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Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist

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1 A human embryonic kidney cell line [HEK 293(EBNA)] stably expressing the human recombinant prostaglandin D_2 (PGD_2) receptor (hDP) has been characterized with respect to radioligand binding and signal transduction properties by use of prostanoids and prostanoid analogues. Radioligand binding studies included saturation analyses, the effects of nucleotide analogues, the initial rate of ligand-receptor association and equilibrium competition assays. In addition, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generation in response to ligand challenge was also measured, as this is the predominant hDP signalling pathway.

2 L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate)) was identified as a novel ligand having high affinity for hDP with an inhibitor constant (K_i) of 0.9 nM. This K_i value was comparable to the K_i values obtained in this study for ligands that have previously shown high affinity for DP: PGD_2 (0.6 nM), ZK 110841 (0.3 nM), BW245C (0.4 nM), and BW A868C (2.3 nM).

3 L-644,698 was found to be a full agonist with an EC_{50} value of 0.5 nM in generating cyclic AMP following activation of hDP. L-644,698 is, therefore, comparable to those agonists with known efficacy at the DP receptor (EC_{50}): PGD_2 (0.5 nM), ZK 110841 (0.2 nM) and BW245C (0.3 nM).

4 L-644,698 displayed a high degree of selectivity for hDP when compared to the family of cloned human prostanoid receptors: EP_1 (>25,400 fold), EP_2 (~300 fold), $EP_{3\text{-III}}$ (~4100 fold), EP_4 (~10000 fold), FP (>25,400 fold), IP (>25,400 fold) and TP (>25,400 fold). L-644,698 is, therefore, one of the most selective DP agonists as yet described.

5 PGJ_2 and Δ^{12}-PGJ_2, two endogenous metabolites of PGD_2, were also tested in this system and shown to be effective agonists with K_i and EC_{50} values in the nanomolar range for both compounds. In particular, PGJ_2 was equipotent to known DP specific agonists with a K_i value of 0.9 nM and an EC_{50} value of 1.2 nM.

Keywords: Prostanoid receptor; DP; recombinant; prostaglandin D_2; prostaglandin J_2; selective agonist

Introduction

Prostaglandins, prostacyclin (PGI_2), and thromboxane A_2 (TXA_2) are collectively described as prostanoids. Originally it was proposed (Kennedy *et al*., 1982; Coleman *et al*., 1984) that individual prostanoid receptors existed for each of the primary bioactive prostanoids. This classification described distinct receptors for prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 which were denoted DP, EP (EP_1 and EP_2) FP, IP and TP, respectively. This was followed by a further subdivision of the EP class of receptors into four subtypes: EP_1, EP_2, EP_3 and EP_4 (for review, Coleman *et al*., 1994). The cloning of the human (h) prostanoid receptors currently includes TP (Hirata *et al*., 1991), FP (Abramovitz *et al*., 1994), IP (Boie *et al*., 1994), EP_1 (Funk *et al*., 1993), EP_2 (Regan *et al*., 1994), EP_3 (Adam *et al*., 1994), EP_4 (An *et al*., 1993; Bastien *et al*., 1994) and DP (Boie *et al*., 1995). These receptors form a sub-family within the G-protein-coupled receptor (GPCR) superfamily. During cloning, alternatively spliced isoforms of both hTP (Raychowdhury *et al*., 1995) and hEP_3 (Adam *et al*., 1994; Schmid *et al*., 1995; Kotani *et al*., 1995) were identified.

The endogenous prostanoids demonstrate preference towards individual prostanoid receptors but, in general, there is a marked degree of cross-reactivity between these ligands and the entire receptor family (Dong *et al*., 1986; Sheldrick *et al*., 1988; Armstrong *et al*., 1989; Bunce *et al*., 1990). This has driven the development of selective compounds. Several selective and efficacious prostanoid agonists have been identified, including cicaprost (Dong *et al*., 1986; Armstrong *et al*., 1989) at the IP receptor, GR63799X (Bunce *et al*., 1990) at the EP_3 receptor and butaprost (Abramovitz *et al*., unpublished observations) at the EP_2 receptor. To date, the group of synthetic DP agonists includes BW245C, ZK 110841, RS-93520, RS-93427, 572C85 and 192C86. BW245C is the most comprehensively studied of these synthetic DP agonists (Town *et al*., 1983; Whittle *et al*., 1983; Woodward *et al*., 1990; Fernandes & Crankshaw, 1995; Rangachari *et al*., 1995) but although the efficacy of BW245C at DP is well-established (Town *et al*., 1983; Boie *et al*., 1995), the selectivity of this ligand versus the other prostanoid receptors is not completely defined. Data suggest that BW245C has affinity for at least one other prostanoid receptor that couples to stimulation of adenylate cyclase via the guanine nucleotide binding (G) protein G_s. Some findings suggest that BW245C cross-reacts with EP_2 (Giles *et al*., 1989; Matsugi *et al*., 1995), while other investigators have proposed IP (Trist *et al*., 1989). Another potential limitation associated with BW245C concerns its stability in aqueous solution, since it has been shown to produce an epimer which is less biologically active than the parent compound (Brockwell *et al*., 1981).

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Studies of the DP receptor have been complicated by its relatively low abundance and narrow scope of distribution. Thus, it was the last known human prostanoid receptor to be cloned (Boie *et al*., 1995). In this study, the radioligand binding and signal transduction properties of recombinant hDP have been more completely studied, both with prostanoids and prostanoid synthetic analogues of varying selectivity. Through these analyses and by use of the system of cloned recombinant human prostanoid receptors previously described (Abramovitz *et al*., unpublished observations), a novel selective DP agonist, L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate) (Figure 1), has been identified.

Methods

pCEP4-hDP stable expression in HEK 293(EBNA) cells

Stable expression of the hDP receptor was achieved by transfection of the pCEP4-hDP (Abramovitz *et al*., unpublished observations) plasmid into HEK 293(EBNA) cells [maintained under selection with GENETICIN (G418)] by cationic-liposome mediated transfer with LipofectAMINE reagent (Felgner *et al*., 1987). Cells were maintained in culture for 48 h post transfection and then grown in the presence of 200 μg ml^{-1} hygromycin B for 2 weeks, to select for resistant colonies expressing the hDP receptor. Resistant colonies were expanded and subsequently tested for hDP expression by radioligand binding. The clone with the highest level of binding activity was then used for signal transduction assays.

Cell culture and membrane preparation

HEK 293(EBNA) cells stably expressing hDP (hDP-HEK) were maintained in culture in Dulbecco's modified Eagle's medium growth medium (Dulbecco's modified Eagle's medium containing 10% heat-inactivated foetal bovine serum, 1 mM sodium pyruvate, 20 units ml^{-1} penicillin G, 20 μg ml^{-1} streptomycin sulphate, 250 mg ml^{-1} GENETICIN and 200 mg ml^{-1} hygromycin B). In order to prepare membranes from hDP-HEK cells (all procedures at 4°C), they were first resuspended by Dounce homogenization (pestle B, 10 strokes) in the presence of 2 mM phenylmethylsulphonylfluoride. Cells were next disrupted by nitrogen-cavitation at 800 psi for 30 min on ice. The resulting cell suspension was subjected to two centrifugation steps: $1000 \times g_{max}$ for 10 min followed by $100\,000 \times g_{max}$ for 30 min. The resulting pellet was resuspended to 1/10th the original volume in 10 mM HEPES/KOH (pH 7.4) containing 1 mM EDTA (tetrasodium salt) by Dounce homogenization (pestle A, 10 strokes), and aliquots were stored at −80°C at a protein concentration of 8–10 mg ml^{-1}.

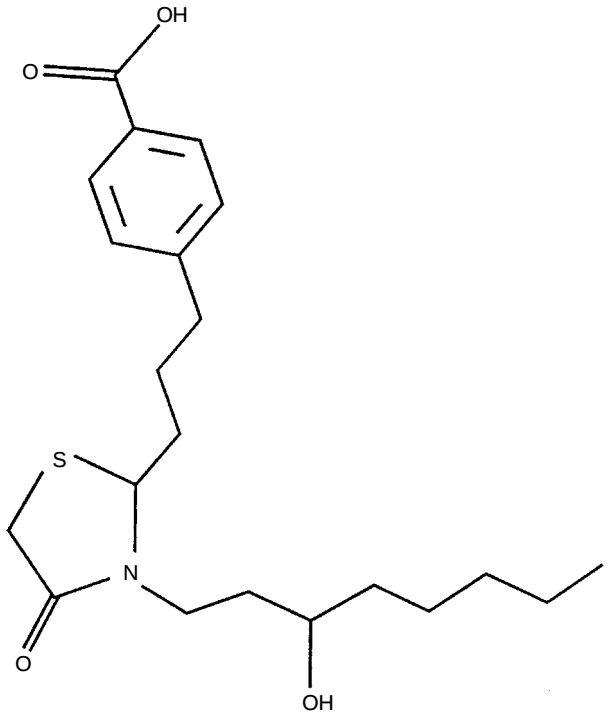

Figure 1 Structure of L-644,698 (racemate).

[^{3}H]-PGD$_2$ binding to hDP-HEK membranes

Radioligand binding assays were performed in 0.2 ml of 10 mM HEPES-KOH (pH 7.4) 1 mM EDTA containing (unless otherwise noted) 0.8 nM [^{3}H]-PGD$_2$ (115 Ci mmol^{-1}) and 10 mM $MnCl_2$. Compounds were added in dimethylsulphoxide (Me_2SO) at 1% (v/v) of the final incubation volume (vehicle concentration was constant throughout). The reaction was initiated by the addition of 30 μg of hDP-HEK membrane protein to all tubes and the samples were incubated at room temperature for 1 h. The reaction was terminated by rapid filtration at 4°C in 3–4 ml of 10 mM HEPES/KOH (pH 7.4) through a GF/C filter (Unifilter) which had been presoaked in the same buffer. Each filter was dried for 1–2 h at 55°C and the residual [^{3}H]-PGD$_2$ bound to the filter (33% efficiency) was determined in 50 μl per well of Ultima Gold scintillation cocktail. Non-specific binding was determined in the presence of 1 μM PGD$_2$.

Analysis of [^{3}H]-PGD$_2$ binding

Rates of association were calculated through a one-site curve-fit analysis, employing the equation $B_t = B_{eq}\text{-}B_{eq}[e^{(-K_{obs}t)}]$; where B_t represents the radioligand bound specifically at time t, B_{eq} represents the radioligand bound specifically at equilibrium and K_{obs} is the observed association rate which is then expressed as K_{obs}/[radioligand].

Specific binding saturation isotherms were deduced by subtracting the nonspecific binding from the total binding, both measured experimentally. The saturation isotherms were transformed by use of nonlinear, least-squares, regression analysis adapted from the work of Feldman (1972) where the equation $[(B_{Max} \times F)/(K_1 + F)] + [(B_{Max} \times F)/(K_2 + F)]$ represents the radioligand specifically bound, B_{Max} is the maximal number of binding sites, K is the equilibrium dissociation constant and F is the concentration of free radioligand. The analyses were performed by use of Accufit Two-Site saturation software (Beckman Instruments).

Sigmoidal curves from equilibrium competition assays were analysed by custom designed software which employs a non-linear least-squares fitting routine based on the four parameter logistic equation: $y = (m1 - m2)(1 + (m0/m3)e^{m4})^{-1} + m2$; where m1 and m2 represent the maximum and minimum of the curve, m3 represents the inflection point (IP), m4 represents the slope of the curve at the inflection point, m0 represents the concentration of the competing ligand and y represents the % [^{3}H]-PGD$_2$-specific binding. K_i values were calculated from the equation $K_i = IP/1 + [radioligand]/K_D$.

Chemical and metabolic stability of [^{3}H]-PGD$_2$ under the experimental conditions

The stability of [^{3}H]-PGD$_2$ exposed to the incubation conditions for 2 h was verified by reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.). Following incubation of [^{3}H]-PGD$_2$ under standard conditions the reaction was terminated with the addition of $2\times$ r.p.-h.p.l.c. solvent (r.p.-h.p.l.c. solvent = 66:15:18:1 (v/v) H_2O:CH_3CN:CH_3OH:CH_3COOH, adjusted to pH 5.6 with 10 N NaOH) to the samples. Samples were allowed to incubate for 10 min at 30°C to dissociate the receptor-bound [^{3}H]-PGD$_2$ and were then subjected to centrifugation at $100\,000\times g$ for 15 min at 4°C. The resulting supernatant fractions, containing both bound and unbound [^{3}H]-PGD$_2$, were then analysed by r.p.-h.p.l.c. with a NovaPak C_{18} column (0.39×15 cm; Waters). [^{3}H]-PGD$_2$ was eluted with a linear gradient from 0 to 70% (v/v) acetonitrile developed over 35 min at a flow rate of 1 ml min^{-1} following an initial 3 min elution with r.p.-h.p.l.c. solvent. The 35 min gradient elution was followed by a final 10 min wash with 90% (v/v) acetonitrile. The profile of radioactivity was monitored with an on-line flow-through radioactivity detector (Berthold). Eluant fractions were subsequently mixed with 5 ml of scintillation fluid for the precise quantitation of the radioactivity recovered.

Cyclic AMP assays with hDP-HEK cells

hDP-HEK cells were harvested at 80% confluence by resuspension in 10 ml of enzyme-free cell dissociation buffer and washed in phosphate-buffered saline by centrifugation ($300\times g_{max}$ for 6 min at room temperature). Cells were then washed in 10 ml of Hank's balanced salt solution (HBSS) by centrifugation under the same conditions as above and resuspended in HBSS at 4×10^6 cells ml^{-1}. Cell viability was determined to be >95%, by trypan blue exclusion. The generation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) was performed in a final incubation volume of 0.2 ml of HBSS containing 100 μM Ro 20-1724 to abrogate cyclic AMP hydrolysis. Compounds were added in Me_2SO kept constant at 1% (v/v) of the final incubation volume. Assays were initiated by the addition of 2×10^4 cells per reaction and samples incubated for 10 min at 37°C with shaking. Reactions were halted by the incubation of samples in boiling water for 3 min and cyclic AMP was subsequently measured by use of a [^{125}I]-cyclic AMP scintillation proximity assay.

Sigmoidal concentration-response curves were analysed by custom designed software to determine EC_{50} values. Maximal stimulation was defined as the quantity of cyclic AMP produced by incubation with 1 μM PGD$_2$. Statistical analysis of the maximal response for each ligand as a percentage of this maximal stimulation was performed with SigmaStat software, version 2.0 (Jandel Scientific). A one-way ANOVA followed by a Tukey test was used to determine full and partial agonists.

Protein assays

Protein concentration was measured by the bicinchoninic acid (BCA) protein assay kit (Pierce) with bovine serum albumin as the standard.

Reagents

PGD$_2$, PGE$_1$, PGE$_2$, PGF$_{2\alpha}$, U46619 (9,11-dideoxy-9α,11α-methanoepoxy-PGF$_{2\alpha}$), PGJ$_2$, Δ^{12}-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ and Ro-20-1724 (4-(3 butoxy-4-methoxybenzyl)-2-imidazolidinone) were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). 13,14-dihydro-15-keto-PGD$_2$ was from Cayman Chemical (Ann Arbor, MI, U.S.A.). BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin)) and BW A868C ((±)-3-benzyl-5-(6-carboxyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) were generous gifts from The Wellcome Foundation Ltd (Beckenham, Kent, U.K.). ZK 110841 ((5Z,13E)-(9R,11R,15S)-9β-chlor-15-cyclohexyl-11,15-dihydroxy-16,17,18,19,20-pentanor-5,13-prostadienoic acid) was a generous gift from Dr D. Crankshaw from the Department of Obstetrics & Gynecology (McMaster University, Hamilton, Ontario, Canada). L-644,698 was synthesized at Merck Research Laboratories by Dr J.B. Bicking. Iloprost and [^{125}I]-cyclic AMP scintillation proximity assay kits were from Amersham (Oakville, ON, Canada). [^{3}H]-PGD$_2$ was from Dupont NEN (Boston, MA, U.S.A.). GTPγS (Guanosine-5′-O-(3-thiotriphosphate)), ATPγS (adenosine-5′-O-(3-thiotriphosphate)), GMP-PNP (guanylyl-imidodiphosphate) and AMP-PNP (adenylyl-imidodiphosphate) were from Boehringer Mannheim Canada (Laval, QC, Canada). Bicinchoninic acid (BCA) protein assays were from Pierce (Rockford, IL, U.S.A.). GENETICIN (G418) and LipofectAMINE were from GIBCO/BRL (Burlington, ON, Canada). Enzyme-free cell dissociation buffer was from Life-Technologies Inc (Gaithersburg, MD, U.S.A.). Hygromycin B was from Calbiochem (La Jolla, CA, U.S.A.). Unifilters and Ultima Gold were from Packard (Meriden, CT, U.S.A.).

Results

Rate of association

The rate of association of [^{3}H]-PGD$_2$ with hDP at room temperature is shown in Figure 2. The initial rate of association was 0.15 ± 0.01 min^{-1} nM^{-1}. Equilibrium was reached within 20 min. Once achieved, equilibrium binding was maintained throughout the 2 h time course. The stability

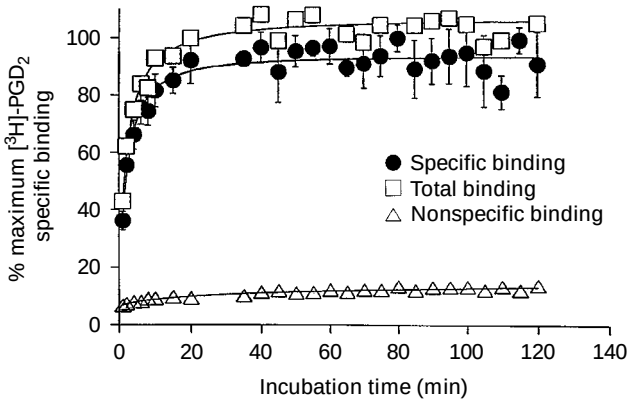

Figure 2 Rates of association of [^{3}H]-PGD$_2$ binding to the human DP receptor expressed on membranes from HEK 293(EBNA) cells. Radioligand membrane binding assays were carried out as previously described under Methods, with the following modifications: the rates of total and nonspecific binding were assessed over a 2 h time course by the sequential addition of HEK 293(EBNA) membranes expressing the hDP receptor to separate incubation tubes. Membranes were added initially at the final time point of 2 h and successively at various other intervals until the initial time point of 1 min. Specific binding was calculated as the difference between nonspecific binding, measured in the presence of 1 μM PGD$_2$, and total binding. Data points are the mean from three separate experiments performed in duplicate; vertical lines show s.e.mean.

of [^{3}H]-PGD_2 under the assay conditions was verified by reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.). [^{3}H]-PGD_2 was incubated under the assay conditions for 2 h, recovered from the incubation media and resolved by r.p.-h.p.l.c. as a single peak with the same retention time (24.5 min) as a control sample of [^{3}H]-PGD_2 analysed under identical conditions (data not shown). Approximately 87% of the [^{3}H]-PGD_2 added to the incubation medium was recovered. [^{3}H]-PGD_2 is, therefore, chemically and metabolically stable during a 2 h incubation under these experimental conditions as determined by r.p.-h.p.l.c.

The association rate of [^{3}H]-PGD_2 specific binding to the hDP receptor was also investigated at 30°C (data not shown). Equilibrium binding was attained more rapidly than under conditions of room temperature, at a rate of 0.31 ± 0.03 min^{-1} nM^{-1}. However, it was not maintained, as demonstrated by a time-dependent decrease in [^{3}H]-PGD_2 specific binding during the 2 h incubation to a value 75% of the maximum level obtained.

Effects of nucleotide analogues

The effects of slowly-hydrolyzable nucleotide analogues on [^{3}H]-PGD_2 specific binding to the hDP receptor were studied (Figure 3). [^{3}H]-PGD_2 specific binding was inhibited in a concentration-dependent manner by GTPγS, ATPγS, GMP-PNP and AMP-PNP. The IC_{50} values (μM) for these compounds were 0.01, 1.67, 1.51 and 347, corresponding to the following rank order of nucleotide analogue potency at the hDP receptor: GTPγS > > ATPγS = GMP-PNP > > AMP-PNP. The effects of GTPγS were studied further in conjunction with saturation analyses as described below.

Saturation analyses

Saturation analysis of [^{3}H]-PGD_2 specific binding to the hDP receptor was performed and two populations of specific binding sites were revealed (Figure 4a): a high affinity site of relatively low abundance with an equilibrium dissociation constant (K_D) of 0.3 ± 0.1 nM and a maximal number of specific binding sites (B_{Max}) of 0.5 ± 0.1 pmol mg^{-1} of membrane protein and a low affinity site of relatively high abundance with a K_D of 13.4 ± 1.2 nM and a B_{Max} of 5.9 ± 0.6 pmol mg^{-1} of membrane protein (all values are the mean $\pm$ s.e.mean, $n = 3$). The nature of these two populations of [^{3}H]-PGD_2 specific binding sites was investigated further with GTPγS. When saturation analysis was conducted in the presence of 100 μM GTPγS (Figure 4b) only the low affinity binding site was identified (K_D of 17.1 ± 1.3 and a B_{Max} of 5.8 ± 0.3 pmol mg^{-1} of membrane protein).

Competition for [^{3}H]-PGD_2 specific binding

Three groups of compounds were investigated for their ability to compete with [^{3}H]-PGD_2 specific binding to hDP in equilibrium competition assays (Table 1): prostanoids, synthetic prostanoid analogues and PGD_2 metabolites. PGD_2 had a K_i of 0.6 ± 0.2 nM. PGE_2, $PGF_{2\alpha}$ and iloprost all had K_i values in the high nanomolar range. Compounds with the highest affinity were the DP specific synthetic ligands: BW245C, BW A868C and ZK 110841, all of which were comparable to PGD_2. PGJ_2 had affinity for the DP receptor that was of the same order as the DP specific synthetic ligands and PGD_2. The other metabolic breakdown products of PGD_2 tested (Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and 13, 14-dihydro-15-keto-PGD_2) had K_i values ranging from 100 nM to 6000 nM.

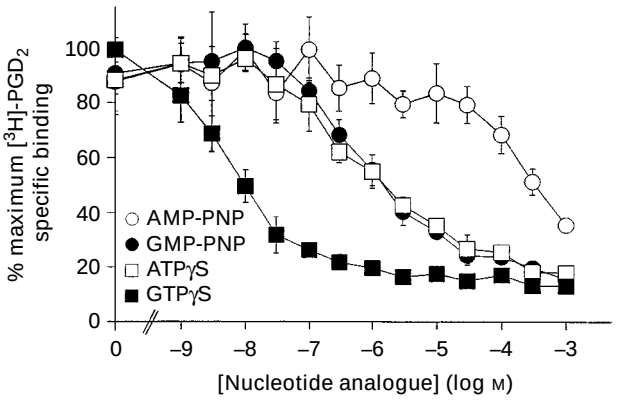

Figure 3 Effects of nucleotide analogues on [^{3}H]-PGD_2 specific binding to the human DP receptor expressed on membranes from HEK 293(EBNA) cells. Radioligand membrane binding assays were carried out as previously described under Methods, in the presence of 0–1000 μM of GTPγS, ATPγS, GMP-PNP or AMP-PNP. The specific binding for each nucleotide analogue concentration was calculated as a percentage of the maximum specific binding obtained in the absence of nucleotide analogue. Data points are the mean from two separate experiments performed in duplicate; vertical lines show s.d.

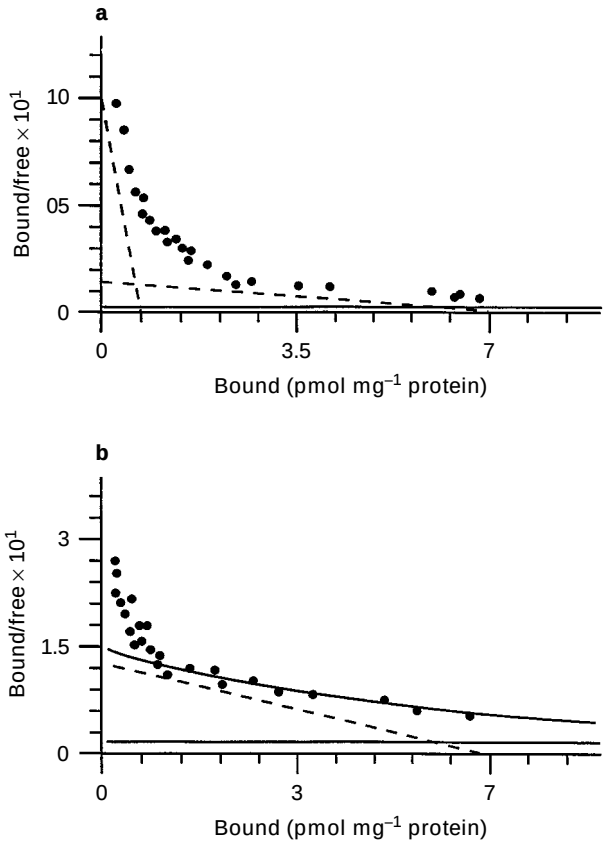

Figure 4 Saturation analysis of [^{3}H]-PGD_2 binding to the human DP receptor expressed on membranes from HEK 293(EBNA) cells. Radioligand membrane binding assays were carried out as previously described under Methods. Total and nonspecific binding were determined over a concentration range of 0.4 nM to 40 nM [^{3}H]-PGD_2 (28.8 Ci mmol^{-1}) in the absence (a) and presence (b) of 100 μM GTPγS. Saturation isotherms were analysed as described in Methods. Note the difference in the scales of the ordinates between the two groups. Nonspecific binding was measured with 100 fold excess (4 μM) PGD_2, as validated in competition assays with [^{3}H]-PGD_2. Data are representative of three separate experiments, each done in duplicate, with membrane stocks prepared from different passages of hDP-HEK cells.

During this series of assays a novel ligand of high affinity and specificity for hDP, denoted L-644,698 (Figure 1), was identified. L-644,698 had high affinity for the hDP receptor with a K_i of 0.9 ± 0.2 nM, which is equal in magnitude to PGD_2, PGJ_2, BW245C, BW A868C and ZK 110841 (Figure 5). In addition, L-644,698 demonstrated considerable selectivity for the hDP receptor, as shown in Table 2. Binding of L-644,698 was detectable at hEP_2, hEP_3 and hEP_4, where the respective K_i values were at least 300 fold, 4100 fold and 10000 fold higher than the K_i value at hDP. L-644,698 did not compete for specific binding to hEP_1, hFP, hIP and hTP at concentrations up to 25.4 μM.

Cyclic AMP generation assays

The ability of prostanoids and synthetic prostanoid analogues to transduce intracellular signals through hDP was explored by measuring stimulation of intracellular cyclic AMP production (Figure 6 and Table 3). PGD_2 had an EC_{50} value of 0.5 ± 0.1 nM for the production of cyclic AMP. The novel selective ligand L-644,698 was equipotent with an EC_{50} value of 0.5 ± 0.1 nM. BW245C and ZK 110841 had EC_{50} values of 0.3 ± 0.1 nM and 0.2 ± 0.1 nM, respectively, whereas BW A868C was less potent with an EC_{50} value of 7.5 ± 0.9 nM. The PGD_2 metabolites all showed varying efficacies, the most notable being PGJ_2 with an EC_{50} value of 1.2 ± 0.1 nM.

The maximal response for each ligand as a percentage of the maximal stimulation achieved with 1 μM PGD_2 was also found in an effort to discriminate between full and partial agonists (Table 3). The majority of compounds were not statistically significantly different from PGD_2 and were thus deemed full agonists, including the novel hDP specific ligand L-644,698. In contrast, BW A868C [documented both as an antagonist (Giles *et al*., 1989) and a partial agonist (Hirata *et al*., 1994; Boie *et al*., 1995)] was significantly different from PGD_2 ($P < 0.05$) since it reached only $68 \pm 4\%$ of PGD_2-mediated maximal stimulation, which is suggestive of partial agonist activity. The only other partial agonist identified was 15-deoxy-$\Delta^{12,14}$-PGJ_2 ($P < 0.05$) with a value of $69 \pm 5\%$ as compared with the PGD_2 control.

Table 1 Inhibitor constants and Hill coefficients for competing ligands at the human DP receptor

	Ligand	K_i (nM)	*Hill coefficient* (n_H)
	PGD_2 (4)	0.6 ± 0.2	0.6 ± 0.2
I	PGE_1	53 ± 8	0.8 ± 0.1
	PGE_2	107 ± 42	0.6 ± 0.1
	$PGF_{2\alpha}$	367 ± 85	0.7 ± 0.1
	Iloprost	269 ± 82	0.7 ± 0.1
	U-46619	1202 ± 64	0.7 ± 0.1
II	PGJ_2 (4)	0.9 ± 0.1	0.9 ± 0.1
	Δ^{12}-PGJ_2	100 ± 13	0.8 ± 0.1
	15-deoxy-$\Delta^{12,14}$-PGJ_2 (4)	280 ± 30	0.1 ± 0.1
	13,14-dihydro-15-keto-PGD_2	6374 ± 1208	0.9 ± 0.1
III	L-644,698 (4)	0.9 ± 0.2	0.6 ± 0.2
	BW 245C (4)	0.4 ± 0.1	0.6 ± 0.2
	BW A868C	2.3 ± 1.4	0.4 ± 0.2
	ZK 110841 (4)	0.3 ± 0.1	0.9 ± 0.1

Inhibitor constant (K_i) values $\pm$ s.e.mean (nM) and Hill coefficients (n_H) values $\pm$ s.e.mean are shown for prostanoids and synthetic prostanoid analogues (I), metabolites of PGD_2 (II) and synthetic prostanoid analogues specific for DP (III). Values were derived from 3 separate experiments unless otherwise noted in parentheses by the ligand name.

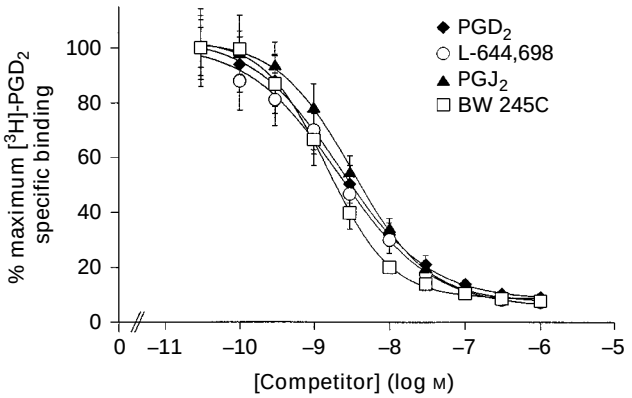

Figure 5 Competition for [^{3}H]-PGD_2 specific binding to the human DP receptor expressed on HEK 293(EBNA) membranes by DP selective agonists. Radioligand membrane binding assays were carried out as previously described under Methods. Assays of competition were conducted under conditions of equilibrium at room temperature, with 0.03–1000 nM PGD_2, BW 245C, PGJ_2 and L-644,698. Data points are the mean from four experiments performed in duplicate; Vertical lines show s.e.mean.

Table 2 Selectivity of L-644,698 and DP agonists at recombinant human prostanoid receptors stably expressed in HEK 293 (EBNA) cells

	K_i (nM)							
Ligand	*hEP_1*	*hEP_2*	*hEP_3*	*hEP_4*	*hDP*	*hFP*	*hIP*	*hTP*
L-644,698	> 25400 (3)	267 ± 39 (3)	3730 ± 738 (3)	*9280 ± 226 (2)	0.9 ± 0.2 (4)	> 25400 (3)	> 25400 (3)	> 25400 (3)
†PGD_2	5820 ± 1801 (3)	2973 ± 100 (3)	421 ± 60 (3)	1483 ± 189 (3)	0.6 ± 0.2 (3)	6.7 ± 0.5 (3)	> 25000 (3)	6602 ± 541 (5)
†BW245C	> 25000 (4)	219 ± 19 (5)	> 25000 (3)	132 ± 26 (4)	0.4 ± 0.1 (4)	> 25000 (3)	> 25000 (3)	13482 ± 1134 (5)
†ZK 110841	1.8 ± 0.2 (4)	6.0 ± 0.6 (4)	522 ± 81 (2)	41 ± 7 (4)	0.3 ± 0.1 (4)	1670 ± 107 (3)	2147 ± 661 (2)	1121 ± 201 (3)
PGJ_2	15678 ± 3711 (5)	989 ± 311 (4)	319 ± 29 (5)	1065 ± 252 (4)	0.9 ± 0.1 (3)	553 ± 226 (4)	> 25000 (2)	6426 ± 1271 (5)

Inhibitor constant (K_i) values $\pm$ s.e.mean (where $n \geq 3$) or s.d. (where $n = 2$) were derived from radioligand binding assays and are shown with the number of determinations (n) indicated in parentheses. *L-644,698 binding to hEP_4, K_i value $\pm$ s.d., data set = 9440 nM, 9120 nM and 43% inhibition at 25.4 μM; ZK 110841 binding to hEP_3, K_i value $\pm$ s.d., data set = 442 nM, 604 nM and 50% inhibition at 2.5 μM and to hIP, K_i value $\pm$ s.d., data set = 2615 nM, 1680 nM, 64% inhibition at 2.5 μM and 70% inhibition at 2.5 μM. †Data from Metters *et al*. (unpublished observations).

Table 3 Efficacies for ligands at the human DP receptor

	Ligand	*EC_{50}* (nM)	*% of maximal stimulation of cyclic AMP production*
	PGD_2 (4)	0.5±0.1	86±2
I	PGE_2	84±1	91±10
	$PGF_{2\alpha}$	264±22	85±10
	Iloprost	377±13	82±2
	U-46619	2903±647	100±12
II	PGJ_2	1.2±0.1	87±1
	Δ^{12}-PGJ_2 (4)	91±18	118±9
	15-deoxy-$\Delta^{12,14}$-PGJ_2	321±15	*69±5
	13,14-dihydro-15-keto-PGD_2	5283±944	78±3
III	L-644,698	0.5±0.1	86±4
	BW 245C	0.3±0.1	90±1
	BW A868C (4)	7.5±0.9	*68±4
	ZK 110841	0.2±0.1	92±1

EC_{50} values±s.e.mean (nM) and maximal responses (percentage of maximally stimulated control values±s.e.mean) are shown for prostanoids and synthetic prostanoid analogues (I), metabolites of PGD_2 (II) and synthetic prostanoid analogues specific for the DP receptor (III). Values were derived from 3 separate experiments unless otherwise noted in parentheses by the ligand name. Maximal stimulation was defined as the cyclic AMP response produced in response to 1 μM PGD_2. *Indicates a value statistically significantly different ($P<0.05$) from the maximal stimulation (100%).

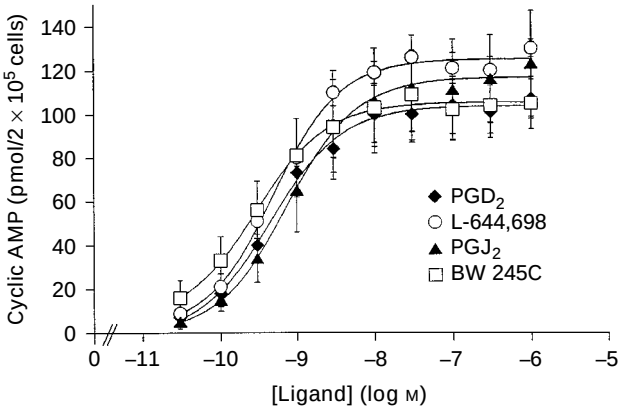

Figure 6 Cyclic AMP production by HEK 293(EBNA) cells expressing the human DP receptor. hDP-HEK cells were incubated in a final volume of 0.2 ml of HBSS containing 100 μM Ro 20-1724, as previously described under Methods. Also, added to the incubation medium was 0.03–1000 nM PGD_2, BW 245C, PGJ_2 or L-644,698. Reactions were initiated with the addition of 2×10^5 cells to the incubation volume, samples were incubated for 10 min at 37°C with shaking and the reaction was terminated by the incubation of the assay plate in boiling water for 3 min. Cyclic AMP was quantified by use of a commercially available scintillation proximity assay. Data points are the mean of three (L-644,698, BW245C, and PGJ_2) or four (PGD_2) experiments each performed in duplicate; vertical lines show s.e.mean.

Discussion

Characterization of a newly-developed hDP-HEK stable cell line has been performed in order to study DP receptor ligands. This characterization includes the radioligand binding properties of hDP determined in cell membrane preparations. In addition, cyclic AMP measurements with hDP-HEK cells have been performed as an index of cell signalling. These features are described below.

Slowly-hydrolyzable nucleotide analogues disrupt GPCR signalling by binding to the G-protein Gα subunit (for review: Gilman, 1987; Neer, 1995). This promotes the sustained dissociation of the G_α subunit from the $G_{\beta\gamma}$ subunits (Hanski *et al*., 1981; Sternweis *et al*., 1981) thereby conferring a low affinity state for agonist binding on the entire receptor population (Rojas & Birmbaumer, 1985; Gilman, 1987). In this study [^{3}H]-PGD_2 specific binding to hDP was inhibited by GTPγS > ATPγS = GMP-PNP > AMP-PNP, indicating a marked difference in agonist affinities between the coupled and uncoupled states of the recombinant hDP receptor. The rank order of potency of the nucleotide analogues probably reflects their structural similarity to guanosine 5′-triphosphate (GTP).

Two populations of hDP specific binding sites were identified by saturation analysis: a high affinity site of relatively low abundance and a low affinity site of relatively high abundance. Further support is lent to the two-site model with the suppression of the high affinity site in the presence of 100 μM GTPγS which allows detection of only the low affinity site. In addition, the calculated Hill coefficient values (n_H) from equilibrium competition radioligand binding assays ranged from 0.4 to 0.9, except for 15-deoxy-$\Delta^{12,14}$-PGJ_2 where n_H was equal to 0.1. This is indicative of more than one binding site since, classically, a single site of binding is indicated by $n_H = 1$.

Competition for [^{3}H]-PGD_2 specific binding to the recombinant hDP receptor identified a novel ligand, L-644,698, with high affinity and selectivity for hDP. The overall rank order of affinities for the ligands tested was: L-644,698 = ZK 110841 = BW245C = PGD_2 = PGJ_2 ⩾ BW A868C >> PGE_1 > Δ^{12}-PGJ_2 = PGE_2 > 15-deoxy-$\Delta^{12,14}$-PGJ_2 = Iloprost > $PGF_{2\alpha}$ >> U46619 > 13, 14-dihydro-15-keto-PGD_2. The selectivity of L-644,698 for the hDP receptor distinguished it from these other compounds of equal affinity. L-644,698 is comparable to BW245C in terms of its selectivity for hDP, but has a slightly different profile relative to the other prostanoid receptors which couple to stimulation of adenylate cyclase. Thus, L-644,698 and BW245C have equal affinities for hIP and hEP_2, but L-644,698 has a 100 fold lower affinity for hEP_4. The other DP agonists tested demonstrated much less selectivity.

L-644,698 is also a potent agonist in signal transduction assays which assessed cyclic AMP production. The following rank order of potency was observed: L-644,698 = ZK 110841 = BW245C = PGD_2 = PGJ_2 ⩾ BW A868C >> Δ^{12}-PGJ_2 = PGE_2 > $PGF_{2\alpha}$ > 15-deoxy-$\Delta^{12,14}$-PGJ_2 = iloprost >> U46619 > 13, 14-dihydro-15-keto-PGD_2. Due to its demonstrated potency and unique profile of selectivity L-644,698 should, therefore, be valuable in studying DP in heterologous systems.

Prostaglandin J_2 was also potent in its ability to stimulate cyclic AMP production and demonstrated full agonism in the recombinant hDP-HEK system. Previously PGJ_2 has demonstrated only partial agonist activity. The latter study was conducted *in vitro* with the DP receptor in human non-pregnant myometrium (Fernandes & Crankshaw, 1995). This discrepancy is probably attributable to the differences in receptor reserve between the two systems. PGJ_2 can be degraded in the presence of albumin, serum or plasma to its biologically active metabolite Δ^{12}-PGJ_2 (Mahmud *et al*., 1984). Δ^{12}-Prostaglandin J_2 was also shown to be a full agonist in this study and was approximately 100 fold less potent than PGJ_2 and 200 fold less potent than PGD_2. This is consistent with a previous study where Δ^{12}-PGJ_2 was shown to be 300 fold less potent than PGD_2 in mediating inhibition of ADP-induced human platelet aggregation (Narumiya & Toda, 1985). Prostaglandin J_2 and, less probably, Δ^{12}-PGJ_2 have the

potential, therefore, to be active *in vivo* at the DP receptor depending on the local concentrations of these ligands.

BW A868C is a hydantoin derivative originally described as a potent DP antagonist with a pK_B value at human platelets ranging from 9.1 to 9.3 when assayed against BW245C (Giles *et al*., 1989; Trist *et al*., 1989; Barraclough *et al*., 1996). Similarly, at DP in human myometrium BW A868C behaved as an antagonist with a pK_B value of 7.3 against ZK 110841 (Fernandes & Crankshaw, 1995). In contrast, BW A868C behaved as a partial agonist in this recombinant hDP receptor system. Similar activity has been previously described with the cloned hDP and mouse (m)DP receptors (Hirata *et al*., 1994; Boie *et al*., 1995). A shift from antagonist to partial agonist activity is usually explained by the low level of receptors in systems expressing endogenous receptors relative to the high receptor reserve obtained in recombinant expression systems. However, the stable cell line expressing mDP had a low receptor number (maximum number of specific binding sites of 93 fmol mg^{-1} membrane protein) which is less than many values observed for tissue preparations (Ito *et al*., 1989). Partial agonism associated with BW A868C is, therefore, observed under conditions of low and high expression of specific binding sites.

BW245C and ZK 110841 were 1.5 and 2.5 times as potent as PGD_2, respectively, in the recombinant hDP-HEK system. In independent studies BW245C was determined to be 3 times more potent than PGD_2 (Town *et al*., 1983) and PGD_2 was found to be 1.5 times more potent than ZK 110841 (Thierauch *et al*., 1988; Schulz *et al*., 1990) at inhibiting ADP-induced platelet aggregation. The values obtained in this study are, therefore, in good agreement with the previous data. However, variations in values obtained do exist for platelet aggregation assays. For example, BW245C has been found to be 8 fold more potent than PGD_2 (Whittle *et al*., 1983; Narumiya & Toda, 1985). In contrast to other studies in which washed platelets were used, in these, platelet-rich-plasma was employed where the plasma and the accompanying albumin would have the potential to metabolize PGD_2, as has been documented (Fitzpatrick & Wynalda, 1983). Studies addressing hDP-mediated inhibition of neutrophil activation are also in agreement with our data. ZK 110841 was 3 fold more potent than PGD_2 in inhibiting superoxide anion formation and equipotent with PGD_2 in inhibiting β-glucuronidase release in neutrophils (Ney & Schrör, 1991). Finally, investigations of PGD_2-mediated effects in the non-pregnant myometrium have documented a biphasic effect (Senior *et al*., 1992; Fernandes & Crankshaw, 1995), where the initial contractile phase occurs through PGD_2 activation of hFP and the secondary relaxation phase results from PGD_2 activation of hDP. These results emphasize the importance of selectivity data in the prediction of *in vivo* ligand activity.

In conclusion, a novel DP-specific agonist, L-644,698, has been described. This synthetic prostanoid analogue is one of the most highly selective DP agonists discovered to date. In addition, the PGD_2 metabolites PGJ_2 and Δ^{12}-PGJ_2 have been shown to be potent at hDP, suggesting that they may have physiological significance *in vivo*. Selectivity data have been presented for some currently accepted DP-specific agonists across the eight human prostanoid receptors cloned to date. Finally, the recombinant hDP receptor has been characterized with available pharmacological tools, both with respect to radioligand binding and signal transduction.

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