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GW627368X ((*N*-{2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)phenyl]acetyl} benzene sulphonamide): a novel, potent and selective prostanoid EP_4 receptor antagonist

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1 *N*-{2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)phenyl]acetyl}benzene sulphonamide (GW627368X) is a novel, potent and selective competitive antagonist of prostanoid EP_4 receptors with additional human TP receptor affinity.

2 At recombinant human prostanoid EP_4 receptors expressed in HEK293 cells, GW627368X produced parallel rightward shifts of PGE_2 concentration–effect (*E*/[A]) curves resulting in an affinity (pK_b) estimate of 7.9 ± 0.4 and a Schild slpoe not significantly different from unity. The affinity was independent of the agonist used.

3 In rings of phenylephrine precontracted piglet saphenous vein, GW627368X (30–300 nM) produced parallel rightward displacement of PGE_2 *E*/[A] curves ($pK_b = 9.2 \pm 0.2$; slope = 1).

4 GW627368X appears to bind to human prostanoid TP receptors but not the TP receptors of other species. In human washed platelets, GW627368X (10 μM) produced 100% inhibition of U-46619 (EC_{100})-induced aggregation (approximate pA_2 ~7.0). However, in rings of rabbit and piglet saphenous vein and of guinea-pig aorta GW627368X (10 μM) did not displace U-46619 *E*/[A] curves indicating an affinity of <5.0 for rabbit and guinea-pig prostanoid TP receptors.

5 In functional assays GW627368X is devoid of both agonism and antagonist affinity for prostanoid $CRTH_2$, EP_2, EP_3, IP and FP receptors. At prostanoid EP_1 receptors, GW627368X was an antagonist with a pA_2 of 6.0, and at prostanoid IP receptors the compound increased the maximum effect of iloprost by 55%. At rabbit prostanoid EP_2 receptors the pA_2 of GW627368X was <5.0.

6 In competition radioligand bioassays, GW627368X had affinity for human prostanoid EP_4 and TP receptors ($pK_i = 7.0 \pm 0.2$ ($n = 10$) and 6.8 ($n = 2$), respectively). Affinity for all other human prostanoid receptors was <5.3.

7 GW627368X will be a valuable tool to explore the role of the prostanoid EP_4 receptor in many physiological and pathological settings.

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Abbreviations: BHK, Syrian Hamster Kidney; cAMP, cyclic adenosine monophosphate; CHO, Chinese Hamster Ovary; compound 8, 3-{[(1*S*)-2-(4,5-diphenyl-1,3-oxazol-2-yl)-2-cyclohexen-1-yl]methyl}-*N*-[(phenylmethyl)sulfonyl]benzamide; compound 11, 6-[(*N*~2~-(1-benzofuran-2-ylcarbonyl)-*N*~5~-{[(phenylmethyl)oxy]carbonyl}-L-ornithyl)amino]hexanoic acid; DMEM-F12, Dulbecco's modified Eagle medium-Ham F12 mix; DMSO, dimethyl sulphoxide; *E*/[A], concentration–effect curve; EDTA, ethylenediaminetetra-acetic acid; EP_4A, (4′-[3-butyl-5-oxo-1-(2-trifluoromethylphenyl)-1,5-dihydro-[1,2,4]triazol-4-ylmethyl]-biphenyl-2-sulfonic acid (3-methyl-thiophene-2-carbonyl)-amide; GW627368X, *N*-{2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl) phenyl]acetyl}benzene sulphonamide; HBSS, Hanks'-buffered saline solution; HEK, human embryonic kidney 293 cells; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; HI-FBS, heat-inactivated foetal bovine serum; HMTB, HEPES-modified Tyrode's buffer; IBMX, isobutylmethylxanthine; n.s.b., non-specific binding; ONO-AE2-227, 2-[2-{2-(1-naphthyl)propanoylamino}phenyl]methylbenzoic acid; PE, phenylephrine; PG, prostaglandin; PMSF, phenylmethylsulphonyl fluoride; PRP, platelet-rich plasma; SFV, Semliki Forest Virus; SPA, scintillation proximity assay; WP, washed platelets

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Introduction

Prostanoids are a group of lipid hormone mediators that are derived from C-20 fatty acids by the action of cyclooxygenases 1, 2 and 3. They consist of the prostaglandins (PG) and the thromboxanes and they elicit a wide variety of biological responses through activation of G-protein-coupled receptors. The prostanoid receptor family consists of eight distinct rhodopsin-like receptor proteins each being the product of an individual gene (Coleman *et al*., 1994; Narumiya *et al*., 1999). These have been termed the DP, EP_1, EP_2, EP_3, EP_4, FP, IP and TP receptors. With the identification of PGD_2 as a potent agonist at the chemoattractant receptor homologous molecule of TH2 cells ($CRTH_2$) receptor (Hirai *et al*., 2001), the total number of prostanoid receptor subtypes is now nine.

In most cases, the myriad biological functions stimulated by PGs are transduced by activation of G-proteins. The prostanoid EP_4 receptor falls into a group of receptors normally associated with elevation of intracellular cyclic adenosine monophosphate (cAMP) levels subsequent to G_s activation. However, in common with both IP and TP receptors (Hirata *et al*., 1994; Wise, 1999), we have generated unpublished data that suggests that EP_4 receptors display promiscuous coupling towards both G_s and G_i G-proteins under certain conditions.

In pharmacological terms, EP_4 receptors are most similar to EP_2 prostanoid receptors. In many physiological settings, EP_2 and EP_4 receptors are colocated making identification of individual roles difficult. Recently, several agonists have been described which should help to discriminate between EP_2 and EP_4 receptors. These include selective EP_2 agonists such as ONO AE1-259 (Yamamoto *et al*., 1999), ONO 8815 (Ogawa *et al*., 2000; Tani *et al*., 2000) and CP533,536 (Li *et al*., 2003), and EP_4 agonists such as ONO-AE1-329 (Yamamoto *et al*., 1999), ONO AE1-437 (Sakata *et al*., 2000) and a series of agonists discovered by Merck (Billot *et al*., 2003).

Antagonists are key to definitive classification of receptors but identifying selective EP_4 receptor antagonists has proved difficult. A commonly used EP_4 antagonist, AH23848 (Brittain *et al*., 1985; Coleman *et al*., 1994), actually has highest affinity for TP receptors and does not discriminate between EP_4 and other human and rat prostanoid receptors. This compound has been superceded by other more potent and selective prostanoid EP_4 receptor antagonists such as EP_4A (Machwate *et al*., 2001), ONO-AE2-227 (Mutoh *et al*., 2002), the diphenyloxazole 'compound 8' and the Nδ-Z-ornithine 'compound 11' described by Hattori *et al*. (2005), and the EP_2 agonist/EP_4 antagonist molecules described by Oxford *et al*. (2005).

N-{2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)phenyl]acetyl}benzene sulphonamide (GW627368X; Figure 1), a potent EP_4 receptor antagonist with additional affinity for human TP receptors, has previously been disclosed in poster form (Giblin *et al*., 2002; Wilson *et al*., 2003). Here we fully describe the binding and functional pharmacology of this molecule at recombinant human, and endogenously expressed animal, prostanoid receptors. This work represents the most complete characterisation of a potent and selective antagonist at human prostanoid EP_4 receptors published to date. Indeed, to our knowledge GW627368X is the most selective EP_4 receptor antagonist so far reported.

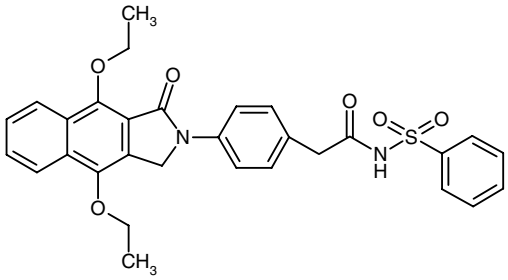

Figure 1 The chemical structure of *N*-{2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)phenyl]acetyl}benzene sulphonamide (GW627368X; Giblin *et al*., 2002).

Methods

Preparation of transiently expressing cell lines

Preparation of EP_1, EP_2, EP_{3I}, EP_4 Semliki Forest Virus (SFV) stocks The coding regions of the human EP_1 (GenBank L22647), EP_2 (GenBank U19487), EP_{3I} (GenBank X83857) and EP_4 (GenBank L25124) receptors were inserted into pSFV using the method described by Marshall *et al*. (1997). pSFV/EP_{1-4} and the pSFV/helper constructs were linearised with *Spe*I, and cleaned using phenol/chloroform/isoamyl alcohol (25 : 24 : 1) followed by two volumes of chloroform/isoamyl alcohol (24 : 1) in phase lock tubes. The DNA was precipitated with $1/10\,v\,v^{-1}$ 3 M sodium acetate (pH 5.2) and two volumes of ethanol, then washed in acidified 70% ethanol before finally being resuspended in Tris/ethylenediaminetetra-acetic acid (EDTA) buffer (10 mM Tris HCl, 5 mM EDTA, pH 7.4) at $1\,mg\,ml^{-1}$. Each DNA sample was checked to ensure that no circular DNA remained. The *in vitro* transcription reactions were performed with the SP6 Message Machine Kit (Ambion) according to the manufacturers instructions. A final GTP concentration of 4 mM was used to avoid [GTP] becoming rate limiting for these long transcripts. Final reaction volumes were all 200 μl. All incubations were for 2 h at 37°C. RNA was aliquoted and stored at −80°C. All reactions yielded sharp single bands when analysed by gel electrophoresis.

BHK cells were cultured in 175 or 500 cm^2 flasks containing Dulbecco's-modified Eagle medium (DMEM)-HAM F12 + 10% FBS + 2 mM Glutamax to 70% onfluence and passaged with Trypsin $0.25\%\,w\,v^{-1}$ + 0.6 mM EDTA. Cells were washed twice in PBS (calcium and magnesium free) and resuspended as a monosuspension in PBS at 1×10^7 cells ml^{-1}. The cell suspension was then kept on ice. RNA stocks were defrosted on ice. Helper RNA and SFV-EP_x RNA were mixed at different test ratios to optimise yields. Each electroporation was completed thus: 10 μl of RNA was added to 490 μl of cell suspension in a 0.2 cm^3 electroporation cuvette on ice and subjected to two pulses of 25 μF (1.5 kV, time constants ~0.75 ms). The resulting cell suspensions from three cuvettes were then transferred into a separate tube containing 50 ml of DMEM-HAM F12 + 10% FBS + 2 mM Glutamax at 37°C and the resulting suspension cultured for 20 h at 27°C in a 175 cm^2 tissue culture flask. The viral stocks were harvested and while still warm, α-chymotrypsin was added to give a final concentration of 500 $\mu g\,ml^{-1}$. After 15 min incubation at

37°C, aprotinin was added to give a final concentration of 500 μg ml^{-1}. The activated SFV stocks were then aliquoted and stored at −80°C.

Infection of CHOK1 cells Cells were cultured at 37°C to 70% confluence in 1800 cm^2 unbreakable roller bottles (0.25 r.p.m.; Cellon) containing DMEM-HAM F12 + 10% FBS + 2 mM Glutamax. Aliquots of activated viral stocks were thawed and diluted 1/4 (v v^{-1}) in medium at 37°C. Spent culture medium was removed from each bottle and 50 ml of virus + medium added before being incubated for 2 h (37°C, 0.5 r.p.m.). Each roller bottle was then supplemented with a further 350 ml of prewarmed medium containing 10^{-6} M indomethacin and incubated for 20 h (33°C, 0.25 r.p.m.). Each roller was then supplemented with a further 200 ml medium + 10^{-6} M indomethacin and incubated for a further 20 h. Cells were harvested as follows: spent cell culture medium was discarded and cells washed with 400 ml PBS before treatment with Hanks'-buffered saline solution (HBSS) + 0.6 mM EDTA (40 ml per roller bottle, 10–20 min, 2 r.p.m.). The resulting cell suspensions from several bottles were pooled together and centrifuged (300 × *g*, 10 min, 4°C), the supernatant discarded, and the cell pellet resuspended in 50 ml cold HBSS + 0.6 mM EDTA for membrane preparation.

Preparation of HEK-DP, FP, IP and TP The coding regions of the hDP gene (GenBank U31098 and U31332), the hFP gene (GenBank L24470), the hIP gene (GenBank L29016) and the hTP gene (GenBank D15056) were cloned into pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) at the *Bam*HI–*Not*I site. HEK293T cells (ECACC, Porton Down, U.K.) were grown to 80% confluence in minimum essential medium alpha + Glutamax supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS) using 500 cm^2 tissue culture-treated triple flasks (Nunc, Roskilde, Denmark). Cell culture was performed at 37°C under a 5% CO_2/air atmosphere. The introduction of cloned pcDNA was achieved using a transfection mixture which was prepared as follows (quantities given are for a single triple flask): 0.8 ml of lipofectamine, and 45 μg of pcDNA were mixed and allowed to stand for 20 min at room temp. The DNA mixture was combined with 9 ml of Optimem and introduced to a culture flask from which medium had been aspirated. Flasks were returned to the incubator for 6 h at the end of which the transfection mixture was removed and 50 ml of normal culture medium reintroduced. Cells were allowed to grow for a further 48 h before being harvested.

Preparation of stable cell lines

CHO-NFAT-EP_1 and FP The coding regions of the hFP (GenBank L24470) and hEP_1 (GenBank L22647) genes were cloned into pCIN3 (Invitrogen, San Diego, CA,. U.S.A.) at the *Eco*RI/*Not*I site and the *Bam*HI fragment, respectively. The vectors were incorporated into CHO cells stably expressing the NFAT-luciferase gene using Lipofectamine®. Cells were grown for 24 h in DMEM-Ham F12 mix (DMEM-F12), supplemented with 10% FBS, 2 mM L-glutamine and 10 μg ml^{-1} puromycin. Subsequent culture was performed using fresh medium that additionally contained 1 mg ml^{-1} neomycin (G418 or geneticin). Individual clones were isolated by dilution cloning and tested for responses to PGE_2 and $PGF_{2\alpha}$. A single clone of each receptor was selected for further study.

CHO-$CRTH_2$, EP_2, EP_{3I} and IP The coding regions of the h$CRTH_2$ gene (GenBank AB008535), the hEP_2 gene (GenBank X83868), the EP_{3I} gene (GenBank X83857) and the hIP gene (GenBank L29016) were cloned into pcDNA3 (Invitrogen) and pCIN3 (hIP) vectors, at the *Bam*HI–*Not*I site. Following the incorporation of plasmids into CHO cells using electroporation, cells were grown for 2 weeks under neomycin selection in Excel 301 medium containing 5% FBS and 400 μg ml^{-1} neomycin. CHO $CRTH_2$ cells were additionally transfected with the chimeric $G\alpha_{16z49}$ G-protein (Mody *et al*., 2000) and were cultured in the presence of 1 mg ml^{-1} neomycin and 400 μg ml^{-1} hygromycin B. Transfection of CHO cells with pCIN3-hIP was achieved using Lipofectamine® and were cultured in DMEM-F12, supplemented with 10% FBS, 2 mM L-glutamine and 400 μg ml^{-1} hygromycin for 24 h before being transferred to fresh medium additionally supplemented with neomycin (1 mg ml^{-1}). Cells were separated using dilution cloning or flow cytometry in order to isolate individual clones in the wells of 96-well plates. Each clone was expanded and pharmacologically characterised. Single clones displaying the largest responses to PGD_2, PGE_2 or to iloprost were selected for further study.

HEK-EP_4 HEK-293 cells expressing the recombinant human EP_4 receptor were obtained from Receptor Biology Inc. (Beltsville, MD, U.S.A.).

Subsequent culture of all cells took place in DMEM-F12, containing 10% HI-FBS and 2 mM L-glutamine in 175 cm^2 flasks. Cells were either passaged into fresh medium or used in an assay once 90% confluency (determined visually) had been achieved.

Membrane preparation

Membranes were prepared from cells grown in 1800 cm^2 roller bottles or 175 cm^2 tissue culture flasks as follows: the cells were harvested into HBSS containing 1 mM EDTA and centrifuged (250 × *g*, 5 min, 4°C). All subsequent steps were performed at 4°C. The cells were homogenised within a glass Waring blender for 2 × 15 s in 200 ml of 50 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES) (pH 7.40) + 10^{-4} M leupeptin + 25 μg ml^{-1} bacitracin + 1 mM EDTA + 1 mM phenylmethylsulphonyl fluoride (PMSF) + 2 μM Pepstatin A, (the latter 2 reagents added as freshly prepared 100 × and 500 × stocks, respectively, in ethanol). The blender was plunged into ice for 5 min after the first burst and for 30 min after the final burst to allow foam to settle. The material was spun at 500 × *g* for 20 min. The supernatant was removed and spun for 36 min at 48,000 × *g*. The resulting pellet was resuspended in the same buffer as above but not containing PMSF and Pepstatin A. The material was then forced through a 0.6 mm gauge needle, made up to the required volume, (usually 4 × the volume of the original cell pellet), and stored as frozen aliquots at −80°C. Protein concentration in membrane samples was determined using the Biorad Protein Assay kit and following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Saturation analysis of radioligand binding

Assessment of binding signal Incremental amounts of membrane were incubated with a fixed concentration of radioligand at or near its assumed K_d for each receptor type. V-bottom 96-well plates (Corning, Koolhovenlaan, The Netherlands) were prepared containing 25 μl [^{3}H]-PGE_2, 25 μl PGE_2 (to determine non-specific binding (n.s.b.)), or 25 μl vehicle (to determine total binding). All reagents were diluted in assay buffer of the following composition: 50 mM HEPES, 10 mM $MgCl_2$, adjusted to pH 7.4 with 1 M KOH(aq). The binding reaction was initiated by the addition of 50 μl of membrane suspension and proceeded for 120–180 min at room temperature. The reaction was terminated by rapid filtration through a pre-soaked 96-well GF/B glass fibre filtermat, which was subsequently dried and treated with Meltilex solid scintillant (Wallac, Turku, Finland). Results were obtained by scintillation counting (1450 Microbeta Trilux liquid scintillation counter, Wallac) using a suitable 1 min [^{3}H] counting protocol in order to generate corrected counts per minute (c.c.p.m.). In each of two experiments, the mean of three data points were used to determine the concentration of membranes giving rise to a specific binding signal of 400–1000 c.c.p.m. on the linear part of the [membrane]/signal relationship.

Determination of radioligand K_d Amounts of membrane giving rise to suitable specific binding signals (see above) were incubated with increasing concentrations of radioligand. The concentration range of each radioligand used varied according to the receptor being studied. A two-fold dilution series ranging from approximately 10-fold below to 10-fold above literature quoted K_d values was used. Binding reactions were conducted as described above in order to generate ccpm data representing total and n.s.b.. Experiments were performed in triplicate twice (except for EP_2 where usable data were only generated in a single experiment).

Competition radioligand-binding studies

The ability of GW627368X to bind to prostanoid receptors was assessed by competitive radioligand displacement using membranes prepared as above and described in Table 2. A combination of filtration and scintillation proximity assay (SPA) formats were used, for which all membranes, beads, compounds and ligands were diluted/suspended in assay buffer of the following composition: 50 mM HEPES, 10 mM $MgCl_2$, adjusted to pH 7.4 with 1 M KOH(aq). Reagents and concentrations suitable for these assays are described in Table 2.

Scintillation proximity assay 96-well SPA plates (Wallac, Turku, Finland) were prepared containing 25 μl of GW627368X, PGE_2, PGD_2, $PGF_{2\alpha}$, iloprost or U-46619 diluted in 0.5 log unit increments, vehicle (to define total binding), and unlabelled displacing ligand for determination of n.s.b. in appropriate wells. The appropriate radioligand was added to all wells (25 μl). The binding reaction was initiated by the addition of 50 μl of a mixture of wheatgerm agglutinin SPA beads (15 mg ml^{-1}) and membrane suspension and allowed to proceed for 120 min at room temperature. Results were obtained by scintillation counting (1450 Microbeta Trilux liquid scintillation counter, Wallac) using a suitable SPA 1 min [^{3}H] counting protocol in order to generate c.c.p.m. Data were generated in three separate experiments.

Filtration-binding assay The low level of EP_1 receptor expression coupled with its relatively low affinity for PGE_2 prevented the use of SPA for this receptor. V-bottom 96-well plates (Corning) were prepared containing GW627368X, PGE_2, [^{3}H]-PGE_2 and vehicle as described above. The binding reaction was initiated by the addition of 50 μl of CHO-EP_1 membranes and then proceeded for 180 min at room temperature. The reaction was terminated by rapid filtration through a 96-well GF/B glass fibre filtermat which was subsequently dried and treated with Meltilex solid scintillant (Wallac). Results were obtained in the manner previously described with the use of a suitable filtermat-counting protocol. Data were generated in three separate experiments.

Cyclic AMP assays

Cells were harvested by treatment with Versene, resuspended in fresh culture medium and plated out to yield approximately 1×10^5 cells per well of a 96-well plate for overnight culture. For assay, the culture medium was replaced with assay medium (CHO-EP_2: DMEM-F12 containing 1 mM L-ascorbate and 300 μM isobutyl-methylxanthine (IBMX); HEK-EP_4: DMEM-F12 containing 300 μM IBMX and 3 μM indomethacin) and incubated for 30 min. Following this, cells were incubated with agonists (CHO-EP_2: three-fold dilution series; HEK-EP_4: four-fold) for 15 min. The reaction was stopped by the aspiration of the assay medium and the addition of ice-cold ethanol. All incubations were carried out at 37°C in a 5% CO_2 atmosphere. Care was taken to ensure the constancy of IBMX, indomethacin and vehicle (dimethyl sulphoxide (DMSO)) concentrations throughout these experiments. The amount of cAMP in each well was then determined by [^{125}-I] cAMP SPA using a proprietary kit (Amersham, Bucks, U.K.) and according to the manufacturer's instructions. Data were generated in duplicate from three separate experiments.

Calcium influx assays

For experiments, cells were removed from flasks with Versene and plated into clear-bottomed, black-walled 96-well plates at ~30,000 cells per well in 200 μl of growth medium. Immediately prior to assay, medium was replaced with 100 μl of assay buffer (145 mM *N*-methyl-D-glucamine, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, 250 μM sulphinpyrazone, 2 mg ml^{-1} BSA, pH 7.4) containing 2 μM Fluo-4 AM and antagonist. Following incubation at 37°C for 90 min plates were washed twice with assay buffer at room temperature, antagonists replaced and plates transferred to a Fluorescence Imaging Plate Reader (FLIPR; Molecular Devices, Sunnywale, CA, U.S.A.) to monitor changes in fluo-4 fluorescence after addition of agonists. Data were generated in duplicate from three separate experiments.

Piglet and rabbit saphenous veins, and guinea-pig aorta

All animals used in this study were handled in accordance with the requirements of the U.K. Home Office, Animals (Scientific Procedures) Act, 1986, and all subsequently issued guidelines.

Large White piglets of either sex (3–6 days old) were humanely killed by captive bolt followed by exsanguination. The saphenous vein was removed from each hind leg of the animal and dissected free of connective and other adhering tissue. Rings of tissue of 5 mm length were suspended between tungsten wire hooks in 5 ml side-arm bubbling tissue baths for isometric force recording containing Krebs solution at 37°C, aerated with 95% O_2 + 5% CO_2. Krebs solution was of the following composition: NaCl 118 mM, $NaHCO_3$ 25 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, glucose 11.1 mM, $CaCl_2$ 1.25 mM and contained indomethacin 3 μM (to block endogenous PG synthesis) and GR32191B 1 μM (to block TP receptors). Tissue was initially collected into Krebs solution additionally supplemented with the monoamine oxidase inhibitor pargyline (500 μM) such that a 30 min exposure to this agent was achieved. Changes in force were detected using FT03C force displacement transducers and recorded digitally on a MacLab data acquisition system running Chart v3.4.2 software (sampling frequency 0.66 Hz; AD Instruments, Hastings, U.K.).

An initial force of $1 \times g$ was applied to each tissue ring for a period of 10 min at the end of which the bathing solution in each bath was replaced. A force of $2 \times g$ was then applied for a period of 30 min followed by exposure to 80 mM KCl to establish the maximum level of force generated by each ring. This concentration of KCl had previously been shown to be maximally effective in these tissues (data not shown). Washout of vasoactive agents was achieved by four exchanges of bathing medium after which basal tone was allowed to re-establish for 10 min prior to the addition of the EP_4 receptor antagonist GW627368X (1 nM–0.3 μM) or vehicle. In order to study the functional effects of prostanoids at relaxant receptors in whole tissues, tone must first be elevated with a suitable spasmogen. This was achieved by the addition of 1 μM phenylephrine (PE) which has previously been shown to represent an EC_{80} concentration of this compound (data not shown). Responses to PE were allowed to stabilise such that an overall antagonist incubation time of 60 min elapsed before the construction of agonist E/[A] curves. In order to maximise the number of experiments performed in tissues from each animal, a single agonist concentration–effect (E/[A]) curve was produced in each ring of tissue by the cumulative addition of compound at 0.5 $\log_{10}$ intervals.

Studies using rabbit saphenous vein and guinea-pig aorta were performed using an analogous procedure. New Zealand White rabbits of either sex (3.5 kg approximately) and Dunkin–Hartley guinea-pigs received a lethal dose of pentobarbital sodium (Euthanal®) prior to tissue excision.

Platelet aggregation

Platelets were obtained from healthy human volunteers using a standard venepuncture technique, in which blood was drawn by syringe through a 19-gauge needle into acid citrate dextrose anticoagulant (8.6:1.4 vv^{-1}). Blood samples were centrifuged at $230 \times g$ for 15 min to obtain platelet-rich plasma (PRP). Washed platelets (WP) were prepared from PRP by centrifugation at $900 \times g$ for 10 min followed by resuspension in HEPES-modified Tyrode's buffer (HMTB; 138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose, pH 7.4) containing 0.05 U ml^{-1} Grade VII apyrase (Sigma, Poole, Dorset, U.K.), 10 U ml^{-1} hirudin (Refludan; Berlex), and 1% platelet-poor plasma. Platelet counts were standardised to 2.5×10^5 platelets μl^{-1} with HMTB. $CaCl_2$ was added to yield a final concentration of 1.25 mM. Platelet aggregation was then performed in a Chrono-Log aggregometer (Chrono-Log Corp., Havertown, PA, U.S.A.) using 250 μl of WP at 37°C with stirring (1000 r.p.m.). GW627368X (0.1, 1 and 10 μM) or DMSO vehicle was incubated with the platelets for 2 min prior to the addition of the prostanoid TP receptor agonist U46619 (Cayman Chemical, Ann Arbor, Michigan, U.S.A.). In each experiment, the concentration of U46619 was titrated so as to be just equal to EC_{100}.

Data analysis

Curve fitting A four-parameter logistic equation of the form:

$$E = \frac{E_m[\mathrm{A}]^{n_H}}{EC_{50}^{n_H} + [A]^{n_H}} \quad (1)$$

was fitted to data from tissue bath studies (grams tension normalised with respect to the PE response; %) and from cAMP assays (pmol cAMP per well determined from the mean of two replicate assay points). Thus, estimates of maximum effect (E_m), curve mid-point (EC_{50}), and Hill slope (n_H) were obtained; other terms in the equation are effect (E) and concentration ([A]).

For data from radioligand competition-binding assays (c.c.p.m.) the following form of the equation was used:

$$B = B_m\left\{1 - \left(\frac{[D]^{n_H}}{IC_{50}^{n_H} + [D]^{n_H}}\right)\right\} + \text{n.s.b.} \quad (2)$$

where B is the binding signal (in c.c.p.m.), B_m is the maximum signal before the addition of unlabelled displacing ligand, [D] is the concentration of the displacing ligand, IC_{50} is the concentration of displacing ligand required to produce a half-maximal reduction in binding signal, n_H is the Hill coefficient, and n.s.b. is the non specific binding level (in c.c.p.m.).

Calculation of affinity estimates – saturation binding The amount of specific radioligand binding to each receptor type was calculated as the difference between total and n.s.b. at each concentration. Three equations were fitted to data:

(1) A hyperbolic plus linear equation fitted to total binding data.

$$\text{c.c.p.m.} = \frac{B_{max} \cdot [B]^{n_H}}{K_d^{n_H} + [B]^{n_H}} + m[B] \quad (4)$$

where c.c.p.m. are corrected counts per minute, B_{max} is the maximum amount of radioligand binding under saturating conditions, [B] is the concentration of radioligand, K_d is the radioligand-binding dissociation constant, n_H is the Hill slope, and m is the slope of the linear n.s.b. relationship.

(2) A linear equation fitted to n.s.b. data and using the value of m to constrain fitting to (4).

$$\text{n.s.b.} = m[B] + c \quad (5)$$

where n.s.b. is non-specific binding, m is the slope of the relationship, [B] is the concentration of radioligand and c is the intercept of the line on the c.c.p.m. axis which should equal background radiation.

(3) A hyperbolic equation fitted to specific binding data.

$$\text{c.c.p.m.} = \frac{B_{\max} \cdot [B]^{n_H}}{K_d^{n_H} + [B]^{n_H}} \quad (6)$$

where terms are as previously defined.

Careful consideration of the values and their associated fitting errors obtained by each fit was made in order to arrive at robust affinity estimates.

Calculation of affinity estimates – competition binding Where the Hill coefficient of a displacement-binding curve was not significantly different from unity, the Cheng & Prusoff (1973) correction was applied to IC_{50} values in order to estimate binding affinity values (pK_i) according to the following equation:

$$pK_i = -\log\left\{\frac{IC_{50}}{1 + \frac{[L]}{K_D}}\right\} \quad (7)$$

where K_D is the dissociation-binding constant, and [L] is the concentration of the radioligand used.

Calculation of affinity estimates – antagonism Constancy of agonist E/[A] curve shape i.p.o. increasing antagonist concentrations was assessed by computerised curve-fitting followed by Student's t-test on asymptotes and slopes. Computed EC_{50} values were fitted to a modification of the Schild equation suitable for nonlinear regression (Lew & Angus, 1985).

$$-\log EC_{50} = -\log([B] + 10^{-pK_b}) - \log c \quad (8)$$

where the constant $-\log c$ is the difference between the agonist control curve EC_{50} and the antagonist pK_b. Single agonist E/[A] curves were generated in each tissue therefore comparisons were made between data generated in different tissues from the same animal in order to obtain a single pK_b estimate. For illustrative purposes, we have presented data graphically as Schild plots (Arunlakshana & Schild, 1959).

Individual estimates of curve parameters and affinity values were obtained from each experiment and then averaged to provide mean data. Quoted values are therefore the mean $\pm$ standard deviation (s.d.) of n separate experiments, each derived from a separate animal, cAMP assay, or competition-binding experiment. As errors around slope estimates are log-normally distributed, slope data is expressed as the geometric mean with 95% confidence intervals.

Materials

Pargyline, indomethacin, PE, PGE_2 (PGE_2), PGD_2, $PGF_{2\alpha}$, U-46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$), ethylinediaminetetra-acetic acid (EDTA), HEPES, PMSF, pepstatin A, leupeptin, bacitracin, Hanks'-buffered saline solution (HBSS), DMEM-Ham F12 mix (DMEM-F12), puromycin, Versene, Krebs' solution 10 × concentrate, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma, Poole, Dorset, U.K. Potassium chloride (KCl), L-ascorbic acid, potassium hydroxide (KOH), magnesium chloride ($MgCl_2$), ethanol and DMSO (all AnalaR grade) were obtained from BDH, Lutterworth, Leics, U.K. Lipofectamine®, FBS, HI-FBS, neomycin, hygromycin and 200 mM L-glutamine were purchased from Gibco-BRL Ltd, Paisley, U.K. Excel 301 medium was obtained from JRH Biosciences, Lenexa, KS, U.S.A. Radiolabelled PGs ([^{3}H]-PGD_2, [^{3}H]-PGE_2, [^{3}H]-$PGF_{2\alpha}$ and [^{3}H]-iloprost trometamol salt), unlabelled iloprost and wheatgerm agglutinin – polyvinyl toluene SPA beads (WGA-PVT SPA beads) were purchased from Amersham, Bucks, U.K., while [^{3}H]-[1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptenoic acid ([^{3}H]-SQ29,548) was purchased from NEN, Hounslow, U.K. GR32191B ([1*R*-[1α(*Z*),2β,3β,5α]]-(+)-7-[5-([1,1′-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptenoic acid hydrochloride salt), and GW627368X were prepared in the Department of Medicinal Chemistry, Glaxo-Wellcome Research and Development, Stevenage, U.K.

Indomethacin, GW627368X and GR32191B were dissolved at 10 mM in DMSO. PGE_2, PGD_2 and $PGF_{2\alpha}$, were dissolved at 10 mM in 100% ethanol and stored at −20°C. Iloprost was supplied as a 1 mM solution in Tris buffer pH 8.0 and was stored in aliquots at −20°C. Radioligands were supplied as ethanolic solutions of differing concentrations which were all stored at −20°C. For tissue bath studies, dilutions of drugs and PE were made freshly on each day of study in Krebs solution containing indomethacin and GR32191B as described above. Diluted compounds were stored in the dark at 4°C for the duration of an experiment. Pargyline was dissolved at 0.5 M in dH_2O and stored at −20°C. Potassium chloride was dissolved at 4 M in Krebs solution and stored at room temperature. For binding and cAMP assays, compounds and radioligands were freshly diluted into assay buffer as described above on each experimental occasion. IBMX was made up at 0.1 M in 0.1 M NaOH and added to DMEM-F12 for use. PMSF and Pepstatin A were used as × 100 and × 500 stocks, respectively, in ethanol.

Results

Characterisation of GW627368X

Competition radioligand binding at human prostanoid receptors Membrane preparations containing a single recombinant human prostanoid receptor were characterised by non-linear curve fitting to saturation-binding data which revealed the presence of a single, saturable population of each receptor type (data for hEP_4 and hTP are shown inset in Figure 2). Estimates of the radioligand dissociation-binding constants (K_d (nM)) and of the level of receptor expression are shown in Table 1. Competition-binding studies using a range of selective agonists and antagonists confirmed that each receptor possessed the expected pharmacology for that receptor type (data not shown).

GW627368X produced concentration-related displacement of radioligand from hEP_4 and hTP receptors with equilibrium dissociation constants (pK_i) values of 7.0 $\pm$ 0.3 ($n = 10$) and 6.8 ($n = 2$), and slope (n_H) values of 0.8 (0.7–1.0) and 1.1, respectively (Table 2 and Figure 2). The maximum level of radioligand displacement generated CCPM values indistinguishable from n.s.b. At all other prostanoid receptors, GW627368X, produced $<50\%$ displacement at 10 μM ($n = 4$).

Effects on human recombinant EP_4-mediated increases in cAMP Basal cAMP production and PGE_2 E/[A] curves at hEP_4 receptors expressed in HEK293 cells were found to be

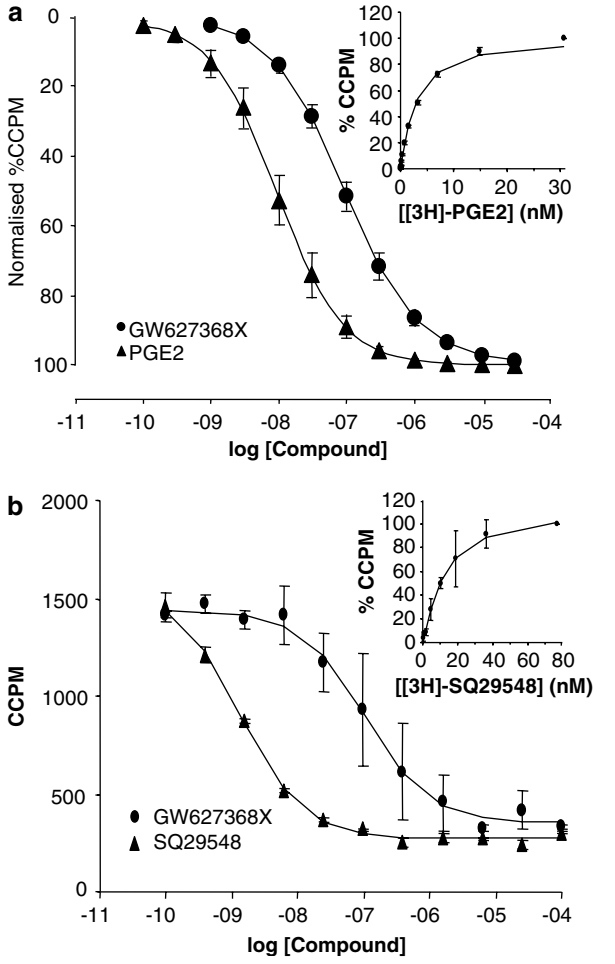

Figure 2 Mean radioligand displacement curves for GW627368X at human prostanoid EP_4 (a) and TP (b) receptors and (inset) saturation curves for the radioligands [^{3}H]-PGE_2 and [^{3}H]-SQ29548, respectively. pK_i values quoted in Table 2 were generated by application of the Cheng–Prusofff correction to IC_{50} values as described in Methods.

altered in the presence of the nonselective COX1/2 inhibitor, indomethacin (3 μM; Figure 3a): curves were left-shifted and steepened (absence of indomethacin: $pEC_{50} = 8.4 \pm 0.2$, $n_H = 0.6 \pm 0.3$; presence of indomethacin (3 μM): $pEC_{50} = 9.1 \pm 0.1$, $n_H = 1.0 \pm 0.3$); basal cAMP production was reduced by 52%.

In this cell line, GW627368X was devoid of agonist activity and actually produced a significant and concentration-related reduction in basal cAMP levels (Figure 3; $pIC_{50} = 6.3 \pm 0.1$, $n_H = 1.9 \pm 0.9$). In the presence of indomethacin (3 μM) GW627368X reduced basal cAMP production to below detectable levels at the lowest concentration tested (0.1 nM). In untransfected HEK293 cells which were not obtained from the same source as the transfected cells, GW627368X (0.1 nM–100 μM) did not produce any alteration in basal cAMP turnover (data not shown). Subsequent experiments in this hEP_4 receptor assay were conducted in the presence of 3 μM indomethacin.

In competition studies against PGE_2 or 11-deoxy-PGE_1 at recombinant human prostanoid EP_4 receptors expressed in HEK293 cells, GW627368X (10 nM–1 μM) produced parallel rightward displacement of PGE_2 E/[A] curves, with no

Table 1 Characteristics of membranes derived from cells transiently expressing prostanoid receptors and of the competition radioligand binding assays using them

	Membranes				*Assay*				
Receptor	*Vector*	B_{max} (pmol mg^{-1})	K_d (nM)	*Protein* (μg per well)	*Assay type*	*Radioligand*	*Specific activity* (TBq mmol^{-1})	*[radioligand]* (nM)	*n.s.b. ligand*	*[n.s.b.]* (μM)
DP	pcDNA3	12.3	11.9	12.0	SPA	[^{3}H]-PGD_2	5.96	1	PGD_2	10
EP_1	SFV-1	3	12	11.3	Filtration	[^{3}H]-PGE_2	6.07	10	PGE_2	100
EP_2	SFV-1	4.3	19	7.8	SPA	[^{3}H]-PGE_2	6.07	10	PGE_2	100
EP_{3I}	SFV-1	30	6	2.3	SPA	[^{3}H]-PGE_2	6.07	3	PGE_2	100
EP_4	SFV-1	1.1–8.8	3	0.7–2.4	SPA	[^{3}H]-PGE_2	6.07	3	PGE_2	100
FP	pcDNA3	12	8.2	18.0	SPA	[^{3}H]-$PGF_{2\alpha}$	7.88	2.1	$PGF_{2\alpha}$	10
IP	pcDNA3	6.5	13	15.0	SPA	[^{3}H]-iloprost	0.63	15	iloprost	1
TP	pcDNA3	6.4	7	15.0	SPA	[^{3}H]-SQ29,548	1.80	6	U-46619	1

B_{max}: estimated receptor expression (pmol mg^{-1}); K_d: the radioligand dissociation-binding constant (affinity) determined by saturation analysis (nM); protein: amount of protein per well for each assay (μg well^{-1}); [radioligand]: concentration of radioligand (nM); n.s.b. ligand: the nonradiolabelled ligand used to displace radioligand from the receptors and therefore determine nonspecific binding; [n.s.b.]: concentration of n.s.b. ligand (μM).

Table 2 The binding properties of GW627368X at human prostanoid receptors

		Reference compound				*GW627368X*			
		pK_i	n_H	*Max displacement* (%)	n	pK_i	n_H	*Max displacement* (%)	n
DP	PGD_2	7.8±0.3	0.8±0.1	100	19	<5.0			2
EP_1	PGE_2	7.3±0.3	0.9±0.3	100	49	<5.1			2
EP_2	PGE_2	7.7±0.3	1.0±0.4	100	58	<5.1			2
EP_3	PGE_2	8.2±0.2	1.1±0.2	100	69	<5.1			2
EP_4	PGE_2	8.1±0.3	1.0±0.4	100	168	7.0±0.3	0.9±0.2	~100	10
FP	$PGF_{2\alpha}$	7.9±0.3	1.0±0.2	100	18	<5.1			2
IP	Iloprost	7.5±0.3	1.2±0.3	100	28	<5.3			2
TP	SQ29,548	8.9	0.9	100	2	6.8	1.1	~100	2

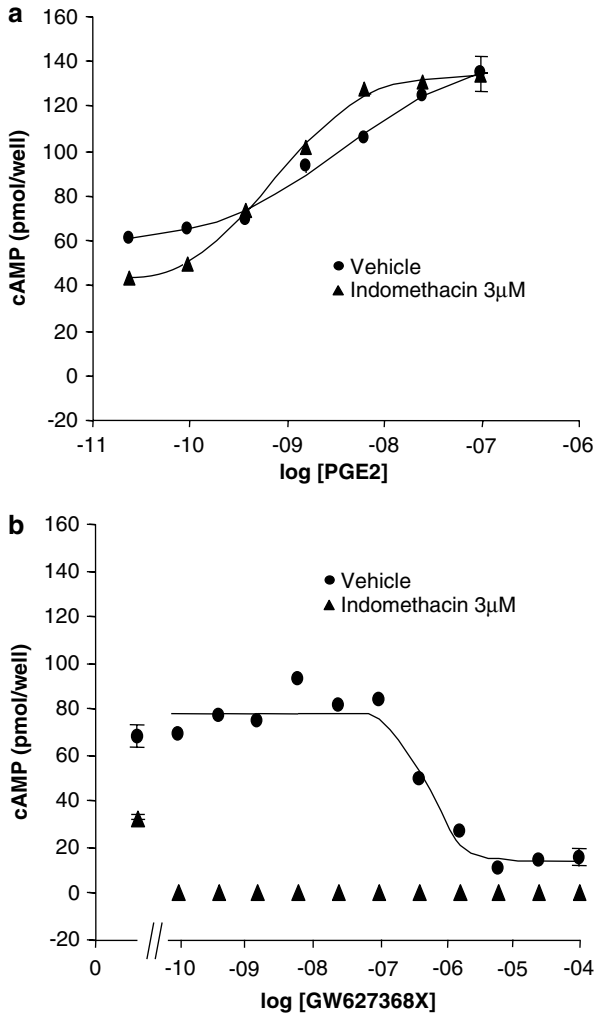

Figure 3 Mean PGE_2 (a) and GW627368X (b) concentration–effect (E/[A]) curves in HEK293 cells expressing recombinant human prostanoid EP_4 receptors in the absence and presence of 3 μM indomethacin.

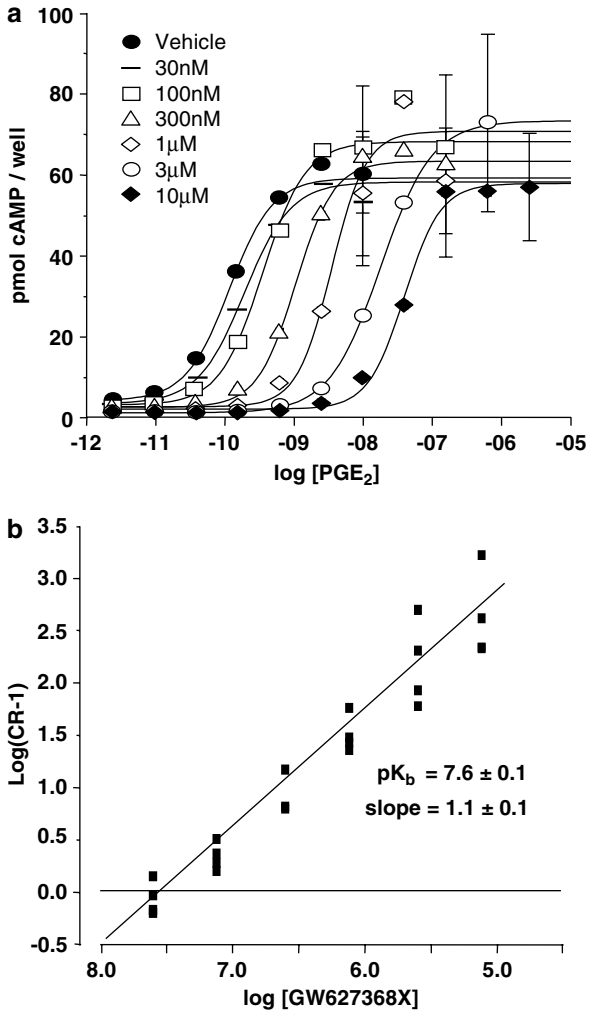

Figure 4 Antagonism of PGE_2 concentration–effect (E/[A]) curves in HEK293 cells expressing recombinant human prostanoid EP_4 receptors by GW627368X. (a) Mean PGE_2 E/[A] curves in the presence of vehicle, and GW627368X 30, 100, 300 nM, 1, 3 and 10 μM $n=3$. (b) Schild plot using data displayed in (a). pK_b and slope values quoted were generated by nonlinear regression as described in Methods.

significant change in Hill slope parameter or maximum effect (Figure 4). Using a modified method of Schild analysis (Lew & Angus, 1985) with PGE_2 as agonist, a pK_b estimate of 7.9±0.4 was generated with a regression slope of 1.2 (0.3–4.5; $n=4$). The requirements of simple competitive antagonism were not violated. In a separate study where the affinity of GW627368X was measured using both PGE_2 and 11-deoxy-PGE_1 as agonists, similar pK_b estimates were obtained (data not shown).

Functional assays at other human recombinant prostanoid receptors Functional assays in CHO cells were developed for $hCRTH_2$, hEP_1, hEP_2, hEP_3, hFP and hIP receptors. Mock-transfected CHO cells were devoid of responses to PGD_2, PGE_2 and $PGF_{2\alpha}$ up to 10 μM agonist concentration, and to iloprost up to 1 μM agonist concentration. The natural

Table 3 The properties of GW627368X in functional assays at human recombinant prostanoid receptors ($n=3$–9)

Recombinant systems	*$CRTH_2$*	*EP_1*	*EP_2*	*EP_{3I}*	*EP_4*	*FP*	*IP*
GW627368X $pA_2/pIC_{50}^1/pK_b^2$	$<5.4^1$	6.0 (n = 2)	<5.0	$<5.0^1$	7.9 ± 0.2^2	<4.5	5.6 ± 0.8
GW627368X max effect as % standard	—	0	43 ± 9	—	-5 ± 1.8	0	5 ± 2
Standard agonist pEC_{50} ipo GW627368X	—	7.1 ± 0.2	7.4 ± 0.3	—	7.3 ± 0.3	7.1 ± 0.3	8.9 ± 0.7
Standard agonist max effect i.p.o. GW627368X as % control (=100)	—	83 ± 21	95 ± 32	—	103 ± 25	46 ± 9	155 ± 53
% Inhibition vs standard agonist EC_{80}	12	—	—	37	—	—	—
Cell type	CHO	CHO	CHO	CHO	HEK	CHO	CHO
Readout	Calcium flux (FLIPR)	CRE-Luc reporter	cAMP	Calcium flux (FLIPR)	cAMP	Calcium flux (FLIPR)	cAMP
Antagonist concentration (μM)	10	10	10	10	10	30	10
Standard agonist	PGD_2	PGE_2	PGE_2	PGE_2	PGE_2	$PGF_{2\alpha}$	Iloprost
Standard agonist pEC_{50}	7.6 ± 0.1	8.7 ± 0.2	7.5 ± 0.3	8.4 ± 0.2	10.3 ± 0.1	7.2 ± 0.3	9.8 ± 0.2

ipo: in the presence of.
Data are mean ± s.d.

agonists produced monophasic *E*/[A] curves at each receptor with Hill slopes in the range 0.7–1.6. Agonist potencies are shown in Table 3.

GW627368X was devoid of agonist action at hEP_1, hEP_2, hFP & hIP receptors (Table 3). Furthermore, as an antagonist of the natural agonist at $CRTH_2$, EP_2 and EP_3 receptors the compound produced no measurable effect at concentrations up to 10 μM. At prostanoid EP_1 and IP receptors 10 μM compound produced rightward agonist *E*/[A] curve shifts resulting in pA_2 estimates of 6.0 ($n=2$) and 5.6 ± 0.8 ($n=3$), respectively. At prostanoid FP receptors, the compound produced no shift of $PGF_{2\alpha}$ *E*/[A] curves but did depress the agonist curve asymptote by 54% at 30 μM.

Effects in piglet and rabbit saphenous veins, and in guinea-pig aorta We have described the prostanoid receptor profile of piglet saphenous vein in a previous publication (Wilson & Giles, 2005). Briefly, relaxatory responses to PGE_2 in this tissue are mediated predominantly by prostanoid EP_4 receptors but, at high concentrations of PGE_2, a significant population of prostanoid EP_2 receptors can be detected. When precontracted with PE (1 μM) PGE_2 produced well-sustained concentration-related relaxations which resulted in monophasic *E*/[A] curves ($pEC_{50}=8.7\pm0.5$; $n_H=2.0$ (0.9–4.2); $\alpha=104.1\pm2.7\%$; Figure 5).

In this EP_4 receptor assay, GW627368X did not produce any change in basal or in PE-elevated tone (Table 4). However, occasional 'spikes' of contraction were observed in 36% of antagonist-treated tissues (Wilson & Giles, 2005). In keeping with the requirements of simple competitive antagonism, parallel rightward displacement of PGE_2 *E*/[A] curves was achieved with GW627368X (1–300 nM) yielding a pK_b estimate of 9.2 ± 0.2 with a slope of 0.8 ± 0.3 (Figure 5, previously published in Wilson & Giles, 2005). No further rightward shift was obtained at concentrations of GW627368X above 300 nM, presumably due to activation of the EP_2 receptor by PGE_2.

In the same tissue, the TP receptor agonist U-46619 produced concentration-related elevations of tissue tone resulting in monophasic *E*/[A] curves ($pEC_{50}=7.0\pm0.5$; $n_H=3.6$ (1.7–7.4), $\alpha=134\pm7$). GW627368X (10 μM) did not alter U-46619 curve potency or maximum effect.

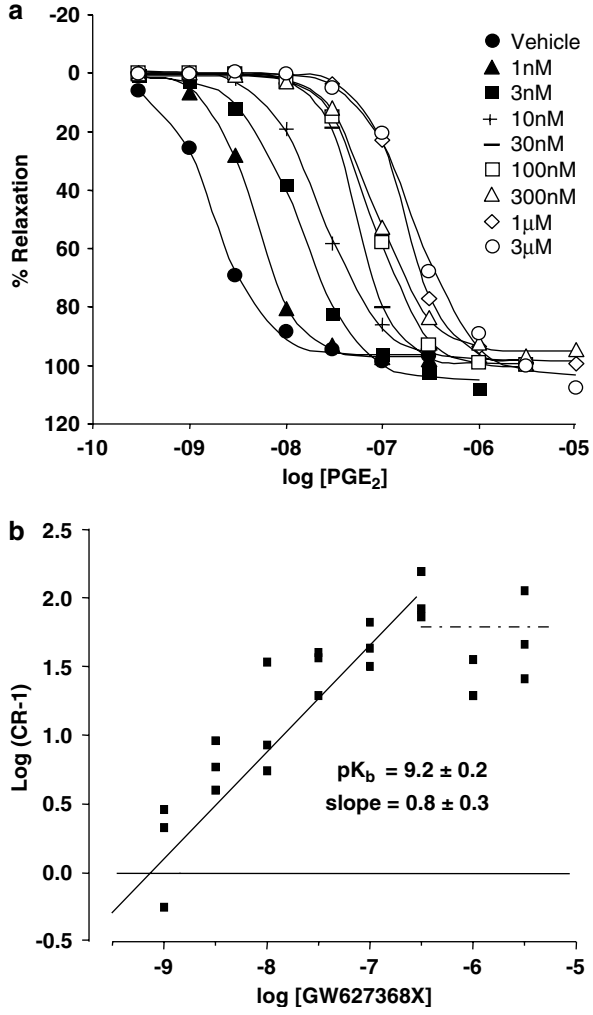

Figure 5 Antagonism of PGE_2 concentration–effect (*E*/[A]) curves in piglet saphenous vein by GW627368X. (a) Mean PGE_2 *E*/[A] curves in the presence of vehicle, and GW627368X 1, 3, 10, 30, 100, 300 nM, 1 and 3 μM ($n=3$). Tissues were incubated with antagonist for 60 min prior to exposure to PGE_2 as described in Methods. (b) Schild plot using data displayed in (a). pK_b and slope values quoted were generated by nonlinear regression as described in Methods. Data have been published previously in Wilson & Giles (2005).

Table 4 The properties of GW627368X in functional isolated tissue bioassays at prostanoid receptors ($n=3$)

Receptor	*EP_2*	*EP_4*	*TP*
Agonist	*PGE_2*	*PGE_2*	*U46619*
Rabbit saphenous vein	<5.0	—	<5.0
Piglet saphenous vein	—	9.2 ± 0.1[a]	<5.0
Guinea-pig aorta	—	—	<5.0
Washed human platelets	—	—	0% inhibition at 1 μM 100% inhibition at 10 μM

Activity at prostanoid EP_4 receptors in piglet saphenous vein has been previously published (Wilson & Giles, 2005) but is included here for completeness. Values are pA_2 except[a] $=pK_b$.

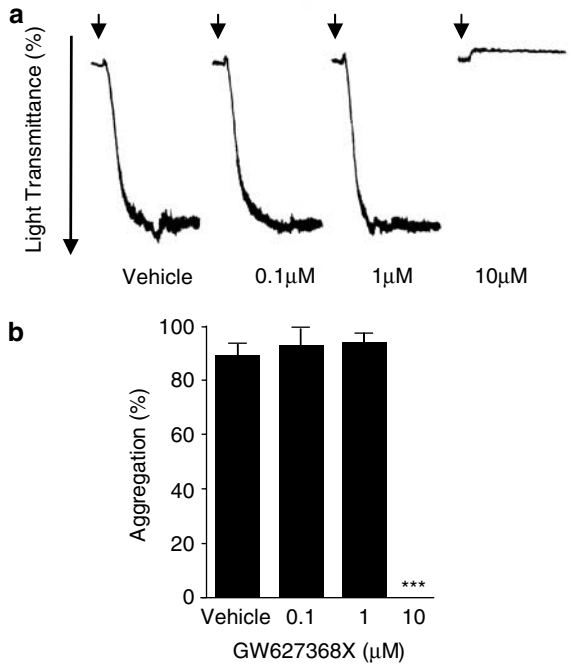

Figure 6 (a) Representative platelet aggregometry tracings showing the effect of GW627368X (0.1–10 μM) on human platelet aggregation induced by the prostanoid TP receptor agonist U-46619 (EC_{100}). Arrows indicate the time of U-46619 addition. Similar results were obtained with three donors. (b) Aggregometry data expressed as mean $\pm$ s.e.m. and analysed by one-way ANOVA with subsequent *post hoc* comparison (Bonferroni); *** $P<0.005$.

In rabbit saphenous vein the most potent response to PGE_2 is *via* the EP_2 receptor (Lydford *et al*., 1996). In PE (1 μM) precontracted rings of rabbit saphenous vein PGE_2 produced well-sustained concentration-related relaxations which resulted in monophasic *E*/[A] curves ($pEC_{50}=8.99\pm0.2$; $n_H=1.1$ (0.8–1.4); $\alpha=102\pm3$). GW627368X (10 μM) did not alter the PGE_2 curve potency or maximum effect, nor did it produce any effect on PE induced tissue contraction. Similarly, the compound was devoid of activity vs U46619 induced contraction (TP receptor assay) in this tissue and also in guinea-pig aorta. Data are summarised in Table 4.

TP-mediated human platelet aggregation GW627368X did produce antagonism of U-46619 induced washed human platelet aggregation (Table 4 and Figure 6). The antagonism was of an all-or-nothing nature, being 0% inhibition at 1 μM compound and 100% at 10 μM compound vs an EC_{100} of agonist (100–300 nM).

Discussion

We have demonstrated that GW627368X is a potent and selective antagonist of human and porcine prostanoid EP_4 receptors with additional affinity for human, but not porcine, prostanoid TP receptors. Furthermore, we have shown that it is possible to clone and express the nine established human prostanoid receptors in assay formats commensurate with the requirements of modern medium throughput, automated drug discovery. It is not our intention to provide a review of the binding and functional pharmacology of each receptor subtype we have used since this has been described extensively in the literature (for reviews see Coleman *et al*., 1994; Narumiya *et al*., 1999; Tsuboi *et al*., 2002). However, certain aspects of the characterisation of our assay systems do require discussion.

A wide body of literature has described the affinity of tritiated radioligands for prostanoid receptors and the K_d estimates we have generated by saturation binding agree well with those already published except for the prostanoid EP_3 and DP receptor subtypes. At least eight human splice variants of prostanoid EP_3 receptors exist (Kotani *et al*., 1995). We have cloned and expressed the EP_{3I} (EP_{3A}) variant to which [^{3}H]-PGE_2 bound with an affinity two- to 10-fold lower than values previously reported (6 nM, present study vs 0.6–2.6 nM (Yang *et al*., 1994; Abramovitz *et al*., 1995, respectively)). Pharmacological characterisation of our prostanoid EP_{3I} clone produced an identical rank order of compound affinities to that previously reported by Adam *et al*. (1994) and Abramovitz *et al*. (2000) at the prostanoid EP_{3C} receptor (data not shown). The reason for the difference in PGE_2-binding affinity is therefore unclear. The most obvious difference between our methodology and that employed by other authors is our choice of the SFV-1/EP_{3I} and CHO cell expression system for this receptor. SFV expression systems are known to generate large amounts of the target protein and to decrease synthesis of normal cellular constituents resulting in marked cytotoxicity (Lundstrom, 2002). Therefore, we may have unintentionally reduced synthesis of accessory proteins needed for high affinity binding of agonist radioligands to receptors. Alternatively, the lower PGE_2 affinity we observed may have reflected an aspect of our assay methodology. For example, other authors have used alternative buffer systems, often at lower pH. The method we present here was optimised to generate the highest agonist affinity that we could achieve. In our hands there was little

difference in PGE_2-binding affinity when buffers of pH 7.4 and 6.0 were used and, arguably, data generated at pH 7.4 is more relevant to physiological situations.

Similarly, in contrast to the findings of Wright *et al*. (1998), [^{3}H]-PGD_2 bound to a single population of prostanoid DP receptors, with a K_d of 11.9 nM and a B_{max} estimate of 12.3 pmol mg^{-1} protein. In the earlier study, biphasic radioligand binding resulted in the identification of high ($K_d = 0.5$ nM, $B_{max} = 0.3$ pmol mg^{-1} protein) and low ($K_d = 5.9$ nM, $B_{max} = 13.4$ pmol mg^{-1}) affinity receptor states. Our data are most consistent with detection of the low affinity receptor state reported by Wright *et al*. but does not agree with the findings of Boie *et al*. (1995) who reported binding to a single receptor population with K_d of 1.5 nM and a B_{max} of 1.2 pmol mg^{-1} protein. Again, this disagreement may relate to the amount of receptor protein expressed since no alteration in the sequence of our receptor clone could be detected.

It is interesting to note that the affinity of GW627368X at prostanoid hEP_4 receptors measured in a radioligand-binding assay (7.0 ± 0.3) is almost 10-fold lower than its antagonist affinity for the same receptor type determined in the functional cAMP assay (7.9). According to the two-state model of receptor behaviour (Leff, 1996), receptors are predicted to adopt one of two macroscopic conformations depending upon their activation state: activated or R* receptors (high agonist affinity) and inactivated or R receptors (low affinity for agonists). A dynamic equilibrium should exist between R and R* receptor states which gives rise to complex agonist-binding curves. We observed monophasic saturation binding of the radioligand to the human prostanoid EP_4 receptors, and monophasic competition-binding curves with both unlabelled agonist (PGE_2) and antagonist (GW627368X) in competition-binding experiments, in each case with slope values close to unity. Therefore, we assume that the experimental conditions we have employed have resulted in the labelling of receptors predominantly in a single affinity state. The low PGE_2-binding affinity and lack of binding curve complexity we observed is suggestive of R state receptors: displacement by GW627368X should therefore have generated a pK_i value in closer agreement with the functional pK_b, also predicted to be the result of interaction with R state receptors. The explanation for the difference we observed is unclear, but we speculate that it may be due to the difference in the microenvironment of the receptor in our membrane-binding assays compared with a whole cell or tissue setting. In competition-binding studies, GW627368X does not discriminate between prostanoid EP_4 and TP receptors having pK_i values of 7.0 and 6.9, respectively. Radioligand displacement curves at these receptors had unit slopes and therefore conformed with the expectations of competition for a single binding site. Curve slopes such as these can also be generated by certain specialised forms of allosteric interaction (Birdsall *et al*., 1995; Lazareno & Birdsall, 1995). However, in this case, allosterism is unlikely to be occurring because functional data at prostanoid EP_4 receptors (see below) is also consistent with simple competition. At other prostanoid receptors, GW627368X (10 μM) produced little or no radioligand displacement and is therefore about 75-fold selective over these receptors.

In functional assays, the potency of PGD_2 at prostanoid $hCRTH_2$ receptors, of PGE_2 at hEP_1, hEP_2 hEP_3 and hEP_4 receptors, of $PGF_{2\alpha}$ at FP receptors and of iloprost at IP receptors was in general agreement with published potency figures for these agonists. In most cases, the true level of receptor expression is unknown and so it is impossible to compare the ability of our functional cell lines to transduce agonist-binding signals into effect in a more meaningful manner. However, agonist rank orders of potency, and antagonist affinities, where available, were appropriate for each receptor subtype.

Competition analysis of GW627368X vs PGE_2 in HEK cells expressing human prostanoid EP_4 receptors and in rings of PSV *in vitro* have demonstrated that GW627368X at concentrations up to 300 nM is a competitive antagonist of prostanoid EP_4 receptors. The affinity of GW627368X for human recombinant prostanoid EP_4 receptors was at least 10-fold less than its affinity for the porcine prostanoid EP_4 receptor. This is likely to be a reflection of interspecies differences in the molecular structure of the human and porcine receptors. However, we have been unable to find published sequence information for the porcine prostanoid EP_4 receptor so we are unable to comment on specific amino-acid residue differences that may underlie the observed difference in affinity. Clearly, though, these data highlight specific areas of pharmacological behaviour that differ between human and porcine prostanoid EP_4 receptors. This may be of particular importance in models relevant to the treatment of human heart conditions (for review see Hughes *et al*., 2003) where extensive use of porcine tissues is made. At concentrations of GW627368X of 1 mM and above, no further shift of PGE_2 E/[A] curves was achieved resulting in dose ratios of zero. This complexity has been discussed by us in an earlier publication (Wilson & Giles, 2005) and is due to the presence of a prostanoid EP_2 receptor population in this tissue, a finding confirmed by Jones & Chan (2005). Agonist independence of antagonist potency was demonstrated using 11-deoxy-PGE_1 as agonist: in this study, identical pK_b's were obtained irrespective of the agonist used, thus confirming the competitive nature of the interaction of GW627368X with prostanoid EP_4 receptors.

The observed concentration-related decreases in basal cAMP generation produced in hEP_4 HEK cells by GW627368X deserve some consideration. Responses such as these may have arisen through one (or more) of several mechanisms: (1) action at a cellular target unrelated to receptors but resulting in reduced cAMP production, for example inhibition of adenylate cyclase; (2) agonism at receptors coupled through $G\alpha_i$ to inhibition of adenylate cyclase (functional antagonism); (3) antagonism of endogenously synthesised PGE_2 acting at prostanoid EP_4 receptors or (4) inverse agonism at prostanoid EP_4 receptors. Non receptor-mediated activity in the molecule would be expected to reveal itself as complexity in the analyses of competition. Since our Schild plots, generated in two very different assay systems, were linear and revealed no such complexity, this seems an unlikely explanation. Similarly, our characterisation data show that GW627368X is devoid of agonist activity at $G\alpha_i$- and $G\alpha_q$-coupled prostanoid receptors, and essentially devoid of antagonist activity at other $G\alpha_s$ coupled prostanoid receptors (the pA_2 of 5.6 at prostanoid IP receptors we regard as an artefact of the increased iloprost maximum effect in the presence of GW627368X). While this does not rule out an effect at an endogenously expressed non prostanoid receptor, we can at least say that an action *via* an endogenous prostanoid receptor seems unlikely. Furthermore, the absence

of responses on untransfected HEK cells suggests that the decreases in basal cAMP are linked to the expression of recombinant human prostanoid EP_4 receptors. Interestingly, we observed that the COX1/2 inhibitor, indomethacin markedly altered the behaviour of both PGE_2 and GW627368X in hEP_4 HEK cells. The concentration of indomethacin we used (3 μM) was around 1000- and 500-fold greater than its IC_{50} at COX-1 and COX-2, respectively, measured in human cell-based functional assays (Palomer *et al*., 2002) and we would expect to achieve near total inhibition of COX1/2 enzyme activity under these conditions. Thus, we have shown that HEK cells synthesise significant amounts of PGE_2 and it therefore follows that the reductions in basal cAMP seen with GW627368X treatment are largely due to inhibition of endogenously synthesised PGE_2 activity. However, even in the presence of indomethacin, we still observed small decreases in basal cAMP generation in response to GW627368X suggesting that the antagonist is able to inhibit cAMP turnover by another mechanism. It is possible that more effective inhibition at all COX enzyme subtypes might totally abolish the effects of GW627368X on basal cAMP turnover but given the level of enzyme occupancy we achieved this seems unlikely. Basal effects of GW627368X were also observed in PSV treated with indomethacin. No obviously compound-related changes in basal tone were observed but spontaneous 'spikes' of contractile activity were observed in 18/54 tissue rings treated with GW627368X possibly indicating the removal of prorelaxatory tone by the antagonist and suggestive of basal prostanoid EP_4 receptor activation. Therefore, though our data does not prove that GW627368X is an inverse agonist at human prostanoid EP_4 receptors, we cannot rule out this possibility.

The compound also produced considerable antagonism of human platelet aggregation induced with the TP receptor agonist, U-46619, with no direct stimulation of aggregation in its own right. U-46619 produces characteristically steep E/[A] curves in platelet aggregation studies, and thus the 'on/off' nature of the antagonism observed is not unexpected given the antagonist concentrations used. The concentration of agonist selected, an EC_{100}, would be expected to shift the antagonist pIC_{50} by c.0.95 log units relative to its true affinity. Assuming a unit agonist E/[A] curve slope and applying a modified Cheng–Prusoff correction to the binding pK_i of 6.9, the approximate IC_{50} for GW627368X of between 6.0 and 5.0 is commensurate with an action at the prostanoid TP receptor. However, affinity for TP receptors appears to be limited only to human receptors since in functional assays using isolated animal tissues, GW627368X was devoid of activity at rabbit, piglet and guinea-pig TP receptors.

In functional assays we have shown that GW627368X is devoid of both agonism and antagonist affinity for prostanoid $CRTH_2$, EP_2, EP_3, IP and FP receptors. At prostanoid EP_1 receptors, GW627368X was an antagonist with a pA_2 of 6.0, and at prostanoid IP receptors the compound increased the maximum effect of iloprost by 55%. The latter effect does not seem related to direct receptor activation since GW627368X had no effect on cAMP levels in the absence of iloprost. Similarly, an effect on adenylate cyclase or on phosphodiesterase enzymes seems unlikely since complexities of behaviour were not observed either by Schild analysis or in the prostanoid EP_2 receptor assay, also performed in CHO cells. Furthermore, high selectivity over rabbit, guinea-pig and piglet prostanoid IP receptors has now been demonstrated elsewhere (Jones & Chan, 2005). These authors interpreted the increased

Table 5 Key pharmacological features of recently discovered potent and selective prostanoid EP_4 receptor antagonists

	Binding				*Functional*		
	Human pK_i	*Rodent* pK_i	*Human* EP_4 pK_b	*Human selectivity*	*Animal* EP_4 pK_b	*Animal* EP_4 pIC_{50}	*Animal selectivity*
GW627368X	EP_4 7.0 TP 6.8 Other <5.3	—	7.9 Competitive Inverse agonist?	EP_1 pA_2 6.0 DP not tested TP pIC_{50} 5.0–6.0 Other <5.6	9.2 (porcine) $\geqslant 8.7$ (rabbit)[a]	—	TP <5.0 rabbit and guinea-pig
EP_4A[b]	EP_4 7.6 EP_3 5.7 TP 6.2 Other <5.3	EP_4 7.5 (rat)	8.4[c] Competitive	—	—	7.0 (rat)	—
ONO-AE2-227[d]	—	EP_4 8.5 (murine) EP_3 7.7 (murine) Other <5.5 (murine)	—	—	—	8.0 (murine)	—
Compound 8[e]	EP_4 9.5 Other <5.5	EP_4 9.1 (rat)	—	—	—	>8.0 (murine)	—
Compound 11[e]	EP_4 9.0 Other <5.0	EP_4 8.3 (rat)	—	—	—	7.0–8.0 (murine)	—

Systematic compound names are given in Abbreviations.
[a]Data taken from Jones & Chan (2005).
[b]Data taken from Machwate *et al*. (2001).
[c]Data taken from Davis *et al*. (2004).
[d]Data taken from Mutoh *et al*. (2002).
[e]Data taken from Hattori *et al*. (2005).

maximum effect of another prostanoid IP receptor agonist, taprostene, in the presence of GW627368X as a 'breakthrough effect' of the agonist (where the concentration of agonist is sufficient to overcome receptor blockade by GW627368X) at prostanoid EP_4 receptors in mixed receptor population tissue strips. CHO cells have been shown to possess endogenously expressed prostanoid EP_4 receptors which could also give rise to a breakthrough effect (Crider *et al*., 2000). However, *E*/[A] curves were monophasic in the presence and absence of GW627368X, and the effect observed was to increase the maximum response compared to that in the absence of antagonist where presumably any prostanoid EP_4 receptor-mediated responses would still contribute. We also observed no effect of PGE_2 on untransfected CHO cells (data not shown) so a breakthrough effect seems unlikely and we can offer no explanation for this phenomenon. At human prostanoid FP receptors, the compound produced no shift of $PGF_{2\alpha}$ *E*/[A] curves but did depress the agonist curve asymptote by 54% at 30 μM. This effect was not concentration related and therefore is unlikely to be a receptor-mediated event. Overall, therefore, GW627368X is 100-fold selective for prostanoid EP_4 receptors over other human prostanoid receptors in functional assays, except for prostanoid TP receptors.

We have demonstrated that GW627368X is a potent, competitive antagonist of prostanoid EP_4 receptors with equal binding affinity, but differential functional affinity, for hEP_4 and hTP receptors and, with the exception of human prostanoid TP receptors, is at least 100-fold selective over other human prostanoid receptors. The utility of GW627368X therefore lies in its application as a tool prostanoid EP_4/TP receptor antagonist in a range of experimental settings, as exemplified in the work of Jones & Chan (2005). High affinity and selectivity for prostanoid EP_4/TP receptors of this order is a significant advance over the classical prostanoid EP_4 receptor antagonists AH23848 and AH22921 (Brittain *et al*., 1985; Coleman *et al*., 1994; 1995) and puts GW627368X into the class of recently discovered antagonists with high affinity and selectivity for prostanoid EP_4 receptors (see Table 5 for brief comparison). Of these molecules, only EP_4A can rightly claim to be antagonist at human prostanoid EP_4 receptors, having a pK_b of 8.4 determined in human middle cerebral artery. Furthermore, EP_4A and GW627368X are the only molecules for which a competitive, or indeed, receptor-mediated, antagonism has been demonstrated. However, in addition to being a prostanoid EP_4 receptor antagonist, EP_4A possesses significant TP and EP_3 receptor-binding affinity and as such appears to be less selective than our molecule. Indeed, as we have demonstrated in this paper, functional assays are often more revealing than binding assays, and so the full selectivity profile of EP_4A has not yet been firmly established. Our molecule has a pK_b of 7.9 ± 0.2 (mean $\pm$ s.d.) vs human prostanoid EP_4 receptors and of 9.2 ± 0.1 (mean $\pm$ s.d.) vs porcine receptors, therefore GW627368X is one of the most potent and selective human prostanoid EP_4 receptor antagonists on record. Finally, while considerable doubt remains over the inverse agonist properties of GW627368X, this aspect of the compound's behaviour may confer additional utility.

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