RESEARCH PAPER

The design, synthesis and pharmacological characterization of novel β₂-adrenoceptor antagonists

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BACKGROUND AND PURPOSE
Selective and potent antagonists for the β₂-adrenoceptor are potentially interesting as experimental and clinical tools, and we sought to identify novel ligands with this pharmacology.

EXPERIMENTAL APPROACH
A range of pharmacological assays was used to assess potency, affinity, selectivity (β₂-adrenoceptor vs. β₁-adrenoceptor) and efficacy.

KEY RESULTS
Ten novel compounds were identified but none had as high affinity as the prototypical β₂-adrenoceptor blocker ICI-118,551, although one of the novel compounds was more selective for β₂-adrenoceptors. Most of the ligands were inverse agonists for β₂-adrenoceptor-cAMP signalling, although one (5217377) was a partial agonist and another a neutral antagonist (7929193). None of the ligands were efficacious with regard to β₂-adrenoceptor-β-arrestin signalling. The (2S,3S) enantiomers were identified as the most active, although unusually the racemates were the most selective for the β₂-adrenoceptors. This was taken as evidence for some unusual enantiospecific behaviour.

CONCLUSIONS AND IMPLICATIONS
In terms of improving on the pharmacology of the ligand ICI-118,551, one of the compounds was more selective (racemic JB-175), while one was a neutral antagonist (7929193), although none had as high an affinity. The results substantiate the notion that β-blockers do more than simply inhibit receptor activation, and differences between the ligands could provide useful tools to investigate receptor biology.

Abbreviations
HEK293, human embryonic kidney cells

Introduction

The β₂-adrenoceptor is one of the prototypical members of a superfamily of transmembrane proteins known as G-protein coupled receptors (GPCR). The β₂-adrenoceptor mediates the effects of catecholamines by predominantly coupling to the stimulating G-protein (Gs) and then subsequent activation of adenylate cyclase, increases in cAMP and activation of protein kinase A. More recently, signalling through the inhibitory G-protein (Gi) (Daaka *et al.*, 1997; Zamah *et al.* 2002) and G-protein-independent signalling have also been

demonstrated (Azzi *et al.*, 2003; Baker *et al.* 2003b). There is substantial evidence for the potential benefits of inhibiting β_2-adrenoceptor signalling in heart failure (CIBIS Investigators and Committees, 1994; Packer *et al.*, 1996; MERIT-HF Study Group, 1999). There is also evidence from both basic and clinical studies that action at β_2-adrenoceptors may be an important component of this beneficial effect (Poole-Wilson *et al.* 2003; Harding and Gong, 2004).

The recent description of crystal structures of the β_2- and β_1-adrenoceptors has given new insights into enduring issues in GPCR biology (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007; Warne *et al.*, 2008). This work is the potential springboard to understanding in molecular detail the basis of agonism, antagonism and inverse agonism and how ligand selectivity might be achieved between two similar GPCR. A series of ligands that are selective and have well-defined pharmacologies is clearly important for the successful implementation of such work.

There is a relative paucity of suitably selective β_2-adrenoceptor ligands for use as experimental tools to probe the receptor and to investigate the role of β_2-adrenoceptor-blockade in experimental and clinical settings. The experimental compound ICI-118,551 is the only β_2-adrenoceptor antagonist that is both potent and selective for the receptor (Bilski *et al.*, 1983; Baker, 2005). However, it has a complex pharmacological profile that includes inverse agonism for β_2-adrenoceptor-Gs signalling (Azzi *et al.*, 2003; Baker *et al.* 2003b), agonism for β_2-adrenoceptor-Gi (Gong *et al.*, 2002), activation of MAPK signalling (Azzi *et al.*, 2003), ion channel blockade (Teixeira *et al.*, 2004) and a direct antiapoptotic action (Mikami *et al.*, 2008). Therefore, experimental or clinical effects of ICI-118,551 may be obscured by events independent of simple blockade of β_2-adrenoceptor. Other problems with ICI-118,551 that make it ill-suited to *in vivo* or clinical studies are that it is extensively metabolized by the liver into less selective derivatives and is potentially carcinogenic (Fitzgerald, 1991).

We sought to identify novel ligands that are potent and selective β_2-adrenoceptor agents in order to generate pharmacological tools to elucidate the biological function of the receptor, while avoiding problems with the pharmacologies of existing ligands. Additionally, the role of β_2-adrenoceptors in diseases such as heart failure could be more effectively investigated with such ligands, which could potentially lead to novel pharmacotherapies. To identify novel ligands, compounds were first chosen for potential β_2-adrenoceptor antagonism using virtual field screening, a computational approach that predicts pharmacological similarities (Cheeseright *et al.*, 2006; 2008). These were then pharmacologically characterized for a number of β_2-adrenoceptor-associated parameters, such as potency, affinity and efficacy. Their selectivity for β_2-adrenoceptors over β_1-adrenoceptors was investigated by comparing binding affinity at each receptor. A second approach for selecting potential ligands was to synthesize structural derivatives of ICI-118,551 using information gained from the active compounds in the virtual field screen. Additionally, the enantiospecific binding properties of some of the ligands were investigated, as this was predicted to provide further information regarding β-adrenoceptor function.

Methods

Materials
(–)-Adrenaline (+)-bitartate, histamine dihydrochloride (±)-ICI-118,551 hydrochloride, papaverine hydrochloride (±)-CGP-12177, cAMP, forskolin, 3-isobutyl-1-methylxanthine, Whatman® GF/B filter papers, [^{3}H]-adenine, Dowex 50W × 4 hydrogen form 100–200 mesh and alumina oxide type WN-3 neutral were from Sigma-Aldrich (UK). cDNA clones encoding the genes for human β_1-adrenoceptor and β_2-adrenoceptor were from The Missouri S&T cDNA Resource Centre (USA). GFP-βarr2(1-380) DNA was a kind gift from Julie Pitcher (UCL). 25 mm diameter glass coverslips were from Menzel-Glaser. Cell culture materials were from Invitrogen (UK). (–)-[^{3}H]-CGP-12177 was from GE Healthcare (UK). Ultima Gold™ liquid scintillation cocktail was from Perkin Elmer (UK). Poly-prep chromatography columns were from BIO-RAD (UK). T0502-1048 was purchased from Enamine (Ukraine). The 5731869, 5217377 and 7929193 were purchased from Chembridge (UK).

Virtual field screening
Drugs with very different chemical structures can bind at the same biological site with the same pharmacological outcome. Biological binding sites recognize molecular fields and not structural detail (Cheeseright *et al.*, 2006). Virtual field screening was used to find new chemotypes by comparing the fields of a known active field template derived from ICI-118,551 to search through the fields of 3.4 M commercially available compounds (Cheeseright *et al.*, 2008). Forty compounds with the most similar field patterns to the template were purchased and tested for β_2-adrenoceptor activity and of these four were identified as active pharmacophores.

Synthesis
The details of the synthesis of analogues of ICI-118,551 are given in Appendix S1.

Organ bath studies
Methods were largely based on those described previously (Tanaka *et al.*, 2005). Male and female guinea pigs of 300–600 g weight were killed by CO_2 asphyxiation. The trachea was dissected and immersed in Krebs buffer (25 mM NaHCO$_3$, 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl, 11.1 mM D(+)-glucose). Excess connective and adipose tissue was removed and it was cut into eight separate rings of approximately 2 mm length. These were epithelium-denuded by passing rolled-up tissue paper through the lumen of each ring. Two wire hooks, one connected to a stable foot and the other to a Grass FT03 force-displacement transducer, were passed through the rings, and they were then suspended in 25 mL water-jacketed organ baths containing carbogen bubbled Krebs buffer at 37°C. Applied tension of 2.5 ± 0.1 g was established and the tissue equilibrated by incubation for 60 min, with replacement of buffer every 20 min. After this period, the rings were contracted with 100 μM histamine for 15 min, followed by replacement of buffer. The 60 min equilibration period was then repeated (including replacement of buffer every 20 min), before the rings were contracted once more with 100 μM histamine. Test compounds being inves-

tigated for β_2-adrenoceptor antagonism were added at this point. More than 30 min later, when the tissue had reached a steady state of contraction, the rings were relaxed by addition of cumulative concentrations of adrenaline from 1 nM to a concentration that elicited no further contraction, in half log unit increments. An adrenaline concentration was only applied when the previous concentration had caused a complete steady-state relaxation; 100 µM papaverine was then applied to induce maximal relaxation. The magnitude of each relaxant response from the histamine-induced contractile state was expressed as a percentage of the papaverine response, and concentration–response curves generated by fitting data to sigmoidal concentration–response (variable slope) curve function in GraphPad Prism 4. The adrenaline concentration–response curves in the presence of the range of antagonist concentrations used and control curves were compared globally using the Gaddum–Schild equation with GraphPad Prism 4 in order to determine pA_2 values (a measure of antagonist potency). Owing to the low Schild-plot slope values of the compounds in this analysis, the data were also re-analysed by plotting the log (dose ratio – 1) of only the lowest concentration of antagonist applied and constraining the slope to unity, as described previously (Kenakin, 2006).

Competition binding assay

Human embryonic kidney cells (HEK293) lines stably expressing the human β_1- or β_2-adrenoceptor at 1.64 and 6.64 fmol·µg^{-1} receptor protein, respectively, were developed using previously described methods (Giblin *et al.*, 1999). Once clonal isolates were established, cells were grown to confluency in 175 cm^2 flasks. Culture medium was aspirated and the cells washed twice with ice-cold binding buffer (50 mM TRIS-base, pH 7.4). Cells were harvested into the same buffer, then an equal volume of a hypotonic buffer (10 mM TRIS-base, 10 mM EDTA, pH 7.4) was added and the mixture left on ice for 10 min to hypotonically shock the cells. Tonicity was restored by further addition of an equal volume of a sucrose buffer (10 mM TRIS-base, 500 mM sucrose, pH 7.4). Cells were homogenized with >60 strokes of a glass-on-glass Dounce homogenizer. The resulting homogenate was centrifuged (600× g, 15 min, 4°C) to pellet large cell debris and nuclei. The supernatant was centrifuged (100 000× g, 60 min, 4°C) and the pellet suspended in binding buffer to give a membrane stock, which was stored at –20°C.

Membranes were then thawed and diluted to 7 and 2 µg·mL^{-1} for β_1- and β_2-adrenoceptor expressing cells respectively. These were incubated with 0.3 nM [^{3}H]-CGP-12177 (a roughly K_d concentration for this radioligand) in the presence of a range of concentrations of each test compound for 2 h at room temperature. This period of time is more than sufficient to allow equilibrium binding of the radioligand (Affolter *et al.*, 1985). Additionally, non-specific binding was determined with incubation of a saturating concentration of cold CGP-12177 (3 µM, which is approximately a thousand times more concentrated than its K_d at the receptors). The total volume of each binding reaction was 250 µL. Experiments for β_1- and β_2-adrenoceptors were done in parallel and each treatment was performed in triplicate. Receptor-bound and unbound radioligand were separated by vacuum filtration through Whatman® grade GF/B filter papers, followed by 4 × 2 mL washes with binding buffer. Filter papers were then

immersed in 5 mL scintillation cocktail and at least 15 h later disintegrations min^{-1} (dpm; binding signal) was measured for 2 min per sample with a Packard Tri-Carb 2100TR liquid scintillation counter. Specific binding was determined in each instance by subtraction of the average non-specific binding signal, and then expressed as a percentage of maximal binding. This was then plotted against the corresponding concentration of inhibitor compound. The Hill coefficients were not significantly different from one (not shown) and thus the data were fitted to one-site competition using non-linear regression analysis with GraphPad Prism 4. The Cheng–Prusoff equation was used to calculate K_i values from these curves, and the binding affinity of the compounds for each β-adrenoceptor subtype was expressed as an average pKi ± SD for the number of experiments. Saturation binding analyses of the two cell lines was conducted (see Appendix S1, $n = 3$ in triplicate) to provide K_d values for [^{3}H]-CGP-12177 at the β_1-adrenoceptor (0.28 ± 0.16 nM) and β_2-adrenoceptor (0.15 ± 0.02 nM) expressing cell lines for use in the Cheng–Prusoff equation. The β_2-/β_1-adrenoceptor selectivity ratio was calculated by dividing the K_i at β_1-adrenoceptor by that at β_2-adrenoceptor.

cAMP assay

Methods were largely based on those described previously (Salomon, 1991). β_2-Adrenoceptor expressing HEK293 cells (as described above) were grown to 80–90% confluency in 12-well cell culture plates and incubated in minimum essential media (MEM) containing 5 µCi [^{3}H]-adenine for 2 h at 37°C, 5% CO_2. Cells were then washed with warm PBS and stimulated with reduced serum medium containing 0.1 mg·mL^{-1} bovine serum albumin, 0.1 mM 3-isobutyl-1-methylxanthine, 30 µM forskolin and the desired concentration of test compound(s) for 20 min at 37°C, 5% CO_2. Basal cAMP levels were also determined for each experiment by incubating in the same media without forskolin or test compound. Each treatment was performed in triplicate. This media was then replaced by (1 mL per well) of a chilled 2.5% perchloric acid solution in dH$_2$O containing 1 mM cAMP. The plates were then kept at 4°C for 30 min with periodic agitation; 900 µL of the resulting extract was transferred to a test tube and 90 µL of 4.2 M potassium hydroxide and 400 µL dH$_2$O were added. In total, 100 µL of each sample was then added to a scintillation vial containing 5 mL scintillation cocktail to measure total [^{3}H]-adenine uptake. The remaining volume was transferred to a chromatography column containing 1.2 mL Dowex 50. Once eluted, this was washed with 3 mL dH$_2$O. The column was then directly suspended over a separate column containing 0.6 g alumina and 8 mL dH$_2$O added. Once this had eluted from the alumina column, the column was suspended directly over a scintillation vial containing 5 mL scintillation cocktail. [^{3}H]-cAMP was then eluted into these by addition of 5 mL elution buffer (100 mM TRIS-base, pH 7.5) to the column. At least 15 h later a dpm binding signal was measured for 2 min per sample with a Packard Tri-Carb 2100TR liquid scintillation counter, to determine the amount of [^{3}H]-cAMP. cAMP responses were calculated as % conversion of the [^{3}H]-cAMP extracted from total [^{3}H]-adenine uptake. For each treatment the basal cAMP signal was subtracted and expressed as a % of the response to forskolin alone.

β-Arrestin recruitment assay

The HEK293 cells were seeded onto six-well cell culture plates containing 25 mm diameter glass coverslips and grown to ~40% confluency. They were then simultaneously transiently transfected with DNA for human β_2-adrenoceptor (2 μg per well) and GFP-βarr2(1–380) (a kind gift from Dr Julie Pitcher) using the Lipofectamine™ method. Approximately 24 h later the cells were washed with 3×2 mL HEPES-buffered reduced serum media (opti-MEM without phenol red) and then incubated for 3 h at 37°C, 5% CO_2 in the same media. Each coverslip, with cells adhered to its surface, was then removed from the plate placed in a sealed confocal imaging chamber with 500 μL of the media. Images were taken with ×60 oil immersion objective with a Nikon Eclipse TE300 confocal microscope connected to a computer running Zeiss Laser-Sharp 2000™ software. A single laser line excitation at a wavelength of 488 nm was used, and images were acquired with a LP500 filter. Test ligand was added to a final concentration of 100 μM and the coverslip (remaining in the chamber and covered) was incubated at 37°C, 5% CO_2 for 15 min before further images were taken of the cells. High quality images of a number of cells both before and after ligand treatment were acquired to investigate the effect of ligand exposure on GPF-βarr2(1–380) localization. At least 10 ligand-treated cells were examined thoroughly for a positive response. Each experiment was repeated at least twice.

Molecular modelling

All molecular mechanics modelling used the eXtended Electron Distribution (XED) force field (Vinter, 1994; Chessari et al., 2002; Cejas et al., 2008). Structures were generated using the in-house package 'XEDRAW' and the Discovery Studio Visualiser from Accelrys. Commercially available molecular dynamics and docking software produce less optimal results due to the use of monopolar electrostatics that mishandle aromatic interactions and hydrogen bond directions amongst others. The XED force field used electrostatic multipoles that avoid such approximations

Nomenclature

All drug and receptor nomenclature conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander et al., 2009).

Results

Pharmacological characterization of compounds identified using virtual field screening

Four novel β_2-adrenoceptor antagonists, T0502-1048, 5731869, 5217377 and 7929193 (Figure 1) were identified following a virtual field screen (see *Methods*). These were investigated using Gaddum–Schild analysis, whereby concentration-dependent increases in antagonism are used to quantify antagonist affinity (pA2 values). Adrenaline-mediated relaxation of guinea-pig tracheal smooth muscle was used for these experiments as this is predominantly a β_2-adrenoceptor-driven response (Tanaka et al., 2005). ICI-118,551 was analysed for comparison, and was found to have a pA2 value of 9.49 (Table 1). The novel compounds had pA2 values ranging from 6.63 to 7.78, with 5731869 having the highest affinity (Table 1). The Schild-plot slopes were below unity in all instances (Table 1), which has been proposed to invalidate the analysis (O'Donnell and Wanstall, 1979; Kenakin, 1997). Therefore, an adapted method for pA2 derivation (Kenakin, 2006) was also used as an alternative to obtain the antagonist affinities of these ligands. This involved plotting a linear slope of unity through the log (dose ratio –
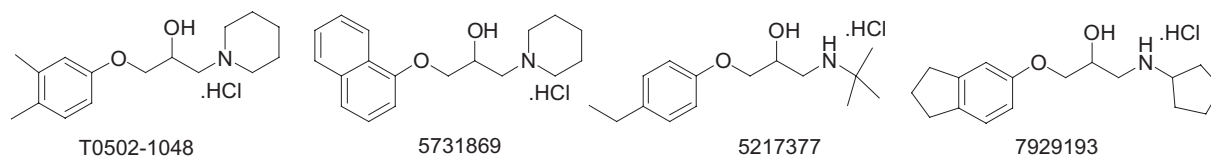

Figure 1

The chemical structures of active compounds isolated using a virtual field screen.

Table 1

Gaddum–Schild analysis of ICI-118,551 and the compounds in Figure 1 in the guinea pig trachea

Compound	n	pA2	Schild-plot slope	pA2 (adapted analysis)
ICI-118,551	4	9.49 ± 0.14	0.61 ± 0.04	8.75
T0502-1408	3	6.63 ± 0.20	0.68 ± 0.09	6.38
5731869	4	7.78 ± 0.17	0.73 ± 0.07	8.06
5217377	4	7.52 ± 0.13	0.88 ± 0.07	7.75
7929193	4	7.01 ± 0.08	0.72 ± 0.03	6.97

Table 2

[^{3}H]-CGP-12177 competition binding data for ICI-118,551 and the compounds in Figure 1

Compound	n	pK$_i$ β$_1$-Adrenoceptor	β$_2$-Adrenoceptor	β$_2$-Adrenoceptor selectivity (ratio)
ICI-118,551	3	6.61 ± 0.09	9.47 ± 0.05	730.87
T0502-1048	3	4.96 ± 0.20	5.99 ± 009	10.79
5731869	3	6.83 ± 0.08	7.68 ± 0.04	7.14
5217377	3	6.04 ± 0.12	7.06 ± 0.07	10.48
7929193	3	6.12 ± 0.11	6.63 ± 0.07	3.24

1) of only the lowest concentration of test compound applied. The re-analysis yielded a decrease in the pA$_2$ values of the ligands and had an effect on their rank order of affinity (Table 1). Compared with control adrenaline concentration–response curves, none of the novel compounds (at the highest concentration applied) had an effect on the maximal relaxation response to adrenaline (ANOVA Dunnett's test, $P >$ 0.05, $n = 3–4$). However, ICI-118,551 caused a small but significant drop in the ability of adrenaline to elicit a maximal response (ANOVA Dunnett's test, $P < 0.05$, $n = 4$).

The interactions of these compounds with β$_2$-adrenoceptors were confirmed by their ability to displace the radioligand [^{3}H]-CGP-12177 to membranes isolated from HEK293 cells stably expressing the receptor. The Cheng–Prusoff correction (Cheng and Prusoff, 1973) was used to calculate the binding affinity of these compounds by the derivation of pK$_i$ values from the concentration-dependent displacement of [^{3}H]-CGP-12177 binding to these membranes. The pK$_i$ values ranged from 5.99 to 7.68, with 5731869 having the highest affinity (Table 2). In comparison, ICI-118,551 had a higher affinity than these compounds (pK$_i$ = 9.47; Table 2).

In order to establish β$_2$-adrenoceptor selectivity, the binding affinity for β$_1$-adrenoceptors was also determined for comparison, using HEK293 cells stably expressing this receptor. For all four compounds, the average pKi value at β$_2$-adrenoceptors was significantly higher compared with β$_1$-adrenoceptors (unpaired t-test, $P \leq 0.05$, $n = 3–5$; Table 2), which identified them as β$_2$-selective ligands. The β$_2$-adrenoceptor selectivity ratios ranged from <4 to >10, with T0502-1048 being the most selective (Table 2). ICI-118,551 was much more selective, with a >700-fold binding preference for β$_2$-adrenoceptors (Table 2). Sample competition binding curves are shown in Figure 2.

The ability of ICI-118,551 and the new compounds to influence intracellular cAMP levels was investigated to determine whether they were inverse agonists, partial agonists or neutral antagonists through β$_2$-adrenoceptors. HEK293 cells stably expressing β$_2$-adrenoceptors were stimulated with 30 μM as it activates adenylate cyclase in a manner that is synergistic with Gs and therefore increases the sensitivity of the assay to changes in Gs-coupling and constitutive receptor activity (Sutkowski *et al.*, 1994; Alewijnse *et al.*, 1997). This made the detection of inverse and partial agonist responses easier. Co-incubation with ICI-118,551, T0502-1048 or 5731869 caused a decrease in the ability of forskolin to elicit

a cAMP response (ANOVA Dunnett's test, $P < 0.01$, $n = 3$; Figure 3), which identified them as inverse agonists. In contrast, 5217377 significantly increased the forskolin response (ANOVA Dunnett's test, $P < 0.01$, $n = 3$; Figure 3) and therefore acted as a partial agonist. The ligand 7929193 acted as a neutral antagonist as it had no effect on forskolin-stimulated cAMP response (ANOVA Dunnett's test, $P > 0.05$, $n = 3$; Figure 3); 100 μM of each ligand was used in these experiments as this was predicted to achieve saturated receptor binding and maximal cAMP responses. Importantly, the respective inverse and partial agonist responses of ICI-118,551 and 5217377 were both sensitive to antagonism by 7929193 (Figure 4), confirming that they are mediated by β$_2$-adrenoceptors, conform to the predictions of receptor activation models and that 7929193 is a true antagonist. Both produced significantly different cAMP responses when in the presence of the antagonist (unpaired t-test, $P < 0.05$, $n = 3$; Figure 4). In these experiments 100 μM 792913 and 1 μM of the efficacious ligands were used to optimize the detection of antagonism.

The compound's ability to evoke a β-arrestin recruitment response at β$_2$-adrenoceptors was also investigated by imaging the subcellular translocation of a fluorescently tagged β-arrestin protein [GFP-βarr2(1–380)]. HEK293 cells were co-transfected with β$_2$-adrenoceptors and the GFP-βarr2(1–380) reporter, and imaged with a confocal microscope. In transfected cells, GFP-βarr2(1–380) was predominantly located in the nucleus with a lesser, but still robust, cytoplasmic signal (Figure 5). After a 15 min incubation (at 37°C) with 100 μM ICI-118,551 or any of the compounds, no change in the subcellular location of GFP-βarr2(1–380) was identified (Figure 5). As a positive control, the ability of 100 μM adrenaline to elicit a response was tested. This treatment produced a noticeable subcellular translocation of GFP-βarr2(1–380) from the cytoplasm to the plasma membrane (Figure 5) in the majority of transfected cells (>75%), which was taken as evidence for β$_2$-adrenoceptor-mediated β-arrestin recruitment. ICI-118,551 inhibited the ability of adrenaline to evoke this response (Figure 5), which suggests it acts as an antagonist.

Pharmacological characterization of some structural derivatives of ICI-118,551

The compounds identified in the virtual field screen lacked desirable levels of potency, affinity and selectivity for β$_2$-adrenoceptors. However, they displayed some notable
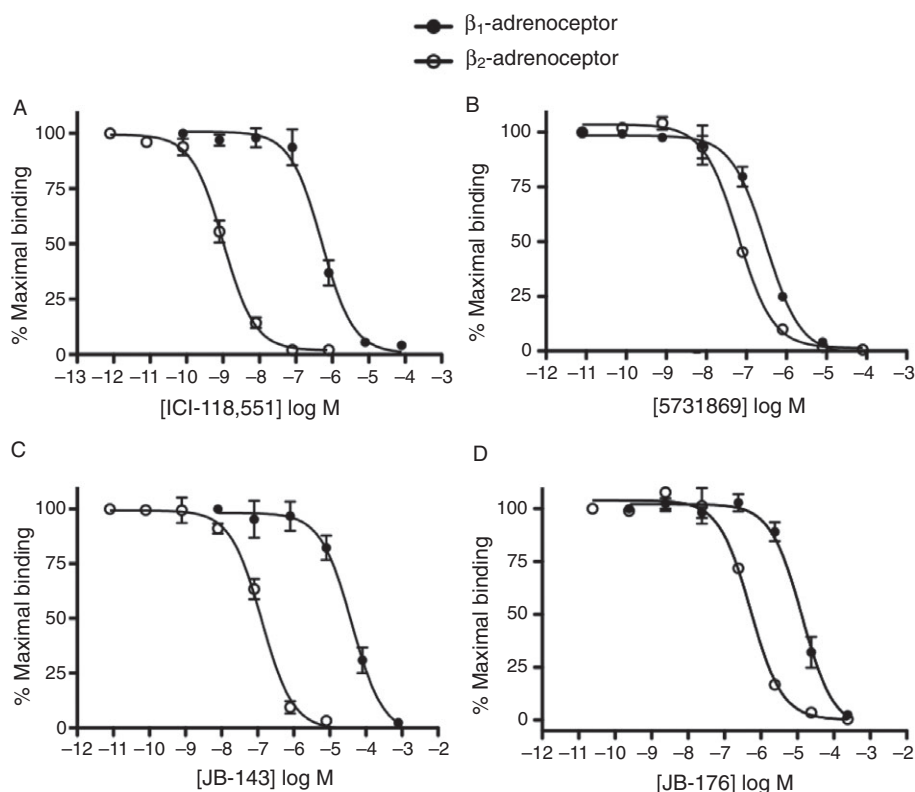
● β₁-adrenoceptor
○ β₂-adrenoceptor

Figure 2

[^{3}H]-CGP-12177 competition binding curves. These curves show the ability of ICI-118,551 (A), 5731869 (B), JB-143 (C) and JB-176 (D) to concentration-dependently displace [^{3}H]-CGP-12177 binding to β₁- and β₂-adrenoceptors. The curves are an average ± SEM of three experiments performed in triplicate.

Table 3

Gaddum–Schild analysis of structural derivatives of ICI-118,551 in the guinea pig trachea

Compound	n	pA$_2$	Schild-plot slope	pA$_2$ (adapted analysis)
JB-141	4	8.86 ± 0.12	0.49 ± 0.02	8.45
JB-142	3	8.36 ± 0.24	0.49 ± 0.04	8.09
JB-143	3	8.38 ± 0.10	0.57 ± 0.02	7.78
JB-163	3	8.93 ± 0.41	0.26 ± 0.03	7.20
JB-175	3	9.06 ± 0.16	0.50 ± 0.03	8.15
JB-176	3	7.23 ± 0.11	0.57 ± 0.03	6.81

structural features, including a piperidine ring in the case of T0502-1048 and 5731869 that are tolerated by β-adrenoceptors. We used this finding to design novel analogues of ICI-118,551 which would incorporate the piperidine or related cyclic amines, while retaining the core structure of ICI-118,551. Thus, six structural derivatives of ICI-118,551 were synthesized (JB-141, JB-142, JB-143, JB-163, JB-175, JB-176; Figure 6).

Gaddum–Schild analysis suggested that these compounds had higher pA$_2$ values compared with the compounds identified by virtual field screening, although none were as

potent as ICI-118,551. The pA$_2$ values ranged from 7.23 to 9.03, with JB-175 displaying the highest affinity (Table 3). All the compounds produced Schild-plot slopes of below unity, with particularly low values of <0.5 for JB-141, JB-142 and JB-163 (Table 3). Re-analysis using only low concentrations of antagonist produced lower pA$_2$ values and changed the rank order of potency of the compounds (Table 3). An example of the concentration-dependent antagonism that produces a low Schild-plot slope value is shown for JB-175 in Figure 7. In contrast to ICI-118,551, none of its structural derivatives had an effect on the ability of adrenaline to elicit

Table 4

[³H]-CGP-12177 competition binding data for structural derivatives of ICI-118,551

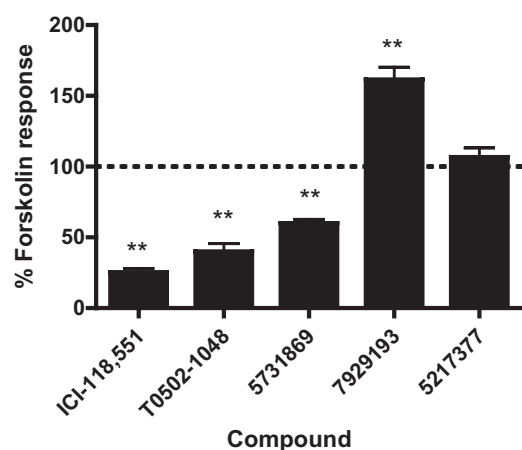| Compound	n	pK$_i$ β$_1$-Adrenoceptor	β$_2$-Adrenoceptor	β$_2$-Adrenoceptor selectivity (ratio)
JB-141	3	5.05 ± 0.14	7.74 ± 0.05	487.86
JB-142	3	5.12 ± 0.18	7.09 ± 0.06	92.04
JB-143	3	4.71 ± 0.12	7.37 ± 0.06	455.95
JB-163	4	5.64 ± 0.10	7.20 ± 0.07	36.55
JB-175	5	5.15 ± 0.07	7.84 ± 0.06	482.26
JB-176	3	5.19 ± 0.09	6.76 ± 0.09	37.55

Figure 3

Efficacy of ICI-118,551 and the Cresset compounds for β$_2$-adrenoceptor-mediated cAMP signalling. HEK239 cells stably expressing β$_2$-adrenoceptors were co-incubated with 30 μM forskolin and 100 μM of each compound, and the resulting cAMP response is expressed as a percentage of the response to forskolin alone. Responses above and below 100% (dotted line) signified partial and inverse agonism respectively. Antagonism was signified by no deviation from this response. **$P < 0.01$ compared with forskolin alone. Data are an average ± SEM of three experiments performed in triplicate.

Figure 4

Antagonism of inverse and partial agonists for the β$_2$-adrenoceptor-mediated cAMP pathway. β$_2$-adrenoceptor-expressing HEK293were incubated with 30 μM forskolin and stimulated with 1 μM ICI-118,551, JB-143 or 5217377 with or without 100 μM 7929193. The resulting cAMP responses from the treatments identified 7929193-mediated antagonism of both inverse and partial agonism. *$P < 0.05$ comparing the effect of antagonist on each ligand response. Data are an average ± SEM of three experiments performed in triplicate.

a maximal relaxant response (ANOVA Dunnett's test, $P > 0.05$, $n = 3$–4).

In [³H]-CGP-12177 competition binding experiments the structural derivatives of ICI-118,551 bound with a range of affinities to β$_2$-adrenoceptors, although none had as high a binding affinity as ICI-118,551 Table 4). JB-175 had the highest affinity with a pK$_i$ of 7.84. These compounds all showed good β$_2$-adrenoceptor selectivity with >30-fold to >480-fold binding preferences (Table 4). JB-141 was the most selective, although this did not reach the high levels of selectivity achieved with ICI-118,551.

In the cAMP assay, 100 μM of all the structural derivatives of ICI-118,551 significantly inhibited the ability of forskolin to elicit a response (ANOVA Dunnett's test, $P < 0.01$, $n = 3$; Figure 8), thus identifying them as inverse agonists. The sen-
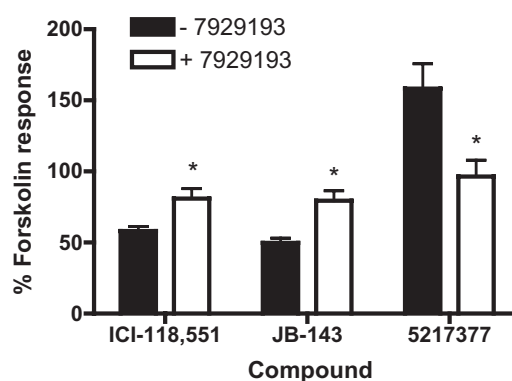
sitivity of the inverse agonism of JB-143 to receptor antagonism (with 7929193) was tested, and its negative responses were significantly less than without antagonism (unpaired t-test, $P < 0.05$, $n = 3$; Figure 4). This was taken as evidence that these are true inverse agonist effects. The pertussis toxin (PTX)-sensitivity of inverse agonism with ICI-118,551 and JB-143 was investigated to determine whether specific β$_2$-adrenoceptor-Gi-coupling with these ligands plays a role in the negative responses. PTX-pretreatment had an effect on the ability of forskolin to elicit a cAMP response alone, so this difference was taken into account when comparing PTX-treated and untreated cells. The negative response to ICI-118,551 or JB-143 was unaffected by PTX (unpaired t-test, $P > 0.05$, $n = 3$; Figure 9). Equivalent inverse agonist, partial agonist and neutral antagonist effects of the novel ligands, were also identifiable without the use of forskolin stimulation (data not shown).

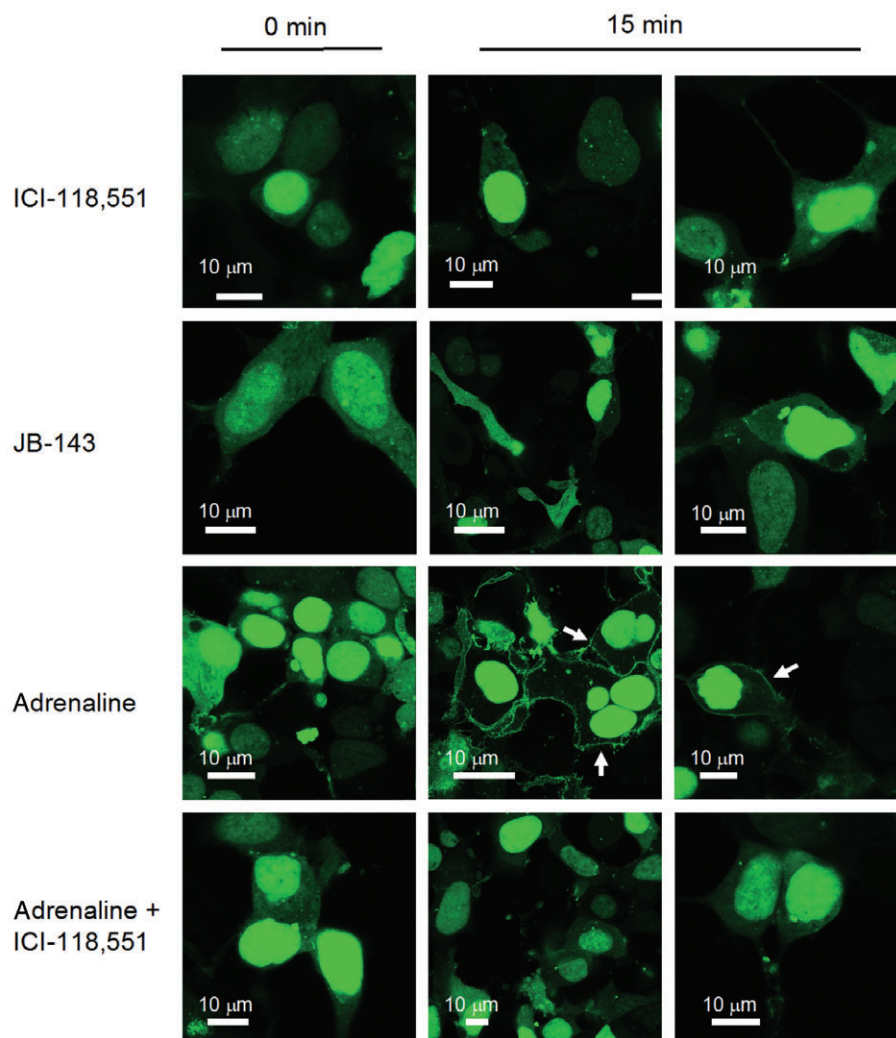None of the derivatives of ICI-118,551 had an effect on the subcellular localization of GFP-βarr2(1–380) (Figure 5).

Figure 5

Ligand-mediated regulation of β2-adrenoceptor-β-arrestin interactions. HEK293 cells were transiently transfected with β2-adrenoceptors and GFP-βarr2(1–380), and subcellular localization of fluorescence was imaged by confocal microscopy. One image before (0 min) and two after (15 min) incubation with ligand are shown. Positive responses (membrane translocation) are highlighted with arrows. Images are a representation of at least 10 groups of cells from at least two separate experiments.
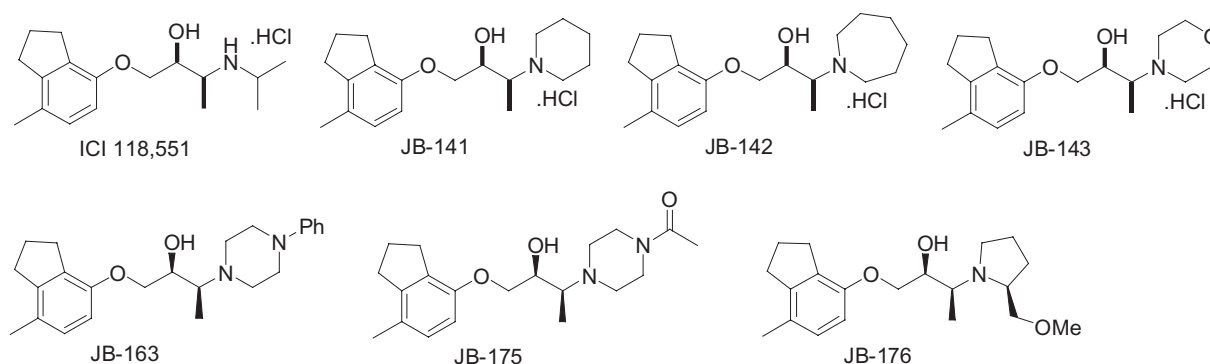

Figure 6

The chemical structures of ICI-118,551 and its derivatives. The compounds JB-163, JB-175 and JB-176 were synthesized and pharmacologically analysed as 2S,3S enantiopure preparations. The remaining compounds are racemates.
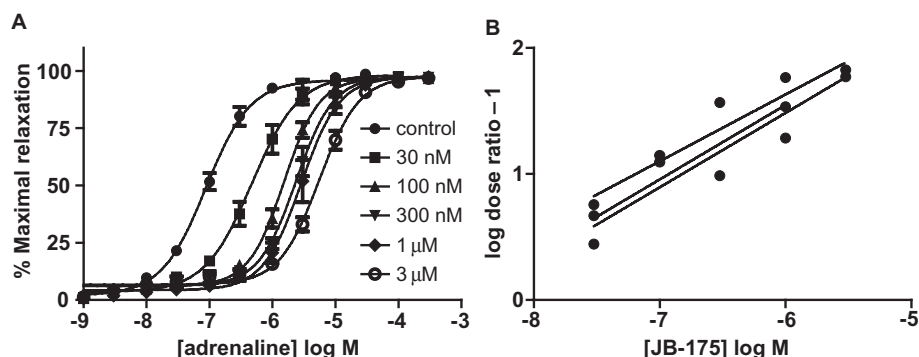
Figure 7

JB-175-mediated antagonism of adrenaline responses in the guinea-pig trachea. (A) Increasing concentrations of JB-175 produce increasing rightward shifts in the concentration–response curve to adrenaline. (B) The Schild-plot of these responses constructed by plotting log (dose ratio – 1) by concentration of JB-175. Results of each individual experiment are plotted together with their respective linear regression lines. This illustrates the low slope of these curves.
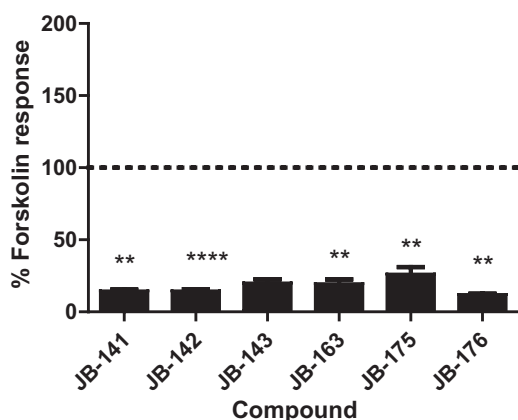

Figure 8

Efficacy of the structural derivatives of ICI-118,551 for β_2-adrenoceptor-mediated cAMP signalling. HEK239 cells stably expressing β_2-adrenoceptors were co-incubated with 30 μM forskolin and 100 μM of each compound, and the resulting cAMP response is expressed as a percentage of the response to forskolin alone. Responses above and below 100% (dotted line) signify partial and inverse agonism respectively. Antagonism was signified by no deviation from this response. **$P < 0.01$ compared with forskolin alone. Data are an average ± SEM of three experiments performed in triplicate.

This suggested that they were not efficacious for this β_2-adrenoceptor pathway, as was the case with the other β-blockers tested.

Stereoselective binding properties of JB-141, JB-143 and JB-175

The stereochemistry of the most potent and selective novel ligands JB-141, JB-143 and JB-175 were investigated with regards to their β-adrenoceptor binding affinities and selectivities, as determined previously for ICI-118,551 (Baker et al., 2010). Racemic (2S,3S)-enantiopure and (2R,3R)-enantiopure preparations of these ligands were subjected to [³H]-CGP-
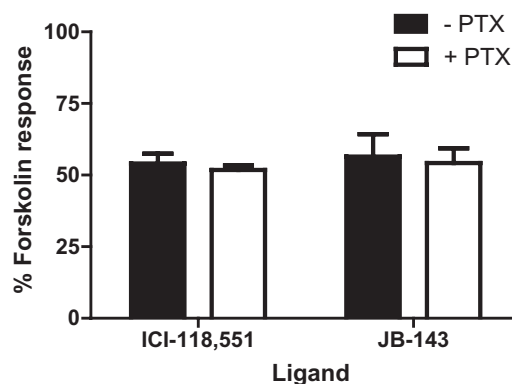
Figure 9

The effect of pertussis toxin (PTX) on the negative cAMP responses of ICI-118,551 and JB-143. HEK239 cells stably expressing β_2-adrenoceptors were pretreated with or without 10 ng·mL⁻¹ PTX for 15 h. They were then co-incubated with forskolin and ICI-118,551 or JB-143, and cAMP responses measured. As PTX had an effect on forskolin responses alone, each ligand-mediated response is expressed as a percentage of the respective forskolin response with or without PTX. Data are an average ± SEM of three experiments performed in triplicate.

12177 competition binding analysis. The (2S,3S) enantiomers of these compounds had far higher affinity for both β_1- and β_2-adrenoceptors than the antipode (ANOVA Bonferroni's test, $P < 0.001$, $n \geq 3$, Table 5). Interestingly, the relative enantiospecific differences in binding affinity appeared to be non-equivalent at the two receptors, which had an effect on the degree of β_2-adrenoceptor selectivity with the enantiopure preparations compared with the racemates (Table 5). The (2S,3S) enantiomers were less selective than the racemates because the relative increases in β_1-adrenoceptor binding were greater than that at β_2-adrenoceptor. Conversely, the (2R,3R) enantiomers were also less selective than the racemates because the relative drops in β_2-adrenoceptor binding were greater than at β_1-adrenoceptors. Therefore, in all instances the racemate was the most selective for

Table 5

[³H]-CGP-12177 competition binding analysis with racemic and enantiopure preparations of some β_2-adrenoceptor selective β-blockers

Compound (preparation)	n	pK$_i$ β_1-Adrenoceptor	β_2-Adrenoceptor	β_2-Adrenoceptor selectivity (ratio)
JB-141				
Racemate	3	5.05 ± 0.14	7.74 ± 0.05	488
(2S,3S)-enantiomer	4	5.70 ± 0.11	8.03 ± 0.08	213
(2R,3R)-enantiomer	3	4.02 ± 0.45	6.37 ± 0.12	222
JB-143				
Racemate	3	4.71 ± 0.12	7.37 ± 0.06	456
(2S,3S)-enantiomer	3	4.98 ± 0.09	7.54 ± 0.05	364
(2R,3R)-enantiomer	3	<3.94	5.92 ± 0.05	>95
JB-175				
Racemate	4	3.98 ± 0.44	6.97 ± 0.10	960
(2S,3S)-enantiomer	5	5.15 ± 0.07	7.84 ± 0.06	482
(2R,3R)-enantiomer	4	3.94 ± 0.47	6.42 ± 0.12	295

The β-adrenoceptor binding affinities (pK$_i$) of racemic, (2S,3S)- and (2R,3R)-enantiopure preparations of JB-141, JB-143 and JB-175 were calculated. From these values, β_2-adrenoceptor-selectivty was derived.

β_2-adrenoceptors. In previous experiments, JB-175 was characterized as a (2S,3S) enantiomer, and upon investigation of the racemate this was found to be more selective for β_2-adrenoceptors than ICI-118,551, having a >950-fold binding preference for this receptor.

Discussion and conclusions

Summary

In an effort to develop novel β_2-adrenoceptor-selective ligands we initially employed virtual field screening. The four novel ligands identified through this route had a variety of efficacies (inverse agonism, partial agonism and neutral antagonism) but showed only modest antagonism, affinity (low μM range) and selectivity (at most 10-fold), and were therefore not promising leads in their own right. However, analysis of the structure of T0502-1048 and 5731869 served to reveal to us that cyclic amines could be tolerated by the β_2-adrenoceptor. We chose to combine this structural motif with the core of ICI-118,551 and we felt confident of accessing new β_2-selective antagonists in this manner. We thus synthesized six novel ICI-118,551 analogues which varied just in the nature of cyclic amine (JB-141, JB-142, JB-143, JB-163, JB-175, JB-176; Figure 6). We found that they were all more pharmacologically similar to ICI-118,551, showing generally greater antagonism, higher affinity, moderate-to-good selectivity and inverse agonism. One of the compounds (racemic JB-175) had higher β_2-adrenoceptor selectivity than ICI-118,551.

Pharmacology

The ligands inhibited adrenaline-mediated responses in the guinea-pig trachea and this response was attributed to β_2-adrenoceptor antagonism because this receptor predominates in this tissue (Carswell and Nahorski, 1983; Tanaka

et al., 2005). Gaddum–Schild analysis suggested that JB-175 had the highest affinity of the novel antagonists (pA$_2$ = 9.06), although this was not as high that for ICI-118,551. These compounds had low Schild-plot slope values and the responses were re-analysed by an alternative method. This produced lower pA$_2$ values and affected the rank order of antagonist affinity of these ligands. Low Schild-plot slope values in the guinea-pig trachea have been demonstrated to be influenced by the use of non-selective β-adrenoceptor agonists, a heterogeneous β-adrenoceptor population in the tissue, and an effect of tissue precontraction, while saturable agonist uptake sites and agonist degradation are also possible contributory factors (O'Donnell and Wanstall, 1979; 1980;Keith et al., 1986; Christopoulos and Kenakin, 2002). A likely explanation for the low slope values in this assay is that at higher concentrations adrenaline activates additional receptor subtypes to drive the functional responses and thus invalidate the assumption of the Schild equation that the agonist and antagonist are acting through a single receptor site. As it has been suggested that the β_1-adrenoceptor is present in this tissue (O'Donnell and Wanstall, 1979; Tanaka et al., 2007), this is the most likely candidate. The observation that the slope values of the novel compounds are relatively variable between the ligands (0.26–0.88; Tables 1 and 3), suggests that pharmacological differences between them may account for at least part of this effect on the analysis. Allosteric receptor interactions, inverse or partial agonism, and off target effects have been identified as potential mechanisms for an unusual Schild-plot, as they can interfere with the assumption that the test compound acts as a neutral competitive antagonist (Ghosh et al., 1999; Christopoulos and Kenakin, 2002). Interestingly, ICI-118,551 acted as an insurmountable antagonist owing to its ability to decrease the maximal responses attainable by the agonist. This is in agreement with a number of previous reports (Hopkinson et al., 2000) and substantiates the notion that unusual

pharmacological properties of β-blockers may influence their antagonist effects.

Radioligand competition binding experiments were used to determine the β-adrenoceptor binding affinities and the β_2 selectivity of the ligands. All the ligands showed a binding preference for β_2- over β_1-adrenoceptors, although this varied greatly. ICI-118,551 had high affinity and selectivity for β_2-adrenoceptors. None of the novel compounds were an improvement on ICI-118,551 in terms of these parameters, although JB-141, JB-143 and JB-175 demonstrated impressive selectivity levels (over 450-fold). Although β_2-adrenoceptor-selective blockade is therefore attainable with these ligands, a far higher concentration compared with ICI-118,551 is needed to achieve this, owing to their lower affinities for β_2-adrenoceptors.

The ligands were tested for efficacy at two β_2-adrenoceptor-mediated pathways; Gs-cAMP and β-arrestin. Within the context of receptor activation models, efficacy is influenced by a ligand's ability to disturb the equilibrium between active (R*) and inactive (R) receptor conformations and therefore evoke a signalling response (Christopoulos and Kenakin, 2002). ICI-118,551 and most of the novel compounds were inverse agonists as their activity at β_2-adrenoceptors caused a decrease in cellular cAMP levels suggesting a selective interaction with R. The 5217377 evoked a positive cAMP response, which suggests that this ligand selectively interacts with and enriches R* and therefore activates the receptor. The 7929193 had no effect on cellular cAMP, which was evidence that it has no binding preference for R or R*, does not influence the receptor equilibrium and is a neutral antagonist. This ligand was particularly interesting as receptor/ligand modelling systems and experimental work have led to the hypothesis that neutral antagonism is a rare occurrence (Kenakin, 2004).

Some β-blockers have been demonstrated to have positive effects (are agonists) for a number of β_2-adrenoceptor-mediated signalling pathways including β-arrestin recruitment, activation of different MAPK cascades and/or Gi-coupling (Azzi et al., 2003; Baker et al., 2003b; Zheng et al., 2005; Wisler et al., 2007; Drake et al., 2008; Wenzel et al., 2009). That these occur in a ligand-specific manner is evidence that there are multiple active β_2-adrenoceptor conformations that differentially interact with β-adrenoceptor ligands. None of these compounds affected the subcellular localization of β-arrestin or had a Gi dependence of action. Indeed, the ability of ICI-118,551 to inhibit adrenaline-mediated β-arrestin responses suggests it acts as an antagonist. These findings substantiate previous reports that ICI-118,551 is without effect alone and can block the effects of efficacious ligands (Wisler et al., 2007; Drake et al., 2008), although they contradict the findings of Azzi et al. (2003), who demonstrated a positive β-arrestin response with this ligand. It is probable that differences in cellular context and assay sensitivity underlie these different observations. For example, putative β-blocker-mediated β-arrestin responses may be smaller than those to agonists, and more sensitive experimental approaches, such as DiscoveRx (McGuinness et al., 2009) or fluorescence resonance energy transfer/bioluminescence resonance energy transfer (Azzi et al., 2003; Wisler et al., 2007), could demonstrate positive responses to these ligands. The efficacy of β-blockers for β_2-adrenoceptor-β-arrestin signalling is impor-

tant as it has been suggested that the improved clinical effects of carvedilol in heart failure pharmacotherapy are influenced by its ability to evoke a β-arrestin response (Wisler et al., 2007).

Stereochemistry

The stereoselectivity of β-blockers is well established for the β_1-adrenoceptor with the (S) enantiomers usually showing greater activity than their antipodes (Morris and Kaumann, 1984; Wahlund et al., 1990). There are data showing that stereoisomers of fenoterol, a β_2 selective agonist, may couple to different G-protein signalling pathways (Woo et al., 2009). It is unclear what the effect of varying the stereochemistry is on inverse agonists/antagonists at the β_2-adrenoceptor. We therefore tested differences between racemates and individual enantiomers of the most promising novel compounds (JB-141, JB-143, JB-175). Radioligand binding experiments provided strong evidence that the (2S,3S) enantiomers of these ligands bound with the highest affinity. Unusually, however, racemic preparations were the most selective for the β_2-adrenoceptor, and this is in line with our findings on ICI-118,551 itself (Baker et al., 2010). This can be explained by the fact that the relative enantiospecific differences in β_1- and β_2-adrenoceptor binding affinity are not equivalent: the gain in affinity of the (2S,3S) enantiomer is greater at β_1-adrenoceptor while the drop in affinity of the (2R,3R) enantiomer is greater at β_2-adrenoceptor. This occurrence is puzzling as the racemates comprise an equimolar combination of the two enantiomers, and their activity is therefore predicted to be an average of these (i.e. an intermediate selectivity). This is clearly not the case, which suggests that some form of functional interaction between the two enantiomers causes their respective binding affinities to be quantitatively different when they are in combination in the racemate. A proposed mechanism for this effect could involve enantiospecific allosteric interactions with either receptor that alters the orthosteric binding properties. An allosteric binding site on β_1-adrenoceptor with affinity for β-blockers has been identified (Baker et al., 2003a; Joseph et al., 2004), while we know of no evidence that such a site exists on β_2-adrenoceptor. This perhaps makes β_1-adrenoceptors a more likely candidate for allosteric interactions. The unusual stereospecific β_2-adrenoceptor selectivities of these ligands may provide novel insights into the activity of racemic preparations of β-blockers, and suggests that the highest receptor selectivity is attainable with the racemate. Indeed, JB-175, was found to be the most selective β_2-adrenoceptor ligand when tested as a racemate, which suggests that it may have a better selectivity than ICI-118,551.

Molecular modelling

In a recent study, the available crystal structures of β_2-adrenoceptors were used to screen chemical libraries using docking based approaches to identify novel ligands (Kolb et al., 2009). They succeeded in doing so although most were inverse agonists and they did not look at β_1/β_2-adrenoceptor selectivity. It is telling that the current crystal structures have been elucidated in the presence of an inverse agonist (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007; Warne et al., 2008). Thus we attempted to model the binding of JB-175 with the published X-ray structures [from

Table 6

Computed binding enthalpies of selected β1- and β2-adrenoceptor ligands

Ligand	Major action	Total	β₂-Adrenoceptor 2RH1			β₁-Adrenoceptor 2VT4			
			LB	coul LB	vdw LB	Total	LB	coul LB	vdw LB
None		2760				2677			
Antagonists									
Cyanopindolol	β₁/β₂-adrenoceptor Ant	2716	−91.8	−26.2	−65.6	2633	−92.2	−27.9	−64.8
Carazolol	β₁/β₂-adrenoceptor In Ag	2723	−98.3	−23.9	−74.4	2641	−90.6	−20.7	−69.9
ICI118551	β₂-adrenoceptor In Ag	2727	−83.6	−12.3	−71.3	2620	−75.2	−8.5	−66.7
JB-175	β₂-adrenoceptor In Ag	2743	−86.7	−12.2	−74.5	2637	−82.1	−9.7	−72.4
JB-143	β₂-adrenoceptor In Ag	2718	−84.4	−13.4	−70.9	2597	−84.7	−7.9	−76.8
Atenolol	β₁-adrenoceptor In Ag	2708	−78.7	−22.6	−56.1	2629	−84.1	−28.4	−55.7
Propranolol	β₂-adrenoceptor In Ag	2699	−85.5	−18.2	−67.2	2622	−81.7	−17.8	−63.8
Agonists									
Salbutamol	β₂-adrenoceptor Ag	2702	−72.8	−19.1	−53.7	2621	−66.3	−14.4	−51.9
Prenalterol	β₁-adrenoceptor Ag	2712	−72.2	−20.9	−51.3	2628	−67.7	−16.6	−51.1
FenoterolRR	β₂-adrenoceptor Ag	2694	−89.4	−17.9	−71.5	2629	−77.4	−15.9	−61.6
FenoterolSR	β₂-adrenoceptor Ag	2687	−94.1	−24.4	−69.7	2628	−77.9	−13.4	−64.5
Adrenaline	β₁/β₂-adrenoceptor Ag	2727	−60.1	−16.4	−43.7	2632	−55.6	−16.6	−39

Each of the 12 ligands in column 1 were placed into the two receptor X-ray structures in place of their native ligands and optimized to a minimum overall energy (columns 3 & 7) using the eXtended Electron Distribution molecular mechanics force field. Once optimized, the overall ligand binding enthalpy was calculated (LB columns 4 & 8) as two contributions; electrostatic (coulombic) enthalpy (coul LB columns 5 & 9) and van der Waals (hydrophobic) enthalpy (vdw LB columns 6 & 10). Antagonist, inverse agonist and agonist are abbreviated to Ant, In Ag and Ag respectively.

protein data bank (PDB) entries 2RH1.PDB (β₂-adrenoceptor/ Carazolol) and 2VT4.PDB (β₁-adrenoceptor/Cyanopindolol)]. Over a dozen ligands were incorporated into each receptor and computationally 'relaxed' to their lowest achievable energetic stable state. The act of computationally optimizing ligands in each receptor yields enthalpies that may indicate the strengths of ligand binding from which selectivity may be inferred. Because real binding strengths include entropy, solvation and other dynamic effects, the calculated enthalpies are approximate, but scrutiny of Table 6 suggests a correlation between major biological action and calculated binding strength. For example, propranolol, which has a small experimental β₂ preference, is electrostatically bound by −18.2 kcal·mol⁻¹ at β₂ and by −17.8 kcal·mol⁻¹ at β₁. Atenolol shows the expected β₁ preference and both tight binders; carazolol and cyanopindolol have strong binding enthalpies. Furthermore the selectivity of ICI-118,551 (and JB compounds) for β₂ over β₁ is also predicted from these calculations. Figure 10 shows the two β-receptors both containing JB-175 after molecular mechanics relaxation over the entire ensembles along with the superposition of the structures by least squares fit of the Cα backbone atoms showing little conformational variation of the backbone or the ligand position (Figure 10C). This insignificant change was observed for all ligands so far replaced into the receptors. This conclusion from molecular modelling is supported by the recent crystal structure of ICI-118,551 bound to the β₂-receptor (Wacker et al., 2010). In addition, the predicted conformation of ICI-118,551 in the binding pocket agrees closely with that

observed in the structure (not shown). Thus the small differences in the inverse agonist bound β₁ and β₂ crystal structures are enough to account for ligand selectivity with inverse agonists and antagonists. Perhaps more surprisingly agonist selectivity is also predicted with adrenaline showing no selectivity and salbutamol confirmed as strongly β₂-selective. The enthalpies for the fenoterol enantiomers, although correctly showing β₂ preference, suggest that the reported less active enantiomer has the higher binding enthalpy. There is much speculation as to the conformation of the agonist activated state (Audet and Bouvier, 2008). However, it is also possible that small variations in ligand structure may also lead to changes in electrostatic forces at specific points around the accessible surface of the receptor. Such subtleties could help explain the differences in biological action, such as ligand-selective agonism, seen with some pharmacophores.

Conclusions

Ten novel β₂-adrenoceptor antagonists were identified and found to have different pharmacological profiles. In terms of improving on the pharmacology of the ligand ICI-118,551, one of the compounds was more selective (racemic JB-175), while one was a neutral antagonist (7929193), although none were as potent. Further work may provide novel ligands that more successfully combine these pharmacologies. Nevertheless, the results substantiate the notion that β-blockers do more than simply inhibit receptor activation, and interesting variation between ligands could provide useful tools to inves-
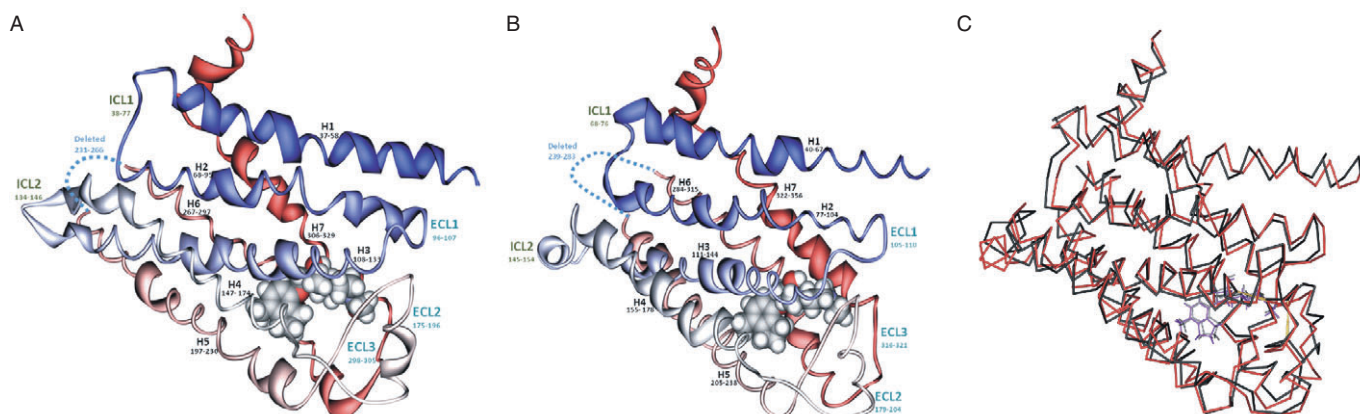
Figure 10

Conformation changes in the β-adrenoceptor on ligand binding. (A) Carazolol was replaced by JB-175 in the β_2-adrenoceptor X-ray structure (2RH1.PDB) (B) Cyanopindolol was replaced by JB-175 in the β_1-adrenoceptor X-ray structure (2VT4.PDB). In each case, the whole ensemble was fully optimized using the eXtended Electron Distribution molecular mechanics force field. The least squares Cα backbone root mean square difference between the fully optimized empty β_2-receptor and the receptor containing JB-175 was 0.49 and that for the β_1-adrenoceptor was 0.23 (C) Superimposition of the two optimized receptors indicates the relatively small change in ligand alignment (red; β_1-adrenoceptor with magenta ligand, black; β_2-adrenoceptor with grey ligand).

tigate the receptor. Additionally, intriguing enantiospecific behaviour of some of the novel ligands provides insights into receptor binding.

Conflict of interest

The authors have no conflicts of interest to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary methods.

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