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# **Identification of key residues in transmembrane 4 responsible for the secondary, low affinity conformation of the human $\beta$ 1- adrenoceptor.**

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**Running title:** identification of secondary conformation of  $\beta$ 1-AR

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## Abbreviations

CHO: Chinese hamster ovary;

CGP12177: (-)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one;

CGP20712A: 2-hydroxy-5-(2-([hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)propyl]amino)ethoxy)benzamide;

CRE-SPAP: name of the reporter gene: cyclic AMP response element-upstream of a secreted placental alkaline phosphatase reporter gene

ICI118551: (-)-1-(2,3-[dihydro-7-methyl-1H-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol;

IBMX: 3-isobutyl-1-methylxanthine

sfm: serum free media

TM: transmembrane

WT: wildtype

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## Abstract

The  $\beta$ 1-adrenoceptor exists in two agonist conformations/states: (1) a high affinity state where responses to catecholamines (and other agonists e.g. cimaterol) are potently inhibited by  $\beta$ 1-adrenoceptor antagonists and (2) a low affinity secondary conformation where agonist responses (particularly CGP12177) are relatively resistant to inhibition by  $\beta$ 1-adrenoceptor antagonists. Although both states have been demonstrated in many species (including human), the precise nature of the secondary state is unknown and does not occur in the closely related  $\beta$ 2-adrenoceptor. Here, using site directed mutagenesis and functional measurements CRE-SPAP production and  $^3\text{H}$ -cAMP accumulation, we examined the pharmacological consequences of swapping transmembrane (TM) regions of the human  $\beta$ 1 and  $\beta$ 2-adrenoceptors, followed by single point mutations, in order to determine the key residues involved in the  $\beta$ 1-adrenoceptor secondary conformation. We found that TM4 (particularly amino acids L195 and W199) had a major role in the generation of the secondary  $\beta$ 1-adrenoceptor conformation. Thus unlike at the human  $\beta$ 1-wildtype adrenoceptor, at  $\beta$ 1-TM4 mutant receptors, cimaterol and CGP12177 responses were both potently inhibited by antagonists. CGP12177 acted as a simple partial agonist with similar  $K_B$  and  $\text{EC}_{50}$  values in the  $\beta$ 1-TM4 but not  $\beta$ 1-wildtype receptors. Furthermore pindolol switched from a biphasic concentration response at human  $\beta$ 1-wildtype adrenoceptors to a monophasic concentration response in the  $\beta$ 1-TM4 mutant receptors. Mutation of these amino acids to those found in the  $\beta$ 2-adrenoceptor (L195Q and W199Y), or mutation of a single residue (W199D) in the human  $\beta$ 1-adrenoceptor, thus abolished this secondary conformation and created a  $\beta$ 1-adrenoceptor with only one high affinity agonist conformation.

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## Introduction

The  $\beta_1$ -adrenoceptor exists in two agonist conformations or states. Catecholamines activate the primary, high affinity, catecholamine conformation and these responses are readily inhibited by  $\beta$ -blockers ( $\beta$ -adrenoceptor antagonists). Much higher concentrations of  $\beta$ -blockers are required to inhibit secondary conformation agonist responses (e.g. response to CGP12177) and this conformation is therefore termed the secondary, low affinity, CGP12177 conformation (Pak and Fishman 1996; Granneman, 2001; Molenaar, 2003; Arch, 2004).

Conventional partial agonists need to saturate receptors to stimulate their maximum response and thus the concentrations required for half receptor occupancy ( $K_B$ ) should normally be equal the concentration required to stimulate half maximum response ( $EC_{50}$ ) value. However, early observations found that several  $\beta$ -blockers with partial agonist activity did not conform to this. The concentration of pindolol, for example, needed for the cardiostimulant effects in feline heart was 10-times greater than that needed to antagonise responses of the full agonist isoprenaline and thus was labelled as non-conventional partial agonist (Kaumann 1973; Kaumann and Blinks 1980; Kaumann and Molenaar 2008). CGP12177 (Staehelin et al., 1983) was also found to be a non-conventional partial agonist with even larger differences between  $K_B$  and  $EC_{50}$  (Kaumann 1983) and therefore has become an important tool in understanding  $\beta_1$ -adrenoceptor pharmacology (Kaumann and Molenaar 2008). In 1996, Pak and Fishman, using transfected cells, showed that CGP12177 inhibited isoprenaline responses at low concentrations (i.e. with high affinity), but required 100-times higher concentrations to stimulate responses. Furthermore, CGP12177 agonist responses required higher concentrations of conventional  $\beta$ -blockers to antagonise them. They therefore proposed a high affinity and a low affinity state or conformation of the  $\beta_1$ -adrenoceptor and this terminology has now been widely adopted (Granneman 2001; Kaumann et al., 2001).

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Studies using transfected cells have determined the site of action of many  $\beta$ -ligands (e.g. Konkar et al., 2000; Baker et al., 2003 Joseph et al., 2004a; Baker 2005a). Pindolol, alprenolol (and several other ligands) have biphasic concentration response relationships (Walter et al., 1984; Baker et al., 2003; Kaumann and Molenaar; 2008; Baker 2010a). The high affinity component (readily blocked by  $\beta$ -blockers) is thought to occur via the high affinity catecholamine conformation whilst the low affinity component (resistant to  $\beta$ -blockade) occurs via the secondary low affinity conformation (Walter et al., 1984; Baker et al., 2003).

These two  $\beta$ 1-adrenoceptor conformations have also been demonstrated in rat, mouse, ferret and human heart (e.g. Kompa and Summers 1999; Kaumann et al., 2001; Lowe et al., 2002; Joseph et al., 2003; Sarsero et al., 2003; Molenaar et al., 2007), human blood vessels (e.g. Mallem et al., 2004; Kozłowska et al., 2003 and 2006) and in whole animals (e.g. Malinowska and Schlicker 1996; Zakrzeska et al., 2005). However the physiological or clinical relevance of this conformation remains unknown, even though plasma concentration of carvedilol (100ng/ml = 300nM) used in human cardiovascular diseases is sufficient to occupy this secondary conformation (Sawangkoon et al., 2000; Baker et al, 2003).

Limited mutagenesis studies have, in passing, noted secondary conformation effects and detected some overlap of the amino acids involved in the catecholamine conformation, although these studies are not all in agreement. D138 (transmembrane 3, TM3) and N363 (TM7) have been suggested by some (Baker et al., 2008), but not all (Joseph et al., 2004b) to be involved in the secondary conformation, as have mutations in TM5 (Kaumann and Molenaar 2008) and TM6 (Baker et al., 2008). However, the precise nature of this secondary conformation remains unknown from its location within the receptor sequence to the physiological relevance or potential clinical implications (Molenaar 2003; Molenaar et al., 2007).

Finally, although the  $\beta$ 1-adrenoceptor of many species (rat, mouse, guinea pig, ferret, cat and human: Kaumann and Molenaar 2008), and the human  $\beta$ 3-adrenoceptor (Baker 2005b) have been shown to exist

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in at least two agonist conformations, this is not true for all  $\beta$ -adrenoceptors. CGP12177 is a conventional partial agonist at the human  $\beta_2$  and turkey  $\beta$ -3C adrenoceptors, (i.e. with similar  $K_B$  values and  $EC_{50}$  values) suggesting interaction via a single conformation (Pak and Fishman 1996; Baker et al., 2002; Baker 2010b).

This study aimed to discover the primary region important for the secondary conformation of the  $\beta_1$ -adrenoceptor. Using a chimeric receptor approach (Isogaya et al., 1999; Kaumann and Molenaar 2008), amino acids in the  $\beta_1$ -adrenoceptor were mutated to those of the  $\beta_2$ -adrenoceptor and the pharmacological consequences observed. Here we locate TM4, and specifically L195 and W199 within the human  $\beta_1$ -adrenoceptor, as the major site responsible for the secondary conformation pharmacology.

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## Materials and Methods

### *Materials*

Molecular biology reagents were from Promega (Madison, WI, USA). Lipofectamine, OPTIMEM, pcDNA3.1 and Top 10F competent cells were from Life Technologies (Paisley, UK). The QuikChange mutagenesis kit was from Stratagene (La Jolla, CA) and foetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK). <sup>3</sup>H-adenine and <sup>14</sup>C-cAMP were from Amersham International (Buckinghamshire, UK) and Ultima Gold XR scintillation fluid from PerkinElmer (Shelton, CT, USA). Cimaterol, CGP 20712A, bisoprolol and carvedilol were from Tocris Life Sciences (Avonmouth, UK). All other reagents were from Sigma Aldrich (Poole, Dorset, UK). Racemic ligands were used throughout.

### *Molecular biology*

The cDNA sequence encoding the wildtype human  $\beta$ 1-adrenoceptor ( $\beta$ 1-WT) in pJG3.6 was a gift from Steve Rees (GlaxoSmithKline, Stevenage). This cDNA was subcloned as a HindIII/XbaI fragment into pcDNA3.1 and the sequence was confirmed by DNA sequencing. Mutations described in Table 1 were generated using QuikChange mutagenesis and BioLine PolyMate Additive for GC-rich templates (Baker et al., 2008). After subcloning in Top 10F' competent cells, each mutant  $\beta$ 1-adrenoceptor cDNA was excised on Hind III/XbaI and subcloned into native pcDNA3.1 containing a neomycin selection marker. All mutations and sequences were confirmed by DNA sequencing using the School of Biomedical Sciences Sequencing Facility. In order to detect the most important areas of the human wildtype  $\beta$ 1-adrenoceptor ( $\beta$ 1-WT) involved in the secondary conformation, we made point mutations in the transmembrane (TM) regions of the receptor such that the each TM region resembled that of the wildtype human  $\beta$ 2-adrenoceptor ( $\beta$ 2-WT). Prediction of the transmembrane regions was performed using ExPASy topology prediction tools ([www.expasy.org](http://www.expasy.org)). For example, 6 point mutations were made in  $\beta$ 1-WT (L63I, L64V, A66S, L71A, A74F, V81T) which effectively converted the TM1 region of this receptor to that of the  $\beta$ 2-WT. This chimeric receptor was called  $\beta$ 1-TM1 (i.e.  $\beta$ 1-adrenoceptor but with TM1 of the  $\beta$ 2-WT, Table 1). This was then replicated for each TM region such that we had eight  $\beta$ 1 receptor



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constructs: the  $\beta$ 1-WT and one with each of the seven TM regions in turn mutated to that of the  $\beta$ 2-adrenoceptor (Table 1). A similar set of mutations were made starting with the  $\beta$ 2-WT and creating seven chimeric receptors each with a TM region of the human  $\beta$ 1-WT (Table 1). These constructs were expressed in CHO-CRE-SPAP cells (cells stably expressing a reporter construct that has six cyclic AMP response elements upstream of a secreted placental alkaline phosphatase reporter gene) and stable cell lines generated (see below).

Once important TM regions for the secondary site were identified, in order to determine which individual amino acids were involved, several single point mutations and chimeric receptors were made (Table 1) and these constructs expressed in CHO-CRE-SPAP cells either as stable mixed populations of cells (CRE-SPAP assays) or in transiently transfected cells ( $^3$ H-cAMP accumulation assays).

**Cell culture:** All CHO cells were grown in Dulbecco's modified Eagle's medium nutrient mix F12 (DMEM/F12) containing 10% foetal calf serum and 2mM L-glutamine in a 37°C humidified 5% CO<sub>2</sub> : 95% air atmosphere.

**Generation of stable cell lines:** CHO cells stably expressing a CRE-SPAP reporter gene were secondarily transfected with the wildtype human  $\beta$ 1 or  $\beta$ 2-adrenoceptor, or one of the full TM chimeric receptors (total 16 cell lines) using lipofectamine and OPTIMEM and selected for 3 weeks using resistance to geneticin (1mg/ml for the receptor) and hygromycin (200 $\mu$ g/ml for the CRE-SPAP reporter). Single clones were identified by dilution cloning and expanded to generate stable cell lines. These cell lines were used to identify the TM regions important for the secondary site. Receptor expression level in these stable cell lines was measured as previously described (Baker 2005b).

**Generation of stable mixed populations of cells:** Here, the same parent CHO-CRE-SPAP cells were transfected with the wildtype human  $\beta$ 1 or  $\beta$ 2-adrenoceptor, or a single point mutation or chimeric receptor and selected for 3 weeks using resistance to geneticin (1mg/ml for the receptor) and hygromycin

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(200µg/ml for the CRE-SPAP reporter). During this time the cells were passaged twice. The cells were then plated into 96-well plates for CRE-SPAP production experiments.

**Generation of transient populations:** For transiently transfected cells used in the  $^3\text{H}$ -cAMP assays, the parent CHO-CRE-SPAP cells were transfected, as above, on day 1, the transfection reagents removed and replaced with media on day 2, the cells plated in to 24-well plates on day 3 and the  $^3\text{H}$ -cAMP accumulation assay performed on day 4.

### ***CRE-SPAP production***

Cells were grown to confluence in, sterile, clear plastic, tissue culture treated 96-well plates. The cells were then serum starved by removal of the media and addition of 100µl serum-free media (sfm) per well for 24 hours before experimentation. At the start of each experiment, the sfm was removed from each well and 100µl sfm or 100µl sfm containing an antagonist at the final required concentration was added to each well and the cells incubated for 30 minutes at 37°C. Agonist in 10µl sfm was then added to each well and the plates incubated for 5 hours. After 5 hours, all drugs and media were removed from each well and 40µl sfm added to each well. The plates were then incubated for 1 hour at 37°C before being transferred to an oven preheated to 65°C and incubated for 30 minutes to destroy endogenous phosphatases. SPAP production was then measured by the addition of 100µl 5mM p-NPP per well (in diethanolamine buffer) and read on a Dynatech MRX plate reader at 405nm.

### ***$^3\text{H}$ -cAMP accumulation***

Cells were grown to confluence in sterile, clear plastic, tissue culture treated 24-well plates. Cells were pre-labelled with  $^3\text{H}$ -adenine by incubation for at least 2 hours with 2µCi/ml  $^3\text{H}$ -adenine in media (0.5ml per well). The cells were washed, then 1ml sfm containing 1mM IBMX (3-isobutyl-1-methylxanthine) was added to each well and the cells incubated for 15 minutes at 37°C. Agonists (in 10µl sfm) were added to each well and the plates incubated for 5 hours in order to maximise the responses (without altering the  $\text{EC}_{50}$  values or % maximum isoprenaline response observed; Baker, 2010a). The assay was

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terminated by adding 50µl concentrated HCl per well, the plates frozen, thawed and <sup>3</sup>H-cAMP separated from other <sup>3</sup>H-nucleotides by sequential Dowex and alumina column chromatography (using <sup>14</sup>C-cAMP to determine column efficiency). Isoprenaline (10µM) was used to define the maximal response in each plate of each experiment.

### ***Data analysis***

Agonist responses were best described by a one-site sigmoidal concentration response curve using the following equation:

$$\text{Response} = \frac{\text{Emax} \times [\text{A}]}{\text{EC}_{50} + [\text{A}]}$$

where Emax is the maximum response, [A] is the agonist concentration and EC<sub>50</sub> is the concentration of agonist that produces 50% of the maximal response.

The affinities of antagonists (K<sub>B</sub> values) were calculated from the rightward shift of the agonist concentration response curve in the presence of a fixed concentration of antagonist using the following:

$$\text{DR} = 1 + \frac{[\text{B}]}{\text{K}_\text{B}}$$

where DR (dose ratio) is the ratio of the agonist concentration required to stimulate an identical response in the presence and absence of a fixed concentration of antagonist [B].

When CGP12177 was used to antagonise the more efficacious agonists, clear partial agonism was seen (e.g. Figure 2). Here, the affinity was initially calculated by the method of Stephenson (1956):

$$\text{K}_\text{B} \text{ partial agonist} = \frac{\text{Y} \times [\text{P}]}{1 - \text{Y}} \quad \text{where } \text{Y} = \frac{[\text{A}_2] - [\text{A}_1]}{[\text{A}_3]}$$

where [P] is the concentration of CGP12177, [A<sub>1</sub>] is the concentration of the agonist at the point where CGP12177 alone causes the same agonist response, [A<sub>2</sub>] is the concentration of agonist causing a given

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response above that achieved by CGP12177 and  $[A_3]$  the concentration of the agonist, in the presence CGP12177, causing the same stimulation as  $[A_2]$ .

The response to pindolol (and at times CGP12177) was best described by a two-site concentration response (e.g. Figure 6b)

$$\% \text{ maximal stimulation} = \frac{[A] \times N}{([A] + EC_{150})} + \frac{[A] \times (100-N)}{([A] + EC_{250})}$$

where N is the percentage of site 1,  $[A]$  is the concentration of agonist and  $EC_{150}$  and  $EC_{250}$  are the respective  $EC_{50}$  values for the two agonist sites.

A two-site analysis was also used for the experiments e.g. Figure 6a:

$$\text{Response} = \text{Basal} + (\text{Ag} - \text{Basal}) \left[ 1 - \frac{[P]}{([P] + IC_{50})} \right] + PAg \left[ \frac{[P]}{([P] + EC_{50})} \right]$$

where basal is the response in the absence of agonist, Ag is the response to a fixed concentration of agonist,  $[P]$  is the concentration of partial agonist (e.g. CGP12177),  $IC_{50}$  is the concentration of competing partial agonist that inhibits 50% of the response of the fixed agonist, PAg is the maximum stimulation by the competing partial agonist and  $EC_{50}$  is the concentration of competing agonist that stimulated a half maximal competing partial agonist response.

A 10 $\mu$ M (maximal) isoprenaline concentration was included in each plate for each separate experiment for CRE-SPAP production and  $^3\text{H}$ -cAMP accumulation, to allow agonist responses to be expressed as a percentage of the isoprenaline maximum for each experiment. Data points in the Figures are presented as mean  $\pm$  s.e.m. of triplicate determinations from a single experiment. Data in the text and Tables are mean  $\pm$  s.e.m. of n separate experiments.

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## Results

### ***Demonstration of the two agonist conformations of the human $\beta$ 1-adrenoceptor – CRE-SPAP production in stable cell lines.***

Cimaterol, a primary catecholamine conformation agonist (Baker 2005a), stimulated a response in cells stably expressing the human wildtype  $\beta$ 1-adrenoceptor ( $\beta$ 1-WT; log  $EC_{50}$  =  $-8.25 \pm 0.14$ ) that was  $95.8 \pm 2.9\%$  that of the response to  $10\mu$ M isoprenaline; top half Table 2a, Figure 1a). As expected, this  $\beta$ 1 response was readily inhibited by the  $\beta$ 1-selective antagonist CGP20712A (log  $K_B$  -9.18) and poorly by the  $\beta$ 2-selective antagonist ICI118551 (log  $K_B$  -6.96). CGP12177, a partial agonist, also inhibited this cimaterol response with high affinity indicating high affinity for the catecholamine conformation of the receptor (log  $K_B$  -9.55; Table 2, Figure 2a). CGP12177 is however also known to activate the secondary conformation of the  $\beta$ 1-adrenoceptor. At the  $\beta$ 1-WT, CGP12177 stimulated a partial agonist response, log  $EC_{50}$   $-8.18 \pm 0.08$ , that was  $73.8 \pm 5.6\%$  that of the maximum to isoprenaline (Figure 1b). This CGP12177 response was also inhibited by antagonists but in each case higher concentrations of antagonist were required yielding lower log  $K_B$  values (e.g. log  $K_B$  for CGP20712A was -7.16; bottom half Table 2a). The fact that 10-500 fold higher concentrations of antagonist were required to inhibit the CGP12177 rather than the cimaterol responses, and the discrepancy between the log  $K_B$  value and the log  $EC_{50}$  value for CGP12177 itself, demonstrate the high affinity and the lower affinity conformations of the human  $\beta$ 1-adrenoceptor.

At the human  $\beta$ 2-adrenoceptor, agonist responses to cimaterol and CGP12177 were also observed, however similar concentrations of antagonist were required to inhibit these responses (yielding similar log  $K_B$  values, Table 3a, Figure 1e and f), and the log  $K_B$  and log  $EC_{50}$  values for CGP12177 were similar suggesting that cimaterol and CGP12177 are acting through the same conformation of the  $\beta$ 2-adrenoceptor.

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***Identification of transmembrane regions important in the secondary conformation of the  $\beta$ 1-adrenoceptor – CRE-SPAP production in stable cell lines.***

To identify areas in the receptor important for the secondary conformation,  $\beta$ 1/ $\beta$ 2 chimeric receptors were examined. These receptors had mutations such that each TM was in turn swapped for that of the other receptor, e.g.  $\beta$ 1-TM1 is the  $\beta$ 1-adrenoceptor sequence containing mutations that make it equivalent to the TM1 region of the  $\beta$ 2-adrenoceptor (Table 1). When the  $\beta$ 1-TM chimeric receptors were examined (each stably expressed in a different cell line), cimaterol stimulated a full agonist response at all  $\beta$ 1/2 receptors ( $\beta$ 1-TM1 through to  $\beta$ 1-TM7; Figure 1c, top half Table 2a) and this response was readily inhibited by CGP20712A and poorly by ICI118551 confirming that despite changing each transmembrane in turn to that of the  $\beta$ 2-adrenoceptor, these chimeric receptors retained a  $\beta$ 1-selective nature. There were some differences in the affinity of antagonists (e.g. affinity of ICI118551 at the  $\beta$ 1-TM2,  $\beta$ 1-TM3,  $\beta$ 1-TM4,  $\beta$ 1-TM6 and  $\beta$ 1-TM7 receptors is substantially higher than at the  $\beta$ 1-WT; top half Table 2a). This could be due to a general trend of these chimeras “losing”  $\beta$ 1-subtype selectivity as judged by a range of ligands or due to a specific interaction between the ligand ICI118551 and specific amino acids in these chimeras. To determine which, several antagonist ligands (bisoprolol a less selective but still a  $\beta$ 1-selective ligand; propranolol and carvedilol slightly  $\beta$ 2-selective ligands) were examined in order to generate a pattern of affinity (top half Table 2a, Figure 1). The affinity of CGP20712A and bisoprolol remained high (similar to  $\beta$ 1-WT, top half Table 2a and very different from values obtained at the  $\beta$ 2-WT; top half Table 3a). Thus, the overall pattern for these chimeras is one of retaining  $\beta$ 1-selective properties (and indeed ICI118551 was later found to interact with a specific amino acid in TM4 rather than a general pattern across several ligands – see below).

When the CGP12177 responses were examined, the data achieved for  $\beta$ 1-TM4 was significantly different from that at the other chimeras ( $p < 0.001$ , bottom half Table 2, Figure 1d). Firstly, the log  $EC_{50}$  for CGP12177 was very different from that at the other receptors (bottom half Table 2a). Secondly, the log  $K_B$  value and log  $EC_{50}$  values for CGP12177 in the  $\beta$ 1-TM4 cells were similar (left column, Table 2b, and

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indeed similar to that seen in the  $\beta$ 2-WT cells; Table 3a). Finally, the affinities of antagonists in the presence of CGP12177 at  $\beta$ 1-TM4 were very similar to those obtained when cimaterol was used as the agonist in these cells (Table 2a). This is most easily seen in Table 2b where the ratios of the antagonist affinities are compared. Whereas the ratio of antagonist affinities for CGP20712A at  $\beta$ 1-WT was log 2.02 (e.g. it has 100 fold different affinity for two conformations), it was log 0.01 at  $\beta$ 1-TM4 suggesting that there is no difference in affinity and that the secondary conformation was not in existence. Thus  $\beta$ 1-TM4 had a pharmacological profile consistent with CGP12177 acting as a partial agonist at a single high affinity conformation of the  $\beta$ 1-adrenoceptor, i.e. in a similar manner to which CGP12177 acts at the single receptor conformation of the  $\beta$ 2-WT adrenoceptor.

When the full  $\beta$ 2-TM4 construct was expressed (11 amino acid changes), no function or binding was detectable despite multiple attempts. Therefore a construct containing 10 amino acid changes (all except the last Y174W) was made which was then used as  $\beta$ 2-TM4 (Table 1 and 3). The data obtained for the  $\beta$ 2/ $\beta$ 1 chimeras (Table 3a) suggest that the agonist responses and antagonist affinities obtained change very little in the  $\beta$ 2-adrenoceptors with whole TM region changes.

Examination of the ratios of antagonist affinities at the two conformations is again helpful for detecting the most important changes – Tables 2b and 3b. Thus although there are some differences for the chimeric receptors (e.g.  $EC_{50}/K_B$  ratio for  $\beta$ 1-TM7 and  $\beta$ 2-TM7), by far the most overwhelming differences are in the  $\beta$ 1-TM4 chimeric receptor and this is clearly shown when comparing the ratios of the log  $K_B$  and log  $EC_{50}$  values at the two conformations, as shown in Tables 2b and 3b. Also, here, although ICI118551 was found to have higher affinity at  $\beta$ 1-TM4 (and indeed  $\beta$ 1-TM7), the ratio of the affinities for the two conformations of the  $\beta$ 1-adrenoceptor clearly shows that  $\beta$ 1-TM4 is different from the other  $\beta$ 1-chimeric receptors.

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Importantly, because these stable cell lines used here have different levels of receptor expression (Table 1), both of these methods of assessing the presence of the secondary conformation do not rely on the receptor expression levels being the same. Antagonist affinity measurements are independent of receptor expression level and, because CGP12177 is a partial agonist in all of these cell lines, its  $K_B$  value in a given cell line should be similar to its  $EC_{50}$  value in that cell line (i.e. ratio of zero) if the two are acting at a single conformation. A further piece of evidence can be obtained from comparing the log  $EC_{50}$  values for CGP12177 over the cell lines although this is more problematic, not least as it is likely to depend on receptor expression levels. Interestingly here, the log  $EC_{50}$  for CGP12177 at  $\beta 1$ -TM4 is markedly more potent (left-shifted) even though its receptor expression level is the lowest of the  $\beta 1$  cell lines and thus, if anything, would be expected to have a less potent (i.e. right -shifted) log  $EC_{50}$  value. Even this softer evidence is therefore pointing to  $\beta 1$ -TM4 as an important region for the secondary conformation.

### ***Identification of the amino acids in TM4 involved in the secondary conformation***

#### ***– CRE-SPAP production in stable mixed populations of cells.***

In order to determine which of the amino acids in TM4 were responsible for this secondary conformation, intermediate stages of the production of the chimeric receptors were examined that had several, but not all, of the amino acids changes required to alter the TM domain from  $\beta 1$  to  $\beta 2$  (see Table 1 for the amino acid changes made). These constructs were expressed in parent CHO-SPAP cells and experiments performed in stable mixed populations of cells (4-24 separate populations of cells were generated for each chimeric receptor). Whereas the affinity for antagonists remained very similar when cimaterol was the agonist (primary catecholamine site, top half of Table 4a, with the exception of ICI118551), when the secondary site conformation was examined, (i.e. with CGP12177 as the agonist), the log  $EC_{50}$  value for CGP12177 became more potent (approaching that for the log  $K_B$  value) in  $\beta 1$ -TM4 stage 5 (bottom half Table 4a). Secondly, the affinity of the other antagonists (log  $K_B$  values) also became higher with  $\beta 1$ -TM4 stage 5 and full  $\beta 1$ -TM4 chimeras (bottom half Table 4a). The affinity for ICI118551 was high at both conformations for  $\beta 1$ -TM4 Stage 4, although the ratio (Table 4b) suggests the retention of both



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conformations of the receptor. This suggests that amino acids L195 and W199 are the most important for the secondary conformation.

To investigate the role of these specific amino acids further, each of these individual single point mutations were examined alone (in stable mixed populations) rather than on the background of the other TM4 mutations (Table 1 and 5, Figure 3 and 4).  $\beta$ 1-L195Q and  $\beta$ 1-W199Y had little effect on the cimaterol catecholamine conformation response (top half Table 5a; Figure 3). However, CGP12177 agonist responses were significantly more potent at  $\beta$ 1-L195Q and  $\beta$ 1-W199Y (bottom half Table 5a, Figure 4). Examining antagonist affinity values, whereas  $\beta$ 1-V189T affected the affinity of ICI118551 at the catecholamine conformation, the affinity of CGP20712A, propranolol and CGP12177 were largely unaffected by any of the mutations (top half of Table 5a, Figure 3). However, the secondary conformation CGP12177 responses were significantly more sensitive to inhibition with the L195Q and W199Y mutations (bottom half Table 5a, Figure 4).

We also examined the impact of these specific mutations in combination with each other to determine whether the effects of each change were additive or whether one single change was sufficient to account for the secondary site. A combination of L195Q and W199Y largely achieved the same effect as the whole TM4 swap, with the exception of the affinity of ICI118551 (Table 5a). However, addition of the V189T mutation (thus making a triple  $\beta$ 1V189T-L195Q-W199Y) effectively normalised the affinity of ICI118551 to that observed with the full TM4 swap (Figure 5a and b). This suggests that  $\beta$ 1V189T-L195Q-W199Y has also lost the secondary conformation.

In addition to the simple substitution of  $\beta$ 2-adrenoceptor residues for their equivalents in the  $\beta$ 1-adrenoceptor, we also examined the effects of different substitutions at position 199. Thus at position 199, as well as mutating the tryptophan (W) to tyrosine (Y, as in the human  $\beta$ 2-adrenoceptor) it was also mutated to another aromatic residue (phenylalanine, F), a hydrophobic residue (leucine, L), a basic

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residue (lysine, K), an amide (asparagine, N), an acid (aspartic acid, D) and the small amino acid alanine (A). Substitution of W for other residues had little effect on the nM EC<sub>50</sub> for CGP12177 observed with the tyrosine (Y) residue from the  $\beta$ 2-adrenoceptor and most appeared to have lost, or at least compromised, the secondary conformation (Figure 5c and d, Table 5a). Interestingly, the W199K and W199L mutations decreased the EC<sub>50</sub> of cimaterol (top half Table 5a) whilst increasing the efficacy of CGP12177 which then appeared as a virtual full agonist in these two mutations (bottom half Table 5a).

***Confirmation of loss of the secondary site with mutations V189T, L195Q and W199Y – <sup>3</sup>H-cAMP accumulation in transiently transfected cells.***

So far, identification of the role of amino acids at positions 195, 199 and possibly 189 in the secondary conformation pharmacology of the  $\beta$ 1-adrenoceptor has relied on observation of (a) different affinity values for antagonists in the presence of cimaterol and CGP12177 and (b) the comparison between the partial agonist log EC<sub>50</sub> value and log K<sub>B</sub> value for CGP12177. To confirm that these amino acids were indeed important, evidence was sought using a different assay to explore two further strands of evidence – the stimulation resulting from CGP12177 in the presence of a fixed concentration of cimaterol and the effect on the response to pindolol. To examine whether cimaterol and CGP12177 were competing at the same site of the receptor, CGP12177 concentration responses were examined in the presence of different fixed concentrations of cimaterol. Low concentrations of CGP12177 competed with cimaterol causing a reduction in the stimulatory response. However, higher CGP12177 concentrations stimulated responses, creating a “dip” in the curve, for the two conformation  $\beta$ 1-WT adrenoceptor (Pak and Fishman, 1996; Figure 6a). This “dip” was absent in the single conformation  $\beta$ 2-WT adrenoceptor and at  $\beta$ 1-TM4 (Figure 6c and e; Table 6). The  $\beta$ 1-V189T mutation alone had little effect on the two conformations (Figure 7a, Table 6), although the “dip” appeared reduced in both L195Q and W199Y mutations alone. However, the “dip” was absent from  $\beta$ 1-V189T-L195Q-W199Y and  $\beta$ 1-W199D (Figure 8a and c; Table 6) in keeping with a single conformation receptor. Interestingly, in the transiently transfected cells, the  $\beta$ 1-WT <sup>3</sup>H-cAMP accumulation response to CGP12177 was best described by a two-component response (Figure 6a,

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Table 6). Hints that the CGP12177 response may, at times, be biphasic have been seen before (e.g. Figure 4 of Baker et al., 2013) and are also potentially present in the stable  $\beta$ 1-WT cell lines and stable mixed  $\beta$ 1-WT population data (Figure 1b and 4a respectively). Interestingly, mutations L195Q and W199Y (or W199D) removed the biphasic response of CGP12177 (Figure 7c and e, Figure 8c, Table 6). Finally, concentration responses to the biphasic ligand pindolol were examined (Figures 6b, d and f, Figure 7 b, d and f, Figure 8b and d, Table 6). Both the racemic and S-isomer of this ligand have previously been shown to stimulate agonist responses via both conformations of the  $\beta$ 1-adrenoceptor (Walter et al., 1984; Baker 2010a). The data obtained with this agonist were consistent with the observations made with CGP12177 (apart from W199Y which continued to exhibit two-site pharmacology with pindolol; Table 6). However, this was not the case with W199D or combinations of W199Y with L195Q and/or V189T (Table 6).

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## Discussion

The human  $\beta 1$ -WT exists in at least two agonist conformations: a high affinity catecholamine conformation and a low affinity secondary (CGP12177) conformation (Pak and Fishman, 1996; Kaumann and Molenaar, 2008). This secondary conformation does not occur in the closely related human  $\beta 2$ -WT (Pak and Fishman, 1996; Baker et al., 2002). This study attempted to identify the key area responsible for this secondary conformation using a  $\beta 1/\beta 2$ -chimeric receptor approach (Isogaya et al., 1999; Kaumann and Molenaar 2008).

At the  $\beta 1$ -WT, the secondary conformation was clearly delineated from the primary catecholamine conformation by two pieces of evidence. Firstly, the cimaterol responses (catecholamine conformation agonist) were readily inhibited by antagonists whereas CGP12177 responses required much greater antagonist concentrations, yielding lower  $\log K_B$  values (Table 2). Secondly, CGP12177 readily inhibited the cimaterol responses at low concentrations ( $\log K_B$  -9.55), but required high concentrations to produce its agonist response ( $\log EC_{50}$  -8.18) in keeping with previous studies (e.g. Pak and Fishman, 1996; Lowe et al., 2002; Baker et al., 2003; Joseph et al., 2004a; Baker, 2005). At the  $\beta 2$ -WT, cimaterol and CGP12177 responses were inhibited by similar concentrations of antagonist and the  $EC_{50}/K_B$  discrepancy for CGP12177 was less marked suggesting competition of the two ligands at the same conformation.

When the TM-chimeras were examined, antagonism of cimaterol responses suggested that all receptors ( $\beta 1$ -TM1 to  $\beta 1$ -TM7 and  $\beta 2$ -TM1 to  $\beta 2$ -TM7, Tables 2 and 3) retained their respective pharmacological parent  $\beta 1$  or  $\beta 2$ -subtype, even when one entire transmembrane region was altered (i.e. high affinity for CGP20712A at  $\beta 1$ -adrenoceptors, high affinity for ICI118551 at  $\beta 2$ -adrenoceptors). Significant changes were however seen in the secondary conformation in  $\beta 1$ -TM4. Here, antagonists inhibited the cimaterol and CGP12177 responses to give similar  $\log K_B$  values (Table 3), and the concentration of CGP12177 required to inhibit the cimaterol response (-9.63) approached that required to stimulate the agonist response (-9.27). Taken together this strongly suggests that the secondary conformation is absent in  $\beta 1$ -

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TM4. Although direct comparison of partial agonist efficacy across cell lines is difficult, the log EC<sub>50</sub> for CGP12177 (-9.27) is substantially more potent (left-shifted) at  $\beta$ 1-TM4 than any other  $\beta$ 1-TM-chimeric-receptor (-7.83 to -8.27; Table 2) even though the response is less efficacious (% isoprenaline maximum response achieved) and the receptor expression level is the lowest of the  $\beta$ 1-cell lines. If CGP12177 were simply more efficacious at  $\beta$ 1-TM4, the left-shifted EC<sub>50</sub> would be accompanied by an increase in the maximum response obtained. The increase in potency, but decrease in efficacy, suggests that something different is happening at  $\beta$ 1-TM4, and indeed these values are similar to those obtained for CGP12177 at the  $\beta$ 2-WT and  $\beta$ 2-chimeric-receptors (Tables 2 and 3). This differs from the comment in Kaumann and Molenaar (2008) regarding TM5.

Thus  $\beta$ 1-TM4 appears to be a  $\beta$ 1-adrenoceptor without the secondary conformation. When the  $\beta$ 2-chimeras were examined (Table 3), the antagonist affinities (whether cimaterol or CGP12177 was the agonist), log EC<sub>50</sub> and log K<sub>B</sub> values for CGP12177 were similar for all  $\beta$ 2-chimeric-receptors to that of  $\beta$ 2-WT and  $\beta$ 1-TM4. Thus although the secondary site was lost in  $\beta$ 1-WT, it was not recreated in  $\beta$ 2-WT by the TM4 swap, and therefore other areas of the receptor must contribute to this secondary conformation.

To locate the specific amino acids involved in the secondary conformation, intermediate stages in the generation of  $\beta$ 1-TM4 were examined. The log EC<sub>50</sub> for CGP12177 was more potent and the antagonist affinities were higher for  $\beta$ 1-TM4 stage 5 and the full  $\beta$ 1-TM4 chimeric receptor (Table 1 and 4).

ICI118551 had a slightly different pattern with increased affinity at both conformations from  $\beta$ 1-TM4 stage 4 onwards. This suggests that amino acids L195, W199 and maybe V189 are important for the secondary conformation. These mutations were therefore examined alone. For  $\beta$ 1-L195Q and  $\beta$ 1-W199Y, the antagonist affinities (log K<sub>B</sub>) in the presence of CGP12177 were higher, and the log EC<sub>50</sub> for CGP12177 more potent, than at  $\beta$ 1-WT suggesting that both of these residues are important in the secondary conformation. ICI118551 had higher affinity for  $\beta$ 1-V189T in the presence of both cimaterol

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and CGP12177, suggesting all-round (not conformation specific) higher affinity. This was seen in all receptors containing a V189T mutation (i.e.  $\beta$ 1-TM4,  $\beta$ 1-V189T,  $\beta$ 1-V189T-L195Q,  $\beta$ 1-V189T-W199Y and  $\beta$ 1-V189T-L195Q-W199Y). ICI118551 also showed lower affinity at  $\beta$ 2-TM4 (containing the reverse T164V mutation). V189T is therefore specifically involved in the affinity of ICI118551. CGP20712A and propranolol both had slightly higher affinity in the presence of CGP12177 at  $\beta$ 1-V189T than  $\beta$ 1WT, giving rise to lower ratios (Table 5b) and suggesting a potential minor role for V189T in the secondary conformation.

However, neither L195Q nor W199Y alone gave  $K_B$  or  $EC_{50}$  values that matched those of the  $\beta$ 1-TM4 (Table 5). Double and triple mutations were therefore made. At  $\beta$ 1-V189T-L195Q-W199Y, antagonist affinities and log  $EC_{50}$  values for CGP12177 were the same as for the  $\beta$ 1-TM4 suggesting complete abolishment of the secondary conformation.  $\beta$ 1-L195Q-W199Y also achieved this (with the exception of ICI118551 affinity because this lacks the V189T mutation).  $\beta$ 1-V189T-L195Q and  $\beta$ 1-V189T-W199Y both had the higher ICI118551 affinity, but the secondary conformation affinity for CGP20712A and propranolol did not reach that of  $\beta$ 1-TM4. This suggests that whilst L195Q and W199Y are each important, their effects are additive and that both are needed to completely abolish the secondary conformation. The effect of V189T, whilst important for ICI118551 affinity, was minor for the secondary conformation.

The effect of charge at the W199 site was then assessed. The addition of a negatively charged group (W199D) greatly increased CGP20712A and propranolol affinity in the presence of CGP12177 to that of  $\beta$ 1-TM4,  $\beta$ 1-L195Q-W199Y and  $\beta$ 1-V189T-L195Q-W199Y, suggesting that this single change could remove the secondary conformation (although ICI118551 affinity does not reach that of  $\beta$ 1-TM4 or  $\beta$ 1-V189T-L195Q-W199Y because V189T is missing). Thus the single amino acid change of W199D appeared to create a  $\beta$ 1-adrenoceptor with  $\beta$ 1 pharmacological characteristics (i.e. 100 fold higher affinity for CGP20712A than ICI118551) but that is completely devoid of the secondary conformation.

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Finally, to confirm L195Q, W199Y (and maybe V189T) involvement in the secondary conformation,  $^3\text{H}$ -cAMP accumulation was investigated (Figures 6-8). At low concentrations, CGP12177 inhibited the stimulation of the catecholamine conformation by cimaterol, whilst at higher concentrations CGP12177 stimulated the secondary conformation resulting in a “dip” in the response to cimaterol in the presence of CGP12177 (Figure 6a Pak and Fishman 1996; Konkar et al., 2000; Baker et al., 2003; Baker 2005a). This “dip” was present for  $\beta 1$ -V189T (Figure 7a) but reduced at  $\beta 1$ -L195Q and  $\beta 1$ -W199Y (Figure 7c and 7e). There was however no “dip” in  $\beta 2$ -WT,  $\beta 1$ -TM4,  $\beta 1$ -L195Q-W199Y,  $\beta 1$ -V189T-L195Q-W199Y or  $\beta 1$ -W199D (Figures 6c, 6e, 8a and 8c). At all these receptors, CGP12177 competed with cimaterol to inhibit the cimaterol response at the same as (or just to the right of) that of the  $\text{EC}_{50}$  value, strongly suggesting competition at the same conformation.

Pindolol has a biphasic concentration response curve at the  $\beta 1$ -adrenoceptor. The first (higher affinity) component is thought to occur via the catecholamine conformation whilst the second component via the secondary conformation (Walter et al., 1984; Baker et al., 2003). Therefore, the pindolol response should become a single component response at  $\beta 1$ -TM4 and the other receptors lacking the secondary conformation. Indeed, pindolol was found to have a two-component concentration response curve at  $\beta 1$ -WT and  $\beta 1$ -V189T, but a single component response curve at  $\beta 2$ -WT,  $\beta 1$ -TM4,  $\beta 1$ -L195Q-W199Y,  $\beta 1$ -T189V-L195Q-W199Y, and  $\beta 1$ -W199D confirming the crucial roles of L195 and W199 in the secondary conformation. Interestingly, for all  $\beta$ -adrenoceptors with a known sequence and two pharmacologically confirmed conformations, V, L and W, or similar, are found at equivalent positions for 189, 195 and 199 respectively, whilst those of one-conformation  $\beta$ -adrenoceptors have T, Q and Y (Table 7).

A recent turkey  $\beta 1$ -adrenoceptor crystal structure suggests that TM4 is involved in a  $\beta 1$ -dimer interface with TM5 (Huang et al., 2013). Figure 9a shows that the equivalent key turkey TM4 residues (V172, M178, and W182; Table 7) are largely outward facing, away from the binding pocket, but lie within the

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TM4/TM5 dimer interface (Figure 9b). However, the key binding residues in TM3 (D138) and TM7 (N363), that are essential for both the catecholamine and secondary conformation responses (Baker et al., 2008), are both inward-facing residues within the ligand binding pocket (Figure 9a). It is therefore interesting to speculate that both binding site activation and receptor dimerization are involved in the secondary conformation. However, more work is required to understand how and where molecules bind in order to create the distinct pharmacological patterns observed.

In conclusion, the  $\beta$ 1-adrenoceptor exists in two agonist conformations and the key residues responsible for the secondary low affinity conformation are in TM4. The mutation  $\beta$ 1-V189T has an effect on the affinity of ICI118551 and may have a minor role in secondary conformation pharmacology. However, the amino acids at positions 195 and 199 in TM4 both have a major role in the secondary conformation and together L195Q and W199Y (or indeed W199D alone), abolishes this secondary conformation and creates a  $\beta$ 1-adrenoceptor with only one high affinity agonist conformation. From Huang et al., 2013 data, it is tempting to speculate that oligomerization involving TM4 may underlie the secondary  $\beta$ 1-adrenoceptor conformation.



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## **Authorship contributions**

Participated in research design: Baker

Conducted experiments: Baker, Proudman

Contributed new reagents or analytical tools: Baker, Proudman, Hill

Performed data analysis: Baker

Wrote or contributed to the writing of the manuscript: Baker, Hill

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## Footnotes

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## Figure legends

### Figure 1

CRE-SPAP production in response to cimaterol (a, c and e) and CGP12177 (b, d and f) in the absence and presence of bisoprolol and propranolol in stable cell lines expressing  $\beta$ 1-WT (a and b),  $\beta$ 1-TM4 (c and d) and human  $\beta$ 2-WT (e and f). Bars represent basal CRE-SPAP production, that in response to 10 $\mu$ M isoprenaline or that in response to bisoprolol or propranolol alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 5, b) 4, c) 4, d) 7, e) 6 and f) 10 separate experiments.

This Figure shows that the greater concentrations of antagonist are required to inhibit the secondary conformation CGP12177 responses in the  $\beta$ 1-WT, but that this is not the case for the  $\beta$ 1-TM4 or  $\beta$ 2-WT.

### Figure 2

CRE-SPAP production in response to cimaterol in the absence and presence of CGP12177 in stable cell lines expressing a)  $\beta$ 1-WT, b)  $\beta$ 1-TM4 and c)  $\beta$ 2-WT. Bars represent basal CRE-SPAP production, that in response to 10 $\mu$ M isoprenaline or that in response 1-30nM CGP12177 alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 6, b) 8, and c) 9 separate experiments.

This Figure shows that CGP12177 inhibits cimaterol responses with similar high affinity at  $\beta$ 1-WT,  $\beta$ 1-TM4 and  $\beta$ 2-WT.

### Figure 3

CRE-SPAP production in response to cimaterol in the absence and presence of 10nM and 100nM CGP20712A and 1 $\mu$ M ICI118551 in stable mixed populations of cell lines expressing a)  $\beta$ 1-WT, b)  $\beta$ 1-V189T, c)  $\beta$ 1-L195Q, d)  $\beta$ 1-W199Y and e)  $\beta$ 1-TM4. Bars represent basal CRE-SPAP production, that in response to 10 $\mu$ M isoprenaline or that in response to 10nM or 100nM CGP20712A or 1 $\mu$ M ICI118551

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alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 24, b) 12, c) 12, d) 12 and e) 23 separate experiments.

This Figure shows that CGP20712A inhibits cimaterol responses in all of these receptors with similar ability, however ICI118551 is able to inhibit  $\beta$ 1-V189T and  $\beta$ 1-TM4 to a greater extent than the others.

#### Figure 4

CRE-SPAP production in response to CGP12177 in the absence and presence of 100nM and 1 $\mu$ M CGP20712A and 10 $\mu$ M ICI118551 in stable mixed populations of cell lines expressing a)  $\beta$ 1-WT, b)  $\beta$ 1-V189T, c)  $\beta$ 1-L195Q, d)  $\beta$ 1-W199Y and e)  $\beta$ 1-TM4. Bars represent basal CRE-SPAP production, that in response to 10 $\mu$ M isoprenaline or that in response to 100nM or 1 $\mu$ M CGP20712A or 10 $\mu$ M ICI118551 alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 21, b) 11, c) 11, d) 11 and e) 20 separate experiments.

This Figure shows that secondary conformation CGP12177 responses are more readily inhibited by CGP20712A and ICI118551 at  $\beta$ 1-L195Q,  $\beta$ 1-W99Y and  $\beta$ 1-TM4 than at  $\beta$ 1-WT suggesting that the secondary conformation is compromised in the  $\beta$ 1-L195Q,  $\beta$ 1-W99Y and  $\beta$ 1-TM4 mutants.  $\beta$ 1-V189T whilst remaining more resistant to CGP20712A inhibition (i.e. demonstrating preservation of the secondary conformation) is once again more sensitive to ICI118551 inhibition.

#### Figure 5

CRE-SPAP production in response to cimaterol (a and c) and CGP12177 (b and d) in the absence and presence of 10nM, 100nM or 1 $\mu$ M CGP20712A and 10 $\mu$ M ICI118551 in stable mixed populations of cell lines expressing  $\beta$ 1-V189T-L195Q-W199Y (a and b) or  $\beta$ 1-W199D (c and d). Bars represent basal CRE-SPAP production, that in response to 10 $\mu$ M isoprenaline or that in response to 10nM, 100nM or 1 $\mu$ M CGP20712A or 10 $\mu$ M ICI118551 alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of 8 separate experiments in each case.

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This Figure shows that CGP20712A and ICI118551 are able to inhibit cimaterol and CGP12177 responses to a similar degree at both  $\beta 1$ -V189T-L195Q-W199Y and  $\beta 1$ -W199D, unlike that seen at  $\beta 1$ -WT (Figure 3a and Figure 4a). This suggests that whilst both are primarily  $\beta 1$ -adrenoceptors (given that both are more readily inhibited by CGP20712A than ICI118551) the secondary conformation is compromised in both of these mutant receptors.

#### Figure 6

$^3\text{H}$ -cAMP accumulation in response to CGP12177 (a, c and e) and pindolol (b, d and f) in transiently transfected cells expressing  $\beta 1$ -WT (a and b),  $\beta 1$ -TM4 (c and d) and  $\beta 2$ -WT (e and f). The CGP12177 responses were examined in the absence and presence of 3, 10 and 30nM cimaterol (a and c) and 0.3, 1 and 3nM cimaterol (e). Bars represent basal  $^3\text{H}$ -cAMP accumulation, that in response to 10 $\mu\text{M}$  isoprenaline or that in response to 0.3-30nM cimaterol alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 6, b) 8, c) 4, d) 3, e) 4 and f) 4 separate experiments.

This Figure examines different evidence for the absence or presence of the secondary conformation. At  $\beta 1$ -WT, low concentrations of CGP12177 (e.g. 3nM, -8.5) inhibit the response to cimaterol whereas higher concentrations of CGP12177 cause a stimulatory response (e.g. 100nM, -7 and above). This creates a “dip” in the curve (CGP12177+fixed concentration of cimaterol) and is strongly suggestive of interaction at two different conformations. This is absent at  $\beta 1$ -TM4 and  $\beta 2$ -WT suggesting single conformation interaction. Similarly the response to pindolol is biphasic at  $\beta 1$ -WT but monophasic at  $\beta 1$ -TM4 and  $\beta 2$ -WT again suggesting two conformations at  $\beta 1$ -WT but only one conformation at  $\beta 1$ -TM4 and  $\beta 2$ -WT.

#### Figure 7

$^3\text{H}$ -cAMP accumulation in response to CGP12177 (a, c and e) and pindolol (b, d and f) in transiently transfected cells expressing  $\beta 1$ -V189T (a and b),  $\beta 1$ -L195Q (c and d) and  $\beta 1$ -W199Y (e and f). The

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CGP12177 responses were examined in the absence and presence of 3, 10 and 30nM cimaterol. Bars represent basal  $^3\text{H}$ -cAMP accumulation, that in response to 10 $\mu\text{M}$  isoprenaline or that in response to 3, 10 and 30nM cimaterol alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 6, b) 5, c) 3, d) 3, e) 4 and f) 4 separate experiments.

This Figure shows that both the “dip” in the CGP12177+cimaterol curve and biphasic pindolol response are still present at the  $\beta 1$ -V189T suggesting conservation of the secondary conformation. However both are reduced at  $\beta 1$ -L195Q and  $\beta 1$ -W199Y suggesting some loss of the secondary conformation at  $\beta 1$ -L195Q and  $\beta 1$ -W199Y (see Figure 6a for  $\beta 1$ -WT comparison)

Figure 8

$^3\text{H}$ -cAMP accumulation in response to CGP12177 (a, and c) and pindolol (b and d) in transiently transfected cells expressing  $\beta 1$ -V189T-L195Q-W199Y (a and b) and  $\beta 1$ -W199D (c and d). The CGP12177 responses were examined in the absence and presence of 3, 10 and 30nM cimaterol. Bars represent basal  $^3\text{H}$ -cAMP accumulation, that in response to 10 $\mu\text{M}$  isoprenaline or that in response to 3, 10 and 30nM cimaterol alone. Data points are mean  $\pm$  sem of triplicate determinations. These single experiments are representative of a) 3, b) 4, c) 6 and d) 4 separate experiments.

This Figure shows that the “dip” in the CGP12177+cimaterol curve and biphasic pindolol response, both present at  $\beta 1$ -WT with two conformations (see Figure 6a), are absent in  $\beta 1$ -V189T-L195Q-W199Y and  $\beta 1$ -W199D suggesting that neither of these receptors have the secondary conformation.

Figure 9.

Location of equivalent key residues (V172, M178, W182) in the crystal structure of the turkey  $\beta 1$ -adrenoreceptor described by Huang et al., (2013). This structure was obtained in the ligand-free state and in a lipid membrane-like environment and suggested that TM4 was involved in a dimer interface with TM5. Views of one protomer of the oligomeric structure (PDB; 4GPO) reported by Huang et al., (2013) were generated with Cn3D (NCBI).

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(a) Residues V172, M178, W182 (equivalent to the human V189, L195 and W199) and D121 and N329 (equivalent to D138 and N363, identified in a previous study as essential for both catecholamine and secondary conformation functional responses, Baker et al., 2008) are highlighted in yellow.

(b) View of V172, M178, W182 (highlighted in red) with the key residues for dimerization in TM5 (R205, A206, A210, I218, R229), TM4 (L171) and the proximal region of extracellular loop 2 (W181, R183) identified by Huang et al., (2013) highlighted in purple. Interestingly, V172 and W182 are on the same outward facing surface as the key dimerization residues with W182 sandwiched between the two residues (W181, R183) and V172 is next to another (L171) identified by Huang et al., (2013).

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Table 1

The chimeric  $\beta 1/\beta 2$ -adrenoceptor constructs – the amino acid changes made and where a stable cell line was made, the receptor expression level is given as fmol/mg protein

	Amino acid changes	Fmol/mg protein
$\beta 1$ -WT		556 $\pm$ 39
$\beta 1$ -TM1	L63I, L64V, A66S, L71A, A74F, V81T	372 $\pm$ 47
$\beta 1$ -TM2	M98T, S102C, L110A, T117A, I118H, V119I, V120L	567 $\pm$ 58
$\beta 1$ -TM3	L133F, V137I, L154V	1994 $\pm$ 214
$\beta 1$ -TM4	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T, L195Q, W199Y	233 $\pm$ 18
$\beta 1$ -TM5	R222Q, V230I, C238V, A241V, L245S	359 $\pm$ 30
$\beta 1$ -TM6	V332T, L342I, A343V, V345I, K347H, A348V, F349I	1207 $\pm$ 100
$\beta 1$ -TM7	R357E, L358V, F359Y, V360I, F361L, F362L, L365I, A368V, A371G, I375L	2078 $\pm$ 133
$\beta 1$ -A187G	A187G	
$\beta 1$ -V189T	V189T	
$\beta 1$ -L195Q	L195Q	
$\beta 1$ -W199Y	W199Y	
$\beta 1$ -TM4 stage 1	G177V, L178I, V179I	
$\beta 1$ -TM4 stage 2	G177V, L178I, V179I, C180L, T181M, A184I, I185V	
$\beta 1$ -TM4 stage 3	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G	
$\beta 1$ -TM4 stage 4	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T	
$\beta 1$ -TM4 stage 5	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T, L195Q	
$\beta 1$ -V189T-L195Q	V189T, L195Q	
$\beta 1$ -V189T-W199Y	V189T, W199Y	
$\beta 1$ -L195Q-W199Y	L195Q, W199Y	
$\beta 1$ -V189T-L195Q-W199Y	V189T, L195Q, W199Y	
$\beta 1$ -W199A	W199A	
$\beta 1$ -W199D	W199D	
$\beta 1$ -W199F	W199F	
$\beta 1$ -W199K	W199K	
$\beta 1$ -W199L	W199L	
$\beta 1$ -W199N	W199N	
$\beta 2$ -WT		268 $\pm$ 25
$\beta 2$ -TM1	I38L, V39L, S41A, A46L, F49A, T56V	138 $\pm$ 8
$\beta 2$ -TM2	T73M, C77S, A85L, A92T, H93I, I94V, L95V	405 $\pm$ 41
$\beta 2$ -TM3	F108L, I112V, V129L	1037 $\pm$ 113
* $\beta 2$ -TM4	V152G, I153L, I154V, L155C, M156T, I159A, V160I, G162A, T164V, Q170L	458 $\pm$ 64
$\beta 2$ -TM5	Q197R, I205V, V213C, V216A, S220L	849 $\pm$ 83
$\beta 2$ -TM6	T281V, I291L, V292A, I294V, H296K, V297A, I298F	728 $\pm$ 58
$\beta 2$ -TM7	E306R, V307L, Y308F, I309V, L310F, L311F, I314L, V317A, G320A, L324I	67 $\pm$ 5

\*Making the full change here, i.e with Y174W, rendered the receptor non-functional (no binding and no functional responses). This chimera with 10 of the 11 amino acid substitutions was therefore used as the  $\beta 2$ -TM4 mutant.

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Table 2a

Log EC<sub>50</sub> values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at the human  $\beta$ 1-WT and chimeric  $\beta$ 1/ $\beta$ 2-adrenoceptors where mutations in the  $\beta$ 1-WT mean that each TM region in turn is mutated to that of the  $\beta$ 2-WT (see Table 1). Log K<sub>B</sub> values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. This data was obtained from stable cell lines and n in the Table refers to the number of separate experiments.

This Table shows that the most consistent and highly significant difference between the  $\beta$ 1-WT receptor and all of the TM-swap mutants is that obtained from  $\beta$ 1-TM4 when CGP12177 is the agonist. As CGP12177 agonist responses occur at the secondary low affinity conformation of the  $\beta$ 1-WT receptor, this suggests that the  $\beta$ 1-TM4 receptor mutations alter this conformation in some way.

Cimaterol as agonist – $\beta$ 1 – stable cell lines															
	Cimaterol Log EC <sub>50</sub>	% isop	n	Log K <sub>B</sub> CGP20712A	n	Log K <sub>B</sub> bisoprolol	n	Log K <sub>B</sub> ICI118551	n	Log K <sub>B</sub> propranolol	n	Log K <sub>B</sub> carvedilol	n	Log K <sub>B</sub> CGP12177	n
$\beta$ 1-WT	-8.25 $\pm$ 0.14	95.8 $\pm$ 2.9	8	-9.18 $\pm$ 0.07	4	-8.48 $\pm$ 0.17	5	-6.96 $\pm$ 0.04	5	-8.52 $\pm$ 0.08	12	-9.89 $\pm$ 0.13	5	-9.55 $\pm$ 0.12	8
$\beta$ 1-TM1	-7.91 $\pm$ 0.05*	96.5 $\pm$ 3.3	11	-9.35 $\pm$ 0.10	9	-8.65 $\pm$ 0.12	5	-6.96 $\pm$ 0.05	9	-8.66 $\pm$ 0.07	16	-9.88 $\pm$ 0.08	5	-9.77 $\pm$ 0.07	15
$\beta$ 1-TM2	-8.19 $\pm$ 0.03	98.9 $\pm$ 3.0	15	-8.78 $\pm$ 0.12	8	-8.15 $\pm$ 0.07	7	-7.47 $\pm$ 0.03*	14	-8.52 $\pm$ 0.03	24	-9.53 $\pm$ 0.18	8	-9.90 $\pm$ 0.09	17
$\beta$ 1-TM3	-8.82 $\pm$ 0.03*	102.4 $\pm$ 2.5	9	-9.34 $\pm$ 0.12	9	-8.43 $\pm$ 0.07	4	-7.31 $\pm$ 0.04*	10	-8.53 $\pm$ 0.06	15	-9.92 $\pm$ 0.16	5	-9.49 $\pm$ 0.06	13
$\beta$ 1-TM4	-8.15 $\pm$ 0.02	99.2 $\pm$ 1.8	10	-9.50 $\pm$ 0.13	11	-8.81 $\pm$ 0.04	5	-8.07 $\pm$ 0.06*	9	-8.99 $\pm$ 0.04*	15	-9.81 $\pm$ 0.12	6	-9.63 $\pm$ 0.08	17
$\beta$ 1-TM5	-8.48 $\pm$ 0.05	103.0 $\pm$ 1.7	9	-9.09 $\pm$ 0.04	11	-7.91 $\pm$ 0.03	5	-7.01 $\pm$ 0.06	11	-8.71 $\pm$ 0.04	17	-9.96 $\pm$ 0.09	6	-9.68 $\pm$ 0.09	17
$\beta$ 1-TM6	-8.59 $\pm$ 0.03*	103.2 $\pm$ 2.2	10	-8.52 $\pm$ 0.07	8	-8.10 $\pm$ 0.15	5	-7.92 $\pm$ 0.05*	12	-8.90 $\pm$ 0.05*	18	-10.13 $\pm$ 0.09	6	-9.58 $\pm$ 0.06	14
$\beta$ 1-TM7	-8.54 $\pm$ 0.05	106.1 $\pm$ 2.5	10	-8.45 $\pm$ 0.08	7	-8.19 $\pm$ 0.06	5	-7.80 $\pm$ 0.05*	12	-8.95 $\pm$ 0.05*	18	-10.06 $\pm$ 0.03	3	-9.39 $\pm$ 0.05	13
CGP12177 as agonist – $\beta$ 1 – stable cell lines															
	CGP 12177 Log EC <sub>50</sub>	% isop	n	Log K <sub>B</sub> CGP20712A	n	Log K <sub>B</sub> bisoprolol	n	Log K <sub>B</sub> ICI118551	n	Log K <sub>B</sub> propranolol	n	Log K <sub>B</sub> carvedilol	n		
$\beta$ 1-WT	-8.18 $\pm$ 0.08	73.8 $\pm$ 5.6	10	-7.16 $\pm$ 0.06	14	-5.83 $\pm$ 0.14	4	-5.90 $\pm$ 0.15	9	-6.18 $\pm$ 0.06	13	-7.25 $\pm$ 0.18	7		
$\beta$ 1-TM1	-7.83 $\pm$ 0.05	53.7 $\pm$ 3.9	10	-7.41 $\pm$ 0.13	11	-5.77 $\pm$ 0.18	4	-5.79 $\pm$ 0.09	3	-6.55 $\pm$ 0.12	13	-7.72 $\pm$ 0.13	7		
$\beta$ 1-TM2	-8.27 $\pm$ 0.09	53.5 $\pm$ 3.9	14	-7.13 $\pm$ 0.08	13	-6.72 $\pm$ 0.18	7	-5.87 $\pm$ 0.05	6	-6.87 $\pm$ 0.15*	18	-8.52 $\pm$ 0.15*#	7		
$\beta$ 1-TM3	-8.07 $\pm$ 0.03	70.5 $\pm$ 3.6	9	-7.45 $\pm$ 0.05	12	-5.83 $\pm$ 0.13	4	-5.83 $\pm$ 0.09	7	-6.31 $\pm$ 0.05	16	-7.45 $\pm$ 0.09	6		
$\beta$ 1-TM4	-9.27 $\pm$ 0.06*#	29.2 $\pm$ 1.4*#	10	-9.49 $\pm$ 0.08*#	9	-8.83 $\pm$ 0.21*#	7	-7.93 $\pm$ 0.11*#	11	-8.85 $\pm$ 0.09*#	15	-9.73 $\pm$ 0.21*#	7		
$\beta$ 1-TM5	-7.91 $\pm$ 0.03	60.1 $\pm$ 3.4	10	-6.68 $\pm$ 0.04*	11	-5.31 $\pm$ 0.13	5	-5.71 $\pm$ 0.15	8	-6.23 $\pm$ 0.09	15	-7.53 $\pm$ 0.16	7		
$\beta$ 1-TM6	-7.83 $\pm$ 0.02*	63.5 $\pm$ 2.8	11	-5.97 $\pm$ 0.08*#	12	-5.22 $\pm$ 0.14	3	-5.79 $\pm$ 0.04	11	-6.05 $\pm$ 0.06	19	-7.24 $\pm$ 0.18	7		
$\beta$ 1-TM7	-7.08 $\pm$ 0.02*#	68.5 $\pm$ 3.8	11	-6.86 $\pm$ 0.05	10	-5.67 $\pm$ 0.05	5	-6.67 $\pm$ 0.03*#	13	-6.42 $\pm$ 0.06	16	-7.35 $\pm$ 0.12	6		

\*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the  $\beta$ 1-WT. Thus the log EC<sub>50</sub> for CGP12177 at  $\beta$ 1-TM4 is different from that obtained from the  $\beta$ 1-WT with p<0.001.

#p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing each value with all other values in this set. Thus the log EC<sub>50</sub> value for CGP12177 at  $\beta$ 1-TM4 is different from that obtained for  $\beta$ 1-WT,  $\beta$ 1-TM1,  $\beta$ 1-TM2,  $\beta$ 1-TM3,  $\beta$ 1-TM5,  $\beta$ 1-TM6 and  $\beta$ 1-TM7 with p<0.001 in all cases.

Table 2b

The ratio of the log  $K_B$  value for CGP12177 (as an antagonist of cimaterol, thus at the high affinity conformation) and log  $EC_{50}$  value for CGP12177 (as an agonist of the secondary low affinity conformation) for responses seen at the  $\beta 1$ -WT and  $\beta 1$ -TM-chimeric receptors (data taken from Table 2a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log  $K_B$  for antagonism of cimaterol at high affinity site / log  $K_B$  for antagonism of CGP12177 at low affinity site).

This Table examines the ratio of the affinities of ligands at the two conformations of the  $\beta 1$ -adrenoceptor and thus allows for differences in absolute affinity. For example, ICI118551 has a higher affinity at the  $\beta 1$ -TM7 receptor (Table 2a) than the  $\beta 1$ -WT receptor, however the ratio of the affinity in the presence of cimaterol and CGP 12177 is similar to that at the WT receptor suggesting that although the  $\beta 1$ -TM7 mutation alters the affinity of ICI118551, it does not alter the secondary conformation. The most consistent change is seen with the  $\beta 1$ -TM4 mutation where the ratios are all nearly 0 suggesting no difference in affinity i.e. no secondary conformation.

	Ratio of log $K_B$ CGP12177 / log $EC_{50}$ CGP12177	Ratio of log $K_B$ (cimaterol as agonist) / log $K_B$ (CGP12177 as agonist)				
		CGP20712A	bisoprolol	ICI118551	propranolol	carvedilol
$\beta 1$ -WT	1.37	2.02	2.65	1.06	2.34	2.64
$\beta 1$ -TM1	1.94	1.94	2.88	1.17	2.11	2.16
$\beta 1$ -TM2	1.63	1.65	1.43	1.60	1.65	1.01
$\beta 1$ -TM3	1.42	1.89	2.60	1.48	2.22	2.47
$\beta 1$ -TM4	0.36	0.01	-0.02	0.14	0.14	0.08
$\beta 1$ -TM5	1.77	2.41	2.60	1.30	2.47	2.43
$\beta 1$ -TM6	1.75	2.55	2.88	2.15	2.85	2.89
$\beta 1$ -TM7	2.31	1.59	2.52	1.13	2.53	2.71



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Table 3a

Log EC<sub>50</sub> values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at the human  $\beta$ 2-WT and chimeric  $\beta$ 2/ $\beta$ 1-receptors where mutations in the  $\beta$ 2-WT mean that each TM region in turn is mutated to that of the  $\beta$ 1-WT (see Table 1). Log K<sub>B</sub> values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. This data was obtained from stable cell lines and n in the Table refers to the number of separate experiments.

This Table shows that although there are some changes (e.g.  $\beta$ 2-TM2 has higher affinity for CGP20712A than  $\beta$ 2-WT), there is no highly significant consistent change in one mutant receptor compared to the others.

Cimaterol as agonist – $\beta$ 2 – stable cell lines													
	Cimaterol Log EC <sub>50</sub>	% isop	n	Log K <sub>B</sub> CGP20712A	n	Log K <sub>B</sub> bisoprolol	n	Log K <sub>B</sub> ICI118551	n	Log K <sub>B</sub> propranolol	n	Log K <sub>B</sub> CGP12177	n
$\beta$ 2-WT	-9.05 $\pm$ 0.08	101.5 $\pm$ 3.2	13	-6.03 $\pm$ 0.12	8	-6.85 $\pm$ 0.10	8	-9.56 $\pm$ 0.07	8	-9.65 $\pm$ 0.05	14	-10.00 $\pm$ 0.05	9
$\beta$ 2-TM1	-9.45 $\pm$ 0.06	105.3 $\pm$ 2.8	9	-6.46 $\pm$ 0.10	8	-6.82 $\pm$ 0.09	10	-9.74 $\pm$ 0.08	9	-9.56 $\pm$ 0.06	19	-9.74 $\pm$ 0.11	19
$\beta$ 2-TM2	-9.64 $\pm$ 0.08*	102.1 $\pm$ 1.8	12	-7.20 $\pm$ 0.11*	8	-7.25 $\pm$ 0.10	7	-9.30 $\pm$ 0.11	8	-9.87 $\pm$ 0.03	14	-10.01 $\pm$ 0.06	9
$\beta$ 2-TM3	-9.21 $\pm$ 0.13	98.3 $\pm$ 4.1	7	-6.57 $\pm$ 0.12	7	-7.05 $\pm$ 0.13	6	-9.71 $\pm$ 0.10	7	-9.81 $\pm$ 0.07	11	-10.00 $\pm$ 0.10	10
$\beta$ 2-TM4	-9.66 $\pm$ 0.06*	103.3 $\pm$ 2.4	13	-6.09 $\pm$ 0.09	10	-6.75 $\pm$ 0.03	9	-8.87 $\pm$ 0.04*	10	-9.34 $\pm$ 0.06	14	-9.83 $\pm$ 0.05	9
$\beta$ 2-TM5	-8.97 $\pm$ 0.09	100.6 $\pm$ 2.4	13	-6.63 $\pm$ 0.12	10	-7.04 $\pm$ 0.09	9	-9.50 $\pm$ 0.09	9	-9.46 $\pm$ 0.04	13	-9.82 $\pm$ 0.07	4
$\beta$ 2-TM6	-8.74 $\pm$ 0.05	101.0 $\pm$ 4.7	12	-7.05 $\pm$ 0.15*	7	-7.07 $\pm$ 0.10	9	-8.86 $\pm$ 0.12*	9	-9.52 $\pm$ 0.06	11	-9.59 $\pm$ 0.13	8
$\beta$ 2-TM7	-8.77 $\pm$ 0.06	107.4 $\pm$ 2.9	10	-6.54 $\pm$ 0.04	14	-7.06 $\pm$ 0.09	11	-9.18 $\pm$ 0.07	12	-9.30 $\pm$ 0.06*	17	-9.72 $\pm$ 0.10	13
CGP12177 as agonist – $\beta$ 2 – stable cell lines													
	CGP 12177 Log EC <sub>50</sub>	% isop	n	Log K <sub>B</sub> CGP20712A	n	Log K <sub>B</sub> bisoprolol	n	Log K <sub>B</sub> ICI118551	n	Log K <sub>B</sub> propranolol	n		
$\beta$ 2-WT	-9.35 $\pm$ 0.09	23.5 $\pm$ 3.9	14	-6.12 $\pm$ 0.09	3	-6.60 $\pm$ 0.14	11	-9.31 $\pm$ 0.17	11	-9.02 $\pm$ 0.12	19		
$\beta$ 2-TM1	-9.48 $\pm$ 0.19	26.9 $\pm$ 1.7	9	-6.00 $\pm$ 0.16	9	-6.63 $\pm$ 0.12	12	-9.51 $\pm$ 0.17	12	-9.07 $\pm$ 0.08	20		
$\beta$ 2-TM2	-9.20 $\pm$ 0.09	12.6 $\pm$ 1.9	11	-6.79 $\pm$ 0.11	8	-7.00 $\pm$ 0.09	9	-9.03 $\pm$ 0.11	10	-9.32 $\pm$ 0.10	14		
$\beta$ 2-TM3	-9.19 $\pm$ 0.10	35.9 $\pm$ 3.3	9	-6.21 $\pm$ 0.29	4	-6.53 $\pm$ 0.12	8	-9.47 $\pm$ 0.16	8	-8.95 $\pm$ 0.09	21		
$\beta$ 2-TM4	-9.20 $\pm$ 0.07	38.5 $\pm$ 2.4*	13	-6.05 $\pm$ 0.12	8	-6.34 $\pm$ 0.09	7	-8.42 $\pm$ 0.08*	11	-8.34 $\pm$ 0.08*	18		
$\beta$ 2-TM5	-9.31 $\pm$ 0.07	28.8 $\pm$ 2.0	12	-6.47 $\pm$ 0.15	8	-6.98 $\pm$ 0.22	8	-9.31 $\pm$ 0.08	9	-8.75 $\pm$ 0.10	19		
$\beta$ 2-TM6	-8.81 $\pm$ 0.10	22.4 $\pm$ 1.6	8	-7.31 $\pm$ 0.34	5	-6.74 $\pm$ 0.11	5	-8.39 $\pm$ 0.27	4	-9.11 $\pm$ 0.11	4		
$\beta$ 2-TM7	-9.71 $\pm$ 0.12	41.3 $\pm$ 2.7*	9	-6.19 $\pm$ 0.15	10	-6.59 $\pm$ 0.16	10	-9.09 $\pm$ 0.14	9	-9.02 $\pm$ 0.08	22		

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\* $p < 0.001$  One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the  $\beta 2$ -WT. Thus the log  $K_B$  value for CGP20712A at  $\beta 2$ -TM2 obtained in the presence of cimaterol is different ( $p < 0.001$ ) from that obtained in the  $\beta 2$ -WT receptor.

# $p < 0.001$  One-way ANOVA with post hoc Newman-Keuls comparing each value with all other values in this set (as for Table 2a). Unlike Table 2a for the  $\beta 1$ -receptor. There are no values that fulfil this criterion for the  $\beta 2$ -receptor.

Table 3b

The ratio of the log  $K_B$  value for CGP12177 (as an antagonist of cimaterol) and log  $EC_{50}$  value for CGP12177 for responses seen at the  $\beta 2$ WT and  $\beta 2$ TM-chimeric-receptors (data taken from Table 3a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log  $K_B$  for antagonism of cimaterol / log  $K_B$  for antagonism of CGP12177).

This Table examines the ratio of the affinities of ligands at the  $\beta 2$ -adrenoceptor and thus allows for differences in absolute affinity. ICI118551 has lower affinity at the  $\beta 2$ -TM4 receptor (Table 3a), but this occurred both in the presence of cimaterol and CGP12177 thus the ratio is similar to that at the other receptors. There is no large consistent change with any of the receptors.

	Ratio of log $K_B$ CGP12177 / log $EC_{50}$ CGP12177	Ratio of log $K_B$ (cimaterol as agonist) / log $K_B$ (CGP12177 as agonist)			
		CGP20712A	bisoprolol	ICI118551	propranolol
$\beta 2$ -WT	0.65	-0.09	0.25	0.25	0.63
$\beta 2$ -TM1	0.26	0.46	0.19	0.23	0.49
$\beta 2$ -TM2	0.81	0.41	0.25	0.27	0.55
$\beta 2$ -TM3	0.81	0.36	0.52	0.24	0.86
$\beta 2$ -TM4	0.63	0.04	0.41	0.45	1.00
$\beta 2$ -TM5	0.51	0.16	0.06	0.19	0.71
$\beta 2$ -TM6	0.78	-0.26	0.33	0.47	0.41
$\beta 2$ -TM7	0.01	0.35	0.47	0.09	0.28

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Table 4a

Log EC<sub>50</sub> values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at the β1-WT, the chimeric β1-TM4 receptor (containing the full TM4 mutations) and chimeric β1/β2-adrenoceptors with several amino acids in TM4 mutated to that of the β2-WT. Log K<sub>B</sub> values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. Table 1 lists the detail of each mutation. This data was obtained from between 4 and 24 stable mixed populations of cells for each chimera, and n in the Table refers to the number of separate experiments.

This Table shows that within TM4, it is only the amino acid changes in Stage 5 and the full TM4 (i.e. L195Q and W199Y) that are very important for the alteration of the secondary conformation (i.e. responses to CGP12177 and antagonist affinities obtained in the presence of CGP12177). Stage 4 (addition of V189T to the other changes) affects the affinity of ICI18551. There is no change in the pharmacology of either conformation even when the majority of amino acids have been changed (up to and including Stage 3).

Cimaterol as agonist – stable mixed populations of cells															
	Cimaterol Log EC <sub>50</sub>	% isop	n		Log K <sub>B</sub> CGP20712A	n		Log K <sub>B</sub> ICI118551	n		Log K <sub>B</sub> propranolol	n		Log K <sub>B</sub> CGP12177	n
β1-WT	-8.29 ± 0.04	81.2 ± 2.1	24		-9.36 ± 0.07	35		-7.13 ± 0.06	31		-8.69 ± 0.08	30		-10.01 ± 0.06	33
β1-TM4 Stage 1	-8.26 ± 0.11	85.6 ± 3.9	4		-9.10 ± 0.09	4		-7.04 ± 0.09	6		-8.64 ± 0.14	4		-9.96 ± 0.33	3
β1-TM4 Stage 2	-8.20 ± 0.06	77.8 ± 1.5	12		-9.34 ± 0.10	18		-7.14 ± 0.06	14		-8.79 ± 0.11	12		-9.81 ± 0.09	14
β1-TM4 Stage 3	-8.13 ± 0.07	78.2 ± 3.9	11		-8.95 ± 0.09	13		-7.00 ± 0.07	10		-8.63 ± 0.07	11		-9.93 ± 0.06	15
β1-TM4 Stage 4	-8.08 ± 0.05	76.3 ± 1.5	12		-9.14 ± 0.08	18		-7.62 ± 0.05*	16		-8.59 ± 0.06	12		-9.77 ± 0.06	19
β1-TM4 Stage 5	-8.14 ± 0.06	78.2 ± 3.2	11		-8.97 ± 0.09	16		-7.71 ± 0.07*	15		-8.81 ± 0.04	11		-9.93 ± 0.08	19
β1-TM4	-8.32 ± 0.05	77.3 ± 1.9	23		-9.43 ± 0.09	35		-7.94 ± 0.06*	31		-9.08 ± 0.07	29		-9.98 ± 0.09	35
CGP12177 as agonist – stable mixed populations of cells															
	CGP12177 Log EC <sub>50</sub>	% isop	n		Log K <sub>B</sub> CGP20712A	n		Log K <sub>B</sub> ICI118551	n		Log K <sub>B</sub> propranolol	n			
β1-WT	-8.12 ± 0.07	53.3 ± 1.5	21		-7.37 ± 0.08	37		-5.86 ± 0.08	22		-6.69 ± 0.08	27			
β1-TM4 Stage 1	-8.43 ± 0.06	50.4 ± 2.2	4		-7.32 ± 0.25	5		-6.21 ± 0.50	3		-6.17 ± 0.17	4			
β1-TM4 Stage 2	-8.46 ± 0.07	46.5 ± 1.9	11		-7.55 ± 0.12	17		-6.08 ± 0.11	10		-6.78 ± 0.09	20			
β1-TM4 Stage 3	-8.48 ± 0.08	45.9 ± 3.0	11		-7.13 ± 0.10	18		-5.90 ± 0.11	10		-6.88 ± 0.11	13			
β1-TM4 Stage 4	-7.93 ± 0.12	40.0 ± 1.6*	10		-7.26 ± 0.10	15		-6.57 ± 0.13*	10		-6.98 ± 0.13	18			
β1-TM4 Stage 5	-9.29 ± 0.06*	30.2 ± 2.3*	11		-8.74 ± 0.09*	19		-7.54 ± 0.09*	10		-8.18 ± 0.08*	25			
β1-TM4	-9.43 ± 0.06*	33.9 ± 1.5*	19		-8.98 ± 0.07*	39		-7.64 ± 0.08*	32		-8.39 ± 0.06*	40			

\*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β1-WT, thus the log EC<sub>50</sub> for CGP12177 at β1-TM4 Stage 5 receptor is different from that obtained from the β1-WT with p<0.001

Table 4b

The ratio of the log  $K_B$  value for CGP12177 (as an antagonist of cimaterol, thus at the high affinity conformation) and log  $EC_{50}$  value for CGP12177 (as an agonist of the secondary low affinity conformation) for responses seen at the  $\beta 1$ -WT and  $\beta 1$ -TM4 chimeric receptors (data taken from Table 4a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log  $K_B$  for antagonism of cimaterol at high affinity site / log  $K_B$  for antagonism of CGP12177 at low affinity site).

This Table examines the ratio of the affinities of ligands and shows that the ratio of affinities between the two conformations is much less for  $\beta 1$ -TM4 Stage 5 and beyond suggesting that only the amino acids in Stage 5 and beyond are important for the secondary conformation.

	Ratio of log $K_B$ CGP12177 / log $EC_{50}$ CGP12177	Ratio of log $K_B$ (cimaterol as agonist) / log $K_B$ (CGP12177 as agonist)		
		CGP20712A	ICI118551	propranolol
$\beta 1$ WT	1.89	1.99	1.27	2.00
$\beta 1$ -TM4 Stage 1	1.53	1.78	0.83	2.47
$\beta 1$ -TM4 Stage 2	1.35	1.79	1.06	2.01
$\beta 1$ -TM4 Stage 3	1.45	1.82	1.10	1.75
$\beta 1$ -TM4 Stage 4	1.84	1.88	1.05	1.61
$\beta 1$ -TM4 Stage 5	0.64	0.23	0.17	0.63
$\beta 1$ -TM4	0.55	0.45	0.30	0.69

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Table 5a

Log EC<sub>50</sub> values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at β1-WT, the chimeric β1-TM4 receptor (containing the full TM4 mutations) and chimeric β1/β2-adrenoceptors with single point mutations and then combinations of these mutations. Log K<sub>B</sub> values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. This data was obtained from between 4 and 24 stable mixed populations of cells for each receptor and n in the Table refers to the number of separate experiments

This Table shows that the mutations L195Q and W199Y both alone and in combination have a very significant effect on the responses to CGP12177 and affinity of antagonist measured in the presence of CGP12177 (i.e. the secondary conformation of the β1-receptor). Every time the mutation V189T is present, the affinity of ICI118551 is increased, both at the catecholamine conformation (in the presence of cimaterol) and at the secondary conformation (presence of CGP12177).

Cimaterol as agonist – stable mixed populations of cells											
	Cimaterol Log EC <sub>50</sub>	% isop	n	Log K <sub>B</sub> CGP20712A	n	Log K <sub>B</sub> ICI118551	n	Log K <sub>B</sub> propranolol	n	Log K <sub>B</sub> CGP12177	n
β1-WT	-8.29 ± 0.04	81.2 ± 2.1	24	-9.36 ± 0.07	35	-7.13 ± 0.06	31	-8.69 ± 0.08	30	-10.01 ± 0.06	33
β1-A187G	-8.22 ± 0.08	78.9 ± 2.3	8	-9.23 ± 0.08	14	-7.02 ± 0.07	8	-8.72 ± 0.09	8	-9.99 ± 0.06	17
β1-V189T	-8.20 ± 0.04	80.6 ± 1.8	12	-9.26 ± 0.09	20	-7.73 ± 0.07*	15	-8.61 ± 0.05	14	-9.81 ± 0.08	27
β1-L195Q	-8.20 ± 0.05	80.6 ± 2.7	12	-8.97 ± 0.10	19	-7.12 ± 0.07	15	-8.86 ± 0.11	14	-9.85 ± 0.07	25
β1-W199Y	-8.46 ± 0.06	77.3 ± 2.4	12	-9.66 ± 0.08	19	-7.39 ± 0.07	16	-9.01 ± 0.12	15	-10.06 ± 0.05	26
β1-W199A	-8.63 ± 0.07*	85.4 ± 3.2	8	-9.51 ± 0.14	13	-7.26 ± 0.08	11	-9.12 ± 0.11	13	-10.07 ± 0.16	4
β1-W199D	-8.91 ± 0.07*	87.6 ± 3.5	8	-9.88 ± 0.08	12	-7.48 ± 0.11	10	-9.17 ± 0.10	12	-9.85 ± 0.12	8
β1-W199F	-8.71 ± 0.09*	80.0 ± 2.6	8	-10.14 ± 0.10*	14	-7.19 ± 0.12	12	-9.09 ± 0.13	14	-9.95 ± 0.16	11
β1-W199K	-7.64 ± 0.05*	86.4 ± 3.2	8	-9.46 ± 0.08	14	-6.41 ± 0.06*	9	-8.00 ± 0.12*	10	\$	
β1-W199L	-7.60 ± 0.06*	75.2 ± 2.9	8	-10.06 ± 0.11*	12	-6.90 ± 0.11	11	-8.72 ± 0.06	12	\$	
β1-W199N	-8.88 ± 0.06*	84.6 ± 5.4	8	-9.93 ± 0.09	11	-7.21 ± 0.08	12	-9.22 ± 0.10	13	-10.12 ± 0.12	12
β1-V189T-L195Q	-8.22 ± 0.04	87.0 ± 3.4	8	-9.49 ± 0.09	13	-7.67 ± 0.10*	11	-8.81 ± 0.11	14	-9.58 ± 0.09	12
β1-V189T-W199Y	-8.62 ± 0.07*	84.2 ± 2.1	8	-9.93 ± 0.08	12	-7.85 ± 0.08*	10	-8.80 ± 0.08	12	-10.10 ± 0.15	11
β1-L195Q-W199Y	-8.41 ± 0.06	79.6 ± 2.0	8	-9.68 ± 0.06	13	-7.24 ± 0.06	11	-9.04 ± 0.11	12	-9.82 ± 0.14	9
β1-V189T-L195Q-W199Y	-8.44 ± 0.04	82.7 ± 2.7	8	-9.85 ± 0.08	14	-7.92 ± 0.06*	11	-8.99 ± 0.09	14	-9.79 ± 0.11	12
β1-TM4	-8.32 ± 0.05	77.3 ± 1.9	23	-9.43 ± 0.09	35	-7.94 ± 0.06*	31	-9.08 ± 0.07	29	-9.98 ± 0.09	35

### CGP12177 as agonist – stable mixed populations of cells

\*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the  $\beta 1$ -WT, thus the log  $K_B$  for ICI118551 at  $\beta 1$ -V189T is different from that obtained from the  $\beta 1$ -WT with p<0.001

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Table 5b

The ratio of the log  $K_B$  value for CGP12177 (as an antagonist of cimaterol, thus at the high affinity conformation) and log  $EC_{50}$  value for CGP12177 (as an agonist of the secondary low affinity conformation) for responses seen at  $\beta 1$ -WT,  $\beta 1$ -TM4, and  $\beta 1$ -receptors with point mutations in TM4 (data taken from Table 5a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log  $K_B$  for antagonism of cimaterol at high affinity site / log  $K_B$  for antagonism of CGP12177 at low affinity site).

This Table examines the ratios of the antagonist affinities and shows that the difference in affinity between the antagonists at the catecholamine conformation (in the presence of cimaterol) and secondary conformation (presence of CGP12177) is substantially less with the L195 and W199 mutations suggesting that these are important for the secondary conformation. Although V189T alters the affinity of ICI118551, the ratio of affinity does not change suggesting that V189T does not have a major role in the secondary conformation.

	Ratio of log $K_B$ CGP12177 / log $EC_{50}$ CGP12177	Ratio of log $K_B$ (cimaterol as agonist) / log $K_B$ (CGP12177 as agonist)		
		CGP20712A	ICI118551	propranolol
$\beta 1$ -WT	1.89	1.99	1.27	2.00
$\beta 1$ -A187G	1.78	1.98	1.21	2.00
$\beta 1$ -V189T	1.80	1.62	1.37	1.63
$\beta 1$ -L195Q	0.85	0.44	0.48	1.12
$\beta 1$ -W199Y	0.90	1.20	0.70	1.11
$\beta 1$ -W199A	0.61	0.72	0.38	1.16
$\beta 1$ -W199D	0.37	0.94	0.50	0.30
$\beta 1$ -W199F	1.00	2.04	0.66	1.48
$\beta 1$ -W199K	*	1.33	0.07	0.53
$\beta 1$ -W199L	*	1.94	0.45	1.16
$\beta 1$ -W199N	0.85	1.67	0.54	1.03
$\beta 1$ -V189T-L195Q	0.64	0.85	0.56	0.64
$\beta 1$ -V189T-W199Y	1.03	1.23	0.43	0.98
$\beta 1$ -L195Q-W199Y	0.31	0.51	0.30	0.89
$\beta 1$ -V189T-L195Q-W199Y	0.39	0.64	0.43	0.33
$\beta 1$ -TM4	0.55	0.45	0.30	0.69

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Table 6

Log EC<sub>50</sub> values and % maximum response to isoprenaline for <sup>3</sup>H-cAMP accumulation in response to CGP12177 and pindolol at β1-WT, β2-WT and β1-adrenoceptors with single point mutations or combinations of these mutations. This data was obtained from transiently transfected cells and n in the Table refers to the number of separate experiments, each being conducted in a separate transiently transfected population of cells.

This Table shows that the biphasic responses to CGP12177 and pindolol, and the “dip” in the curve seen when CGP12177 competed with a fixed concentration of cimaterol (see Figures 6-8) observed in the β1-WT are lost with several β1-TM4 mutant receptors, including the β1-L195Q-W199Y, β1-V189T-L195Q-W199Y and β1-W199D receptors.

	CGP12177 as agonist – transiently transfected cells						Pindolol as agonist – transiently transfected cells					
	Log EC <sub>50</sub> 1	Log EC <sub>50</sub> 2	% site 1	% max isop	Competition with cimaterol (dip)	n	Log EC <sub>50</sub> 1	Log EC <sub>50</sub> 2	% site 1	% max isop	n	
β1WT	-9.28 ± 0.17	-7.36 ± 0.21	42.3 ± 7.2	53.0 ± 2.9	yes	6	-9.10 ± 0.04	-5.93 ± 0.18	57.1 ± 3.3	30.0 ± 2.0	8	
β1V189T	-9.37 ± 0.12	-7.16 ± 0.06	45.1 ± 2.7	48.4 ± 4.6	yes	6	-8.87 ± 0.05	-5.78 ± 0.14	67.1 ± 3.3	22.3 ± 2.9	5	
β1L195Q	-9.02 ± 0.09			37.4 ± 1.5	slight	3	-9.14 ± 0.08			27.9 ± 1.6	3	
β1W199Y	-9.28 ± 0.11			41.0 ± 3.2	slight	4	-9.02 ± 0.21	-6.49 ± 0.18	71.7 ± 1.4	32.4 ± 3.0	4	
β1W199D	-9.80 ± 0.06			49.9 ± 1.0	no	6	-8.96 ± 0.23			28.9 ± 1.8	4	
β1L195Q-W199Y	-9.62 ± 0.05			39.9 ± 3.7	no	4	-9.33 ± 0.08			26.2 ± 3.0	4	
β1V189T-L195Q-W199Y	-9.52 ± 0.05			35.3 ± 6.3	no	3	-9.11 ± 0.03			21.8 ± 1.4	4	
β1TM4	-9.54 ± 0.06			30.9 ± 1.8	no	4	-9.25 ± 0.08			23.0 ± 3.1	3	
β2WT	-9.81 ± 0.15			11.2 ± 1.6	no	4	-9.58 ± 0.06			11.6 ± 0.4	4	



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Table 7

Amino acids occurring at positions 189, 195 and 199 or equivalent positions in other  $\beta$ -adrenoceptors from several species.

	UniProtKB/TrEMBL accession number		Position 189		Position 195		Position 199
Two-conformation receptors							
Human $\beta$ 1	P08588		V 189		L 195		W 199
Rat $\beta$ 1	P18090		V 189		L 195		W 199
Mouse $\beta$ 1	P34971		V 189		L 195		W 199
Guinea pig $\beta$ 1	B0FL73		V 189		L 195		W 199
Cat $\beta$ 1	Q9TST6		V 189		L 195		W 199
Turkey $\beta$ 1	P07700		V 172		M 178		W 182
Turkey $\beta$ 4C	P43141		I 154		M 160		W 164
Human $\beta$ 3	P13945		V 168		M 174		W 178
One-conformation receptors							
Human $\beta$ 2	P07550		T 164		Q 170		Y 174
Turkey $\beta$ 3C	*		T 161		Q 167		Y 171

\*Sequence from Figure 1, Baker 2010b

Figure 1

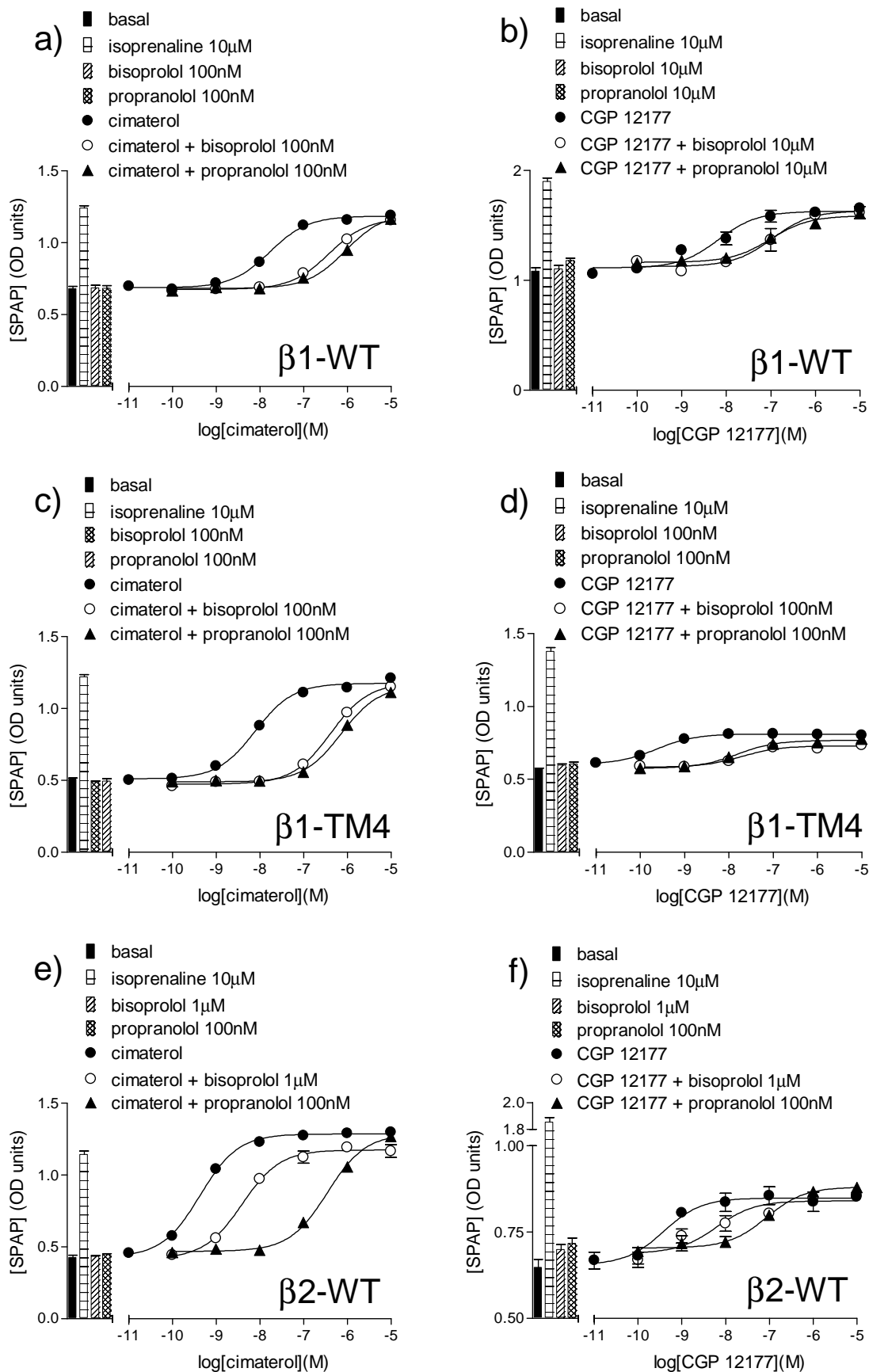


Figure 2

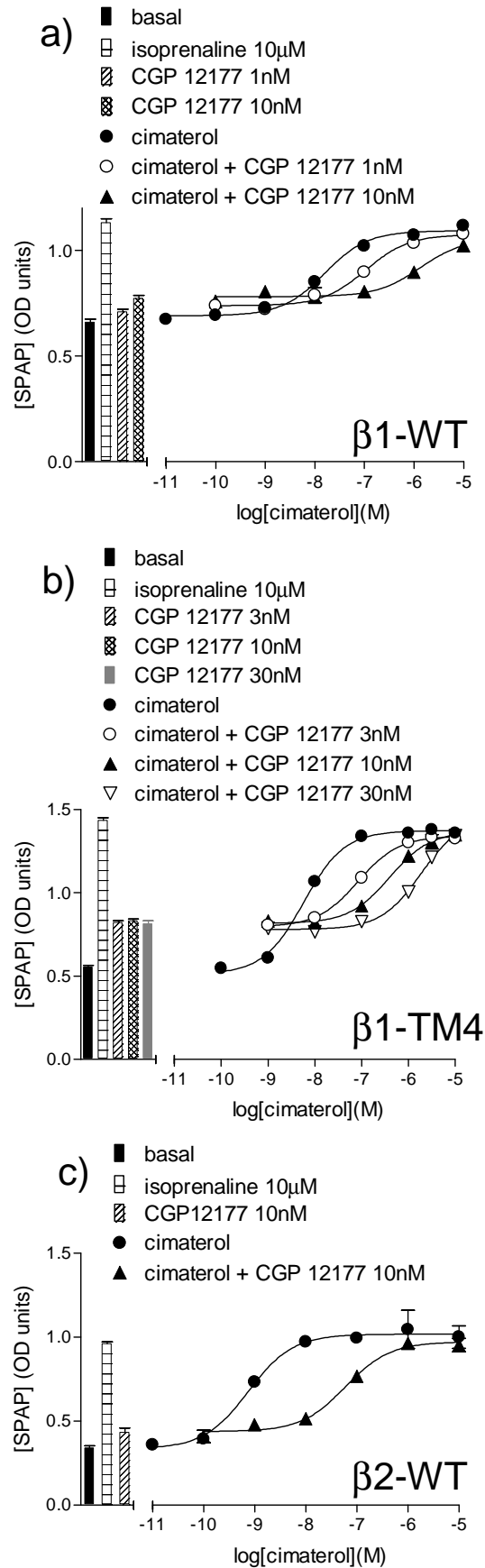


Figure 3

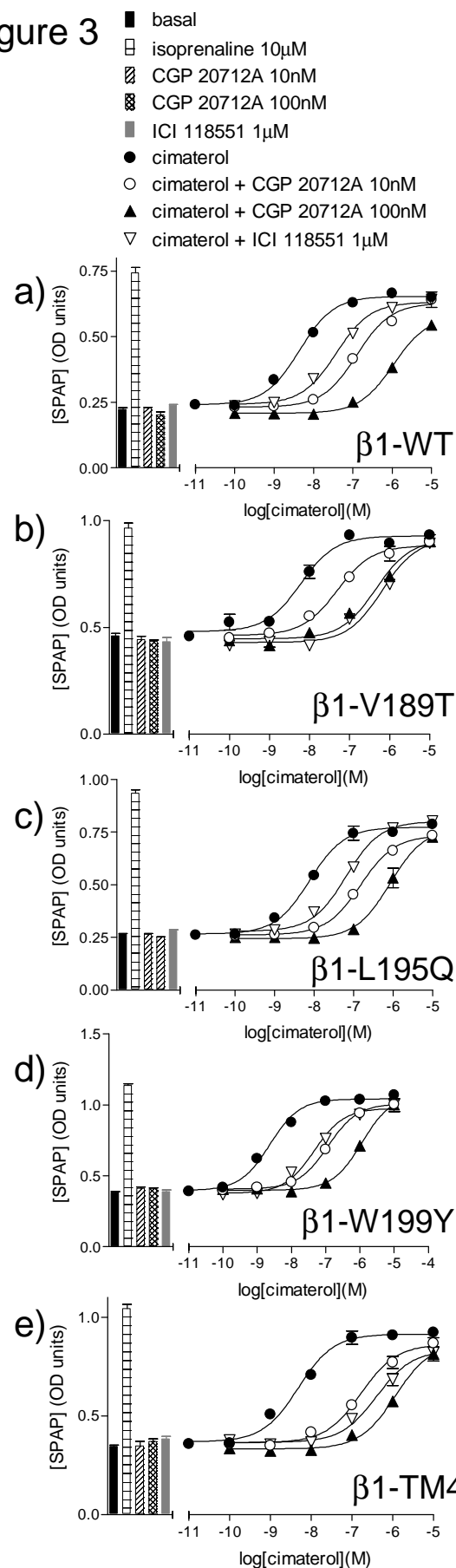


Figure 4

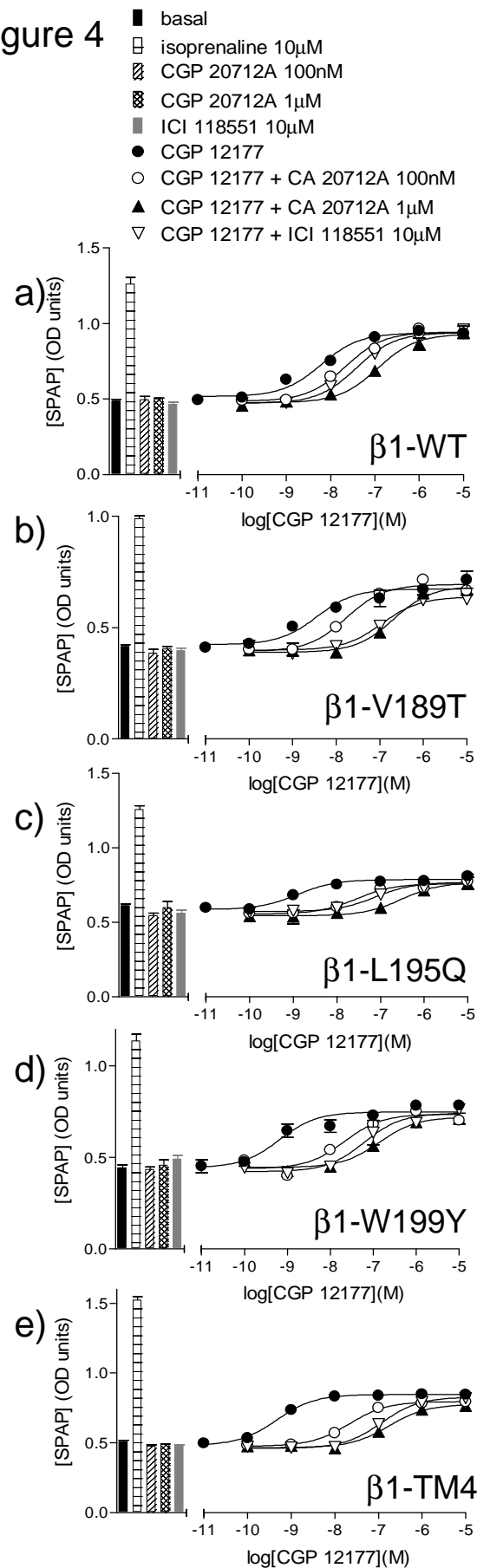


Figure 5

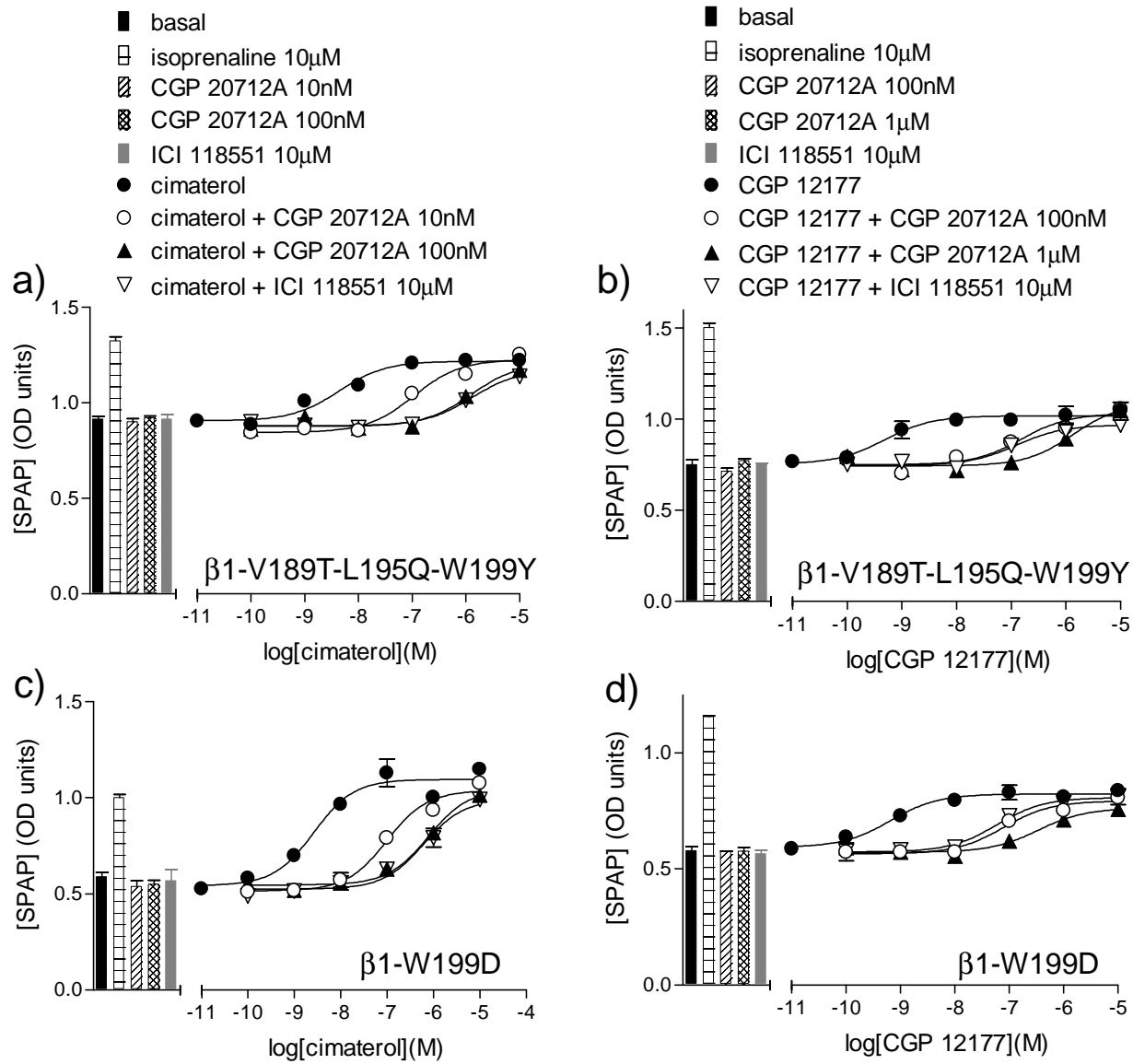


Figure 6

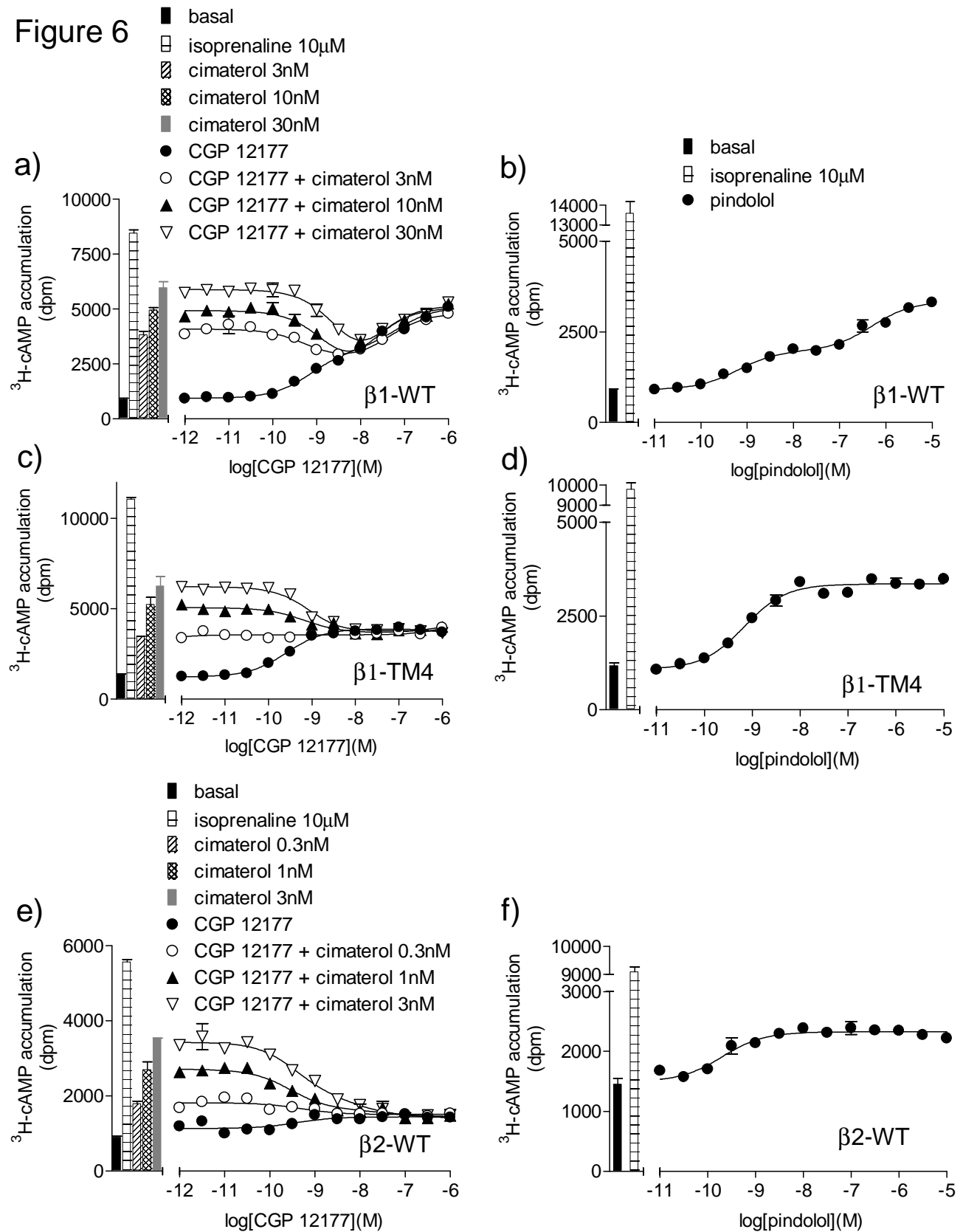


Figure 7

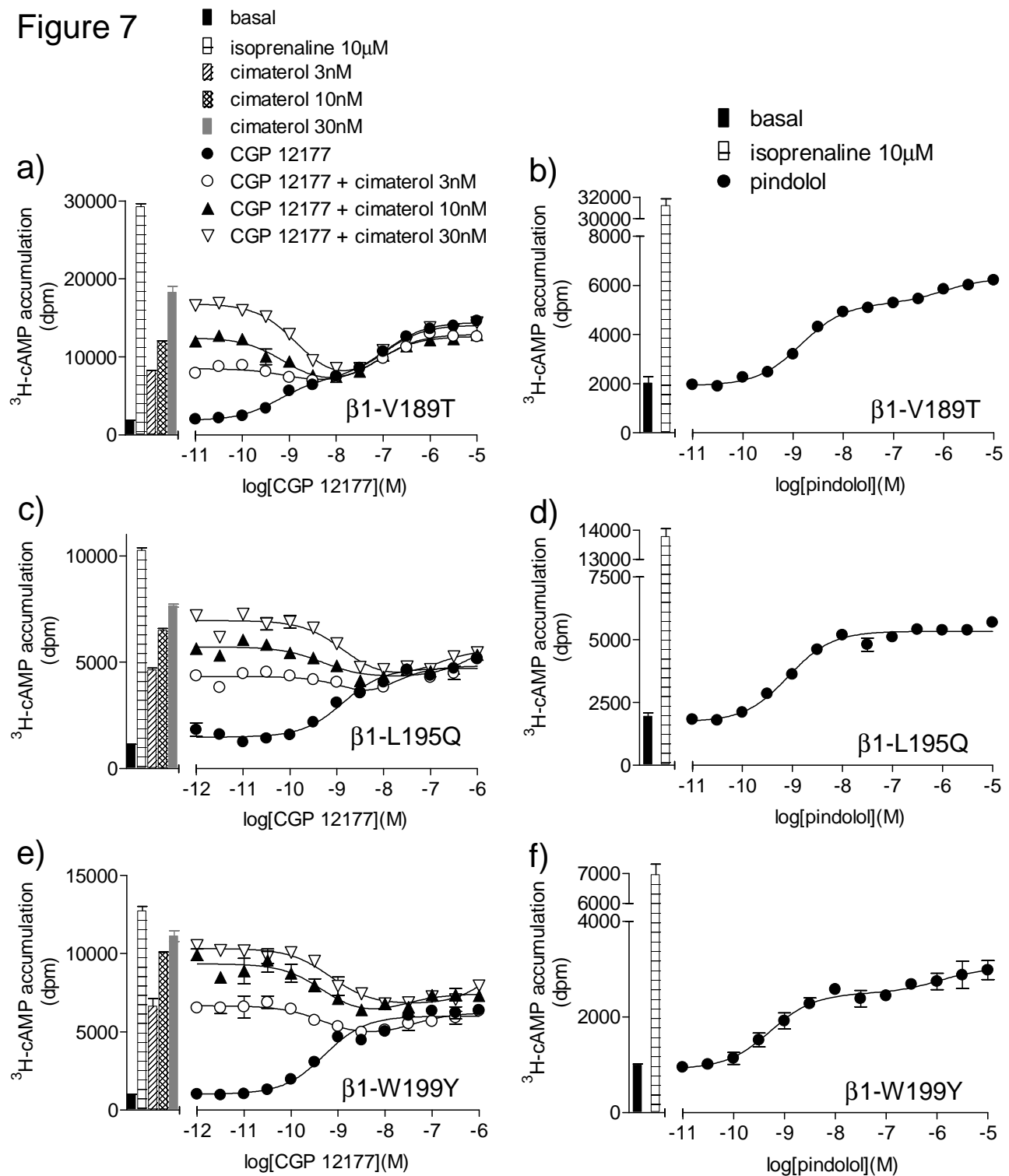




Figure 8

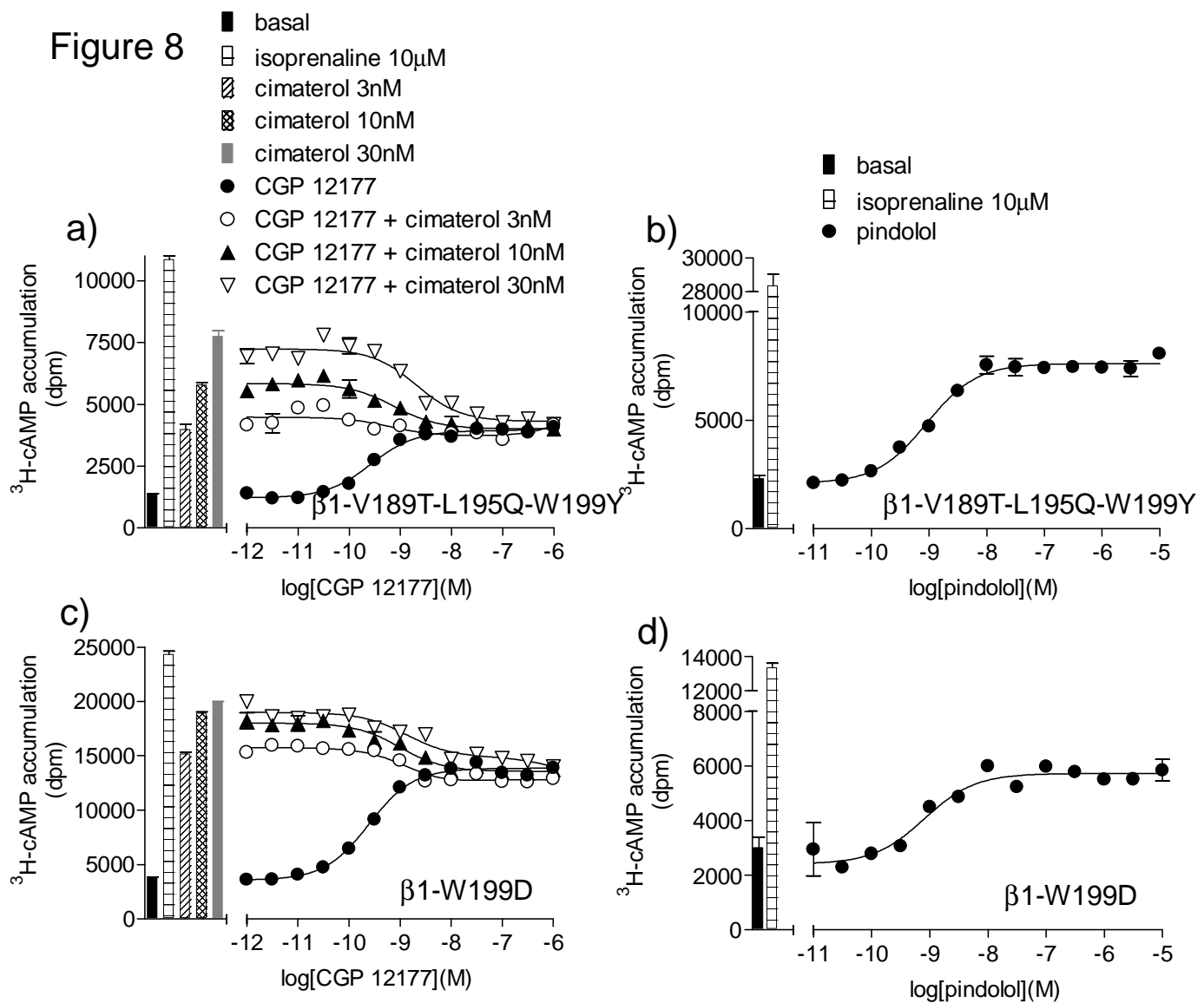


Figure 9.

