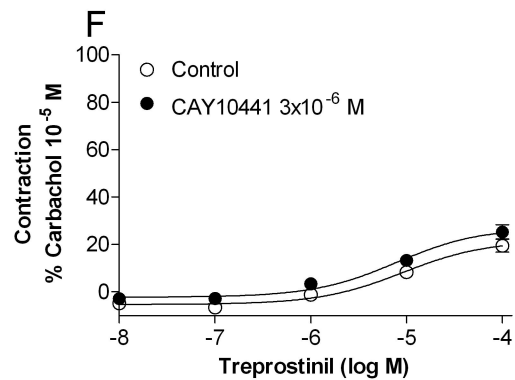
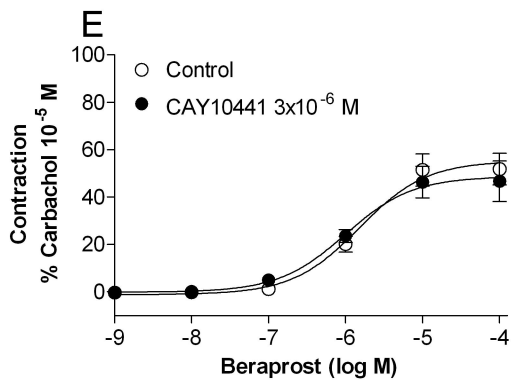
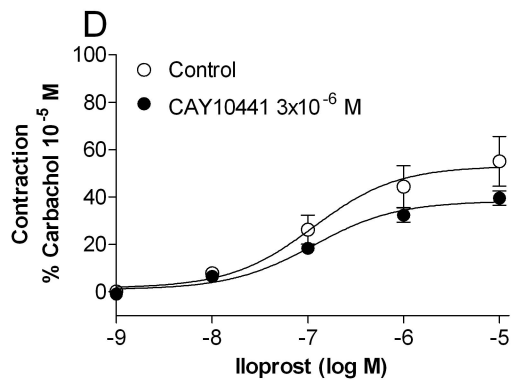
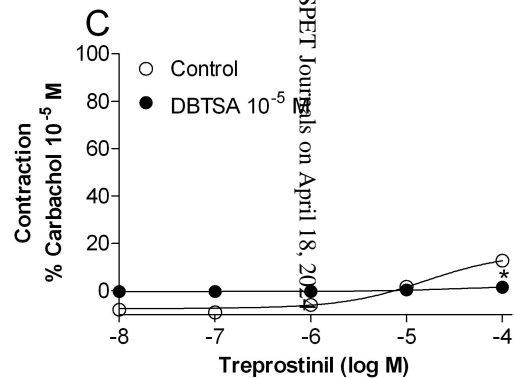
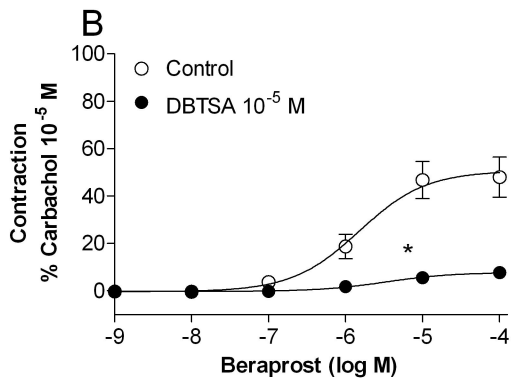
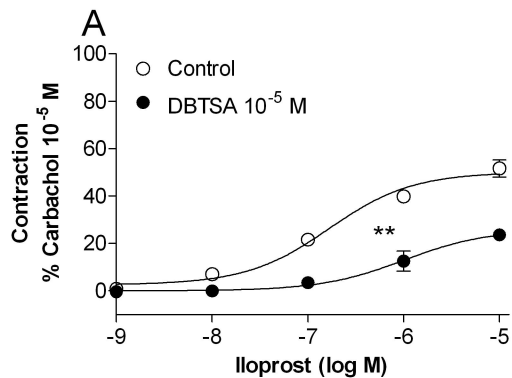


Figure 3

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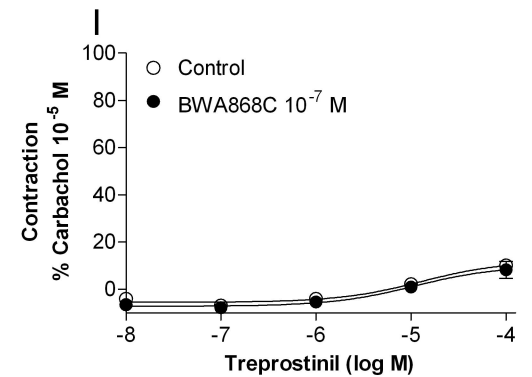
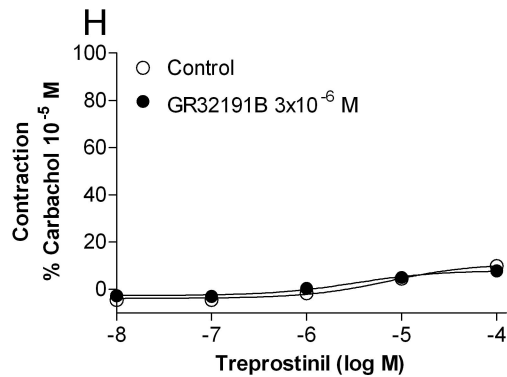
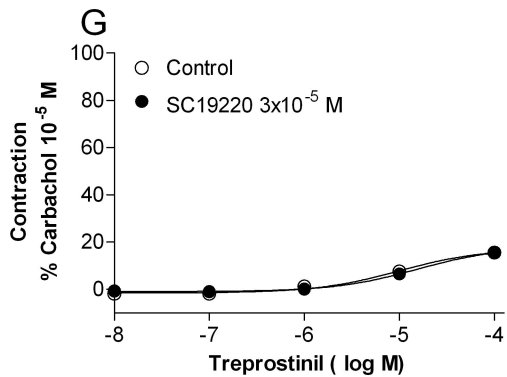
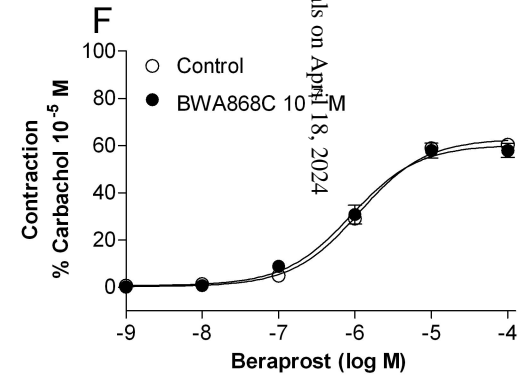
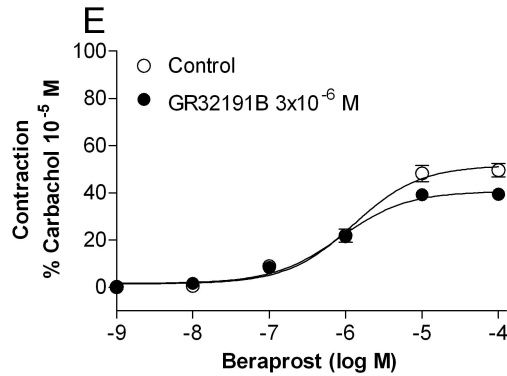
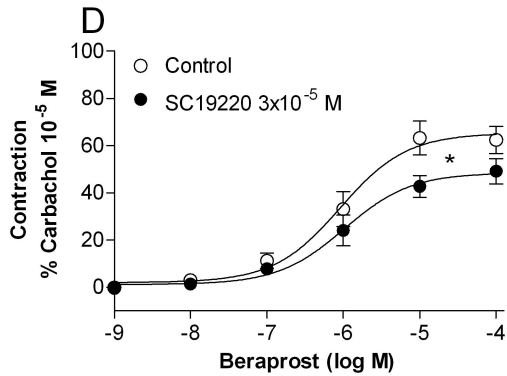
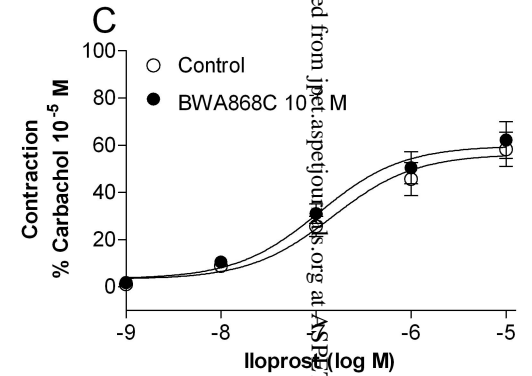
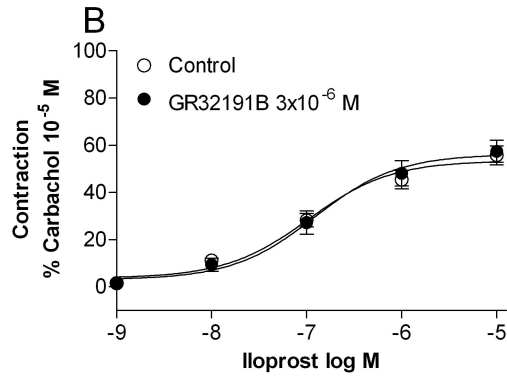
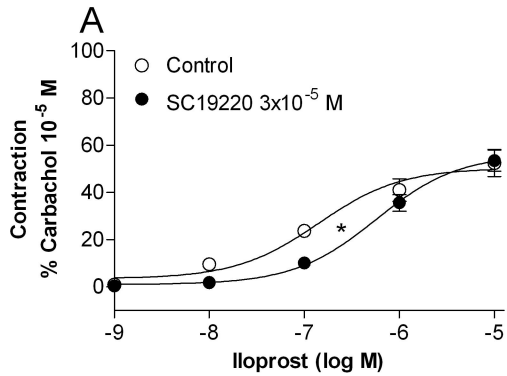


# Figure 4





# Figure 5



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Figure 6

