

# The Isoleucine at Position 118 in Transmembrane 2 Is Responsible for the Selectivity of Xamoterol, Nebivolol, and ICI89406 for the Human $\beta$ 1-Adrenoceptor<sup>§</sup>

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## ABSTRACT

Known off-target interactions frequently cause predictable drug side-effects (e.g.,  $\beta$ 1-antagonists used for heart disease, risk  $\beta$ 2-mediated bronchospasm). Computer-aided drug design would improve if the structural basis of existing drug selectivity was understood. A mutagenesis approach determined the ligand-amino acid interactions required for  $\beta$ 1-selective affinity of xamoterol and nebivolol, followed by computer-based modeling to provide possible structural explanations. <sup>3</sup>H-CGP12177 whole cell binding was conducted in Chinese hamster ovary cells stably expressing human  $\beta$ 1,  $\beta$ 2, and chimeric  $\beta$ 1/ $\beta$ 2-adrenoceptors (ARs). Single point mutations were investigated in transiently transfected cells. Modeling studies involved docking ligands into three-dimensional receptor structures and performing molecular dynamics simulations, comparing interaction frequencies between *apo* and *holo* structures of  $\beta$ 1 and  $\beta$ 2-ARs. From these observations, an ICI89406 derivative was investigated that gave further insights into selectivity. Stable cell line studies determined that transmembrane 2 was crucial for the  $\beta$ 1-selective affinity of xamoterol and nebivolol. Single point mutations determined that the  $\beta$ 1-AR isoleucine (I118) rather than the  $\beta$ 2 histidine (H93) explained selectivity.

Studies of other  $\beta$ 1-ligands found I118 was important for ICI89406 selective affinity but not that for betaxolol, bisoprolol, or esmolol. Modeling studies suggested that the interaction energies and solvation of  $\beta$ 1-I118 and  $\beta$ 2-H93 are factors determining selectivity of xamoterol and ICI89406. ICI89406 without its phenyl group loses its high  $\beta$ 1-AR affinity, resulting in the same affinity as for the  $\beta$ 2-AR. The human  $\beta$ 1-AR residue I118 is crucial for the  $\beta$ 1-selective affinity of xamoterol, nebivolol, and ICI89406 but not all  $\beta$ 1-selective compounds.

## SIGNIFICANCE STATEMENT

Some ligands have selective binding affinity for the human  $\beta$ 1 versus the  $\beta$ 2-adrenoceptor; however, the molecular/structural reason for this is not known. The transmembrane 2 residue isoleucine I118 is responsible for the selective  $\beta$ 1-binding of xamoterol, nebivolol, and ICI89406 but does not explain the selective  $\beta$ 1-binding of betaxolol, bisoprolol, or esmolol. Understanding the structural basis of selectivity is important to improve computer-aided ligand design, and targeting I118 in  $\beta$ 1-adrenoceptors is likely to increase  $\beta$ 1-selectivity of drugs.

## Introduction

Predictable side-effects from known off-target interactions frequently cause adverse drug effects. Thus, drug discovery efforts are increasingly centered around creating compounds that are highly selective for only the clinical target, in the expectation of maximizing clinical effectiveness while minimizing harm (Clarke and Bond, 1998). However, the precise structural basis of selectivity for most ligands and most G-coupled protein receptors is

poorly understood, including the long studied prototypical  $\beta$ -adrenoceptors ( $\beta$ -AR).

A clinical example of where receptor selectivity matters, and indeed careful control of all pharmacological characteristics is required for minimum harm, are  $\beta$ -blockers for cardiovascular disease.  $\beta$ -blockers are important in the management of heart failure and ischemic heart disease, where blockade of the cardiac  $\beta$ 1-AR is thought to be the main therapeutic factor (Cruickshank, 2007). In those with heart failure,  $\beta$ -blockers lower mortality by about 35%, and several different compounds have been shown to be beneficial [e.g., metoprolol, MERIT-HF Study Group, 1999; bisoprolol, CIBIS-II Investigators and Committees, 1999; carvedilol, Packer et al., 2002; (COPERNICUS); nebivolol, Flather et al., 2005 (SENIORS)]. A similar reduction in mortality is seen in ischemic heart disease with a wider range of  $\beta$ -blockers (Baker and Wilcox 2017 and references therein). However, receptor selectivity is a concern: drugs with concomitant  $\beta$ 2-AR antagonism can cause

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**ABBREVIATIONS:** AR, adrenoceptor;  $\beta$ -AR,  $\beta$ -adrenoceptors; CHO, Chinese hamster ovary; EL, extracellular loop; MD, molecular dynamics; TM, transmembrane; WT, wild-type.

bronchospasm in those with asthma and impair the efficacy of  $\beta$ 2-agonist rescue medication (Baker and Wilcox, 2017 and references therein). Therefore, such drugs remain contraindicated, and, as a consequence, potentially life-saving treatment is denied to those with both asthma and heart disease. In the clinical scenario, efficacy, as well as affinity, matters. Xamoterol is a  $\beta$ 1-AR ligand that has a similar degree of  $\beta$ 1-selective affinity as nebivolol, but xamoterol also has partial agonist activity, and its use was associated with increased mortality (Xamoterol in Severe Heart Failure Study Group, 1990). Thus, controlling receptor selectivity and efficacy are clinically important.

Selective molecules are traditionally developed by an iterative medicinal chemistry-pharmacology process from a starting ligand, making small changes to the molecular structure, examining the pharmacological effects, and then synthesizing further analogs until the required characteristics have sufficiently been optimized. This basic medicinal chemistry technique was used in the original design of  $\beta$ -blockers (Black et al., 1965) and is still used to develop novel  $\beta$ -selective molecules (Baker et al., 2017), but it is costly both in terms of time and materials when done in a “trial and error” fashion. Given the recent advances in protein structure determination techniques, computer-aided ligand design approaches offer a real opportunity to reduce both the time and expense of novel drug development. Although there have been some areas where crystal structures have suggested a structural basis for subtype selectivity (e.g., angiotensin receptors, Zhang et al., 2017; melatonin receptors, Stauch et al., 2019), for many target proteins, there are either too few crystal structures with ligands of different selectivities or these structures do not offer straightforward explanations for ligand selectivity between receptor subtypes. Thus, the structural basis for ligand selectivity, even for the prototypical  $\beta$ -AR, remains largely unknown (e.g.,  $\beta$ 2-selectivity for ICI118551, structure PDB 3NY8). This makes rational drug design challenging.

There are, however, a few compounds where the structural basis for selectivity has been wholly or partly deciphered. Mutagenesis (including chimeric mutagenesis) approaches have uncovered suggestions for certain necessary ligand-amino acid interactions (e.g., Frielle et al., 1988; Marullo et al., 1990; Isogaya et al., 1999). The precise amino acid interactions required for the highly  $\beta$ 2-selective affinity of salmeterol were determined from chimeric mutagenesis studies (Baker et al., 2015) and have been confirmed in the  $\beta$ 2-salmeterol crystal structure (Masureel et al., 2018).

This was an exploratory study with the aim of discovering the precise ligand-amino acid interactions that are important for the  $\beta$ 1- versus  $\beta$ 2-selectivity of two moderately selective  $\beta$ 1-AR ligands, xamoterol and nebivolol. We used a chimeric receptor mutagenesis approach, beginning with the whole receptor, then narrowing down to identify the amino acid(s) important for the selective affinity of these two ligands. This was followed by computational structure-based techniques to explain the molecular basis for the selective affinity of these ligands for the human  $\beta$ 1-AR over the  $\beta$ 2-AR. Having identified the important amino acids, studies were widened to determine whether this single amino acid could explain the selective affinity of other moderately selective compounds (ICI89406, bisoprolol, betaxolol, and esmolol).

## Materials and Methods

### Materials

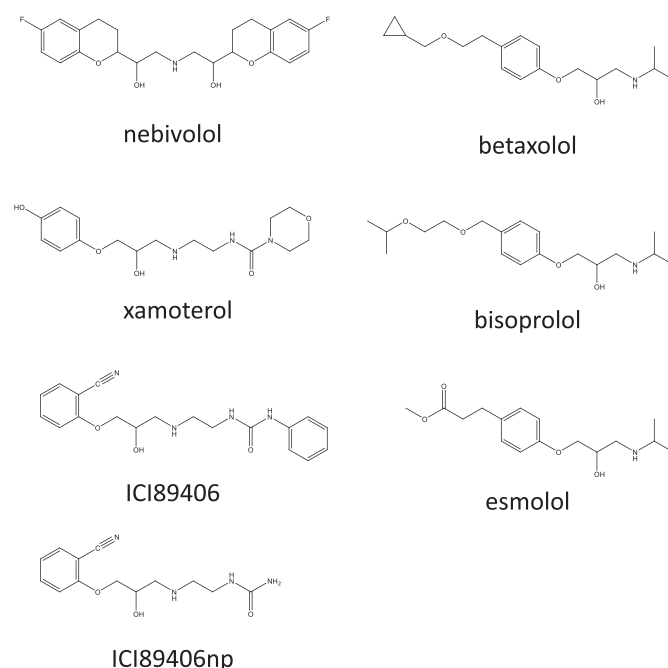
Molecular biology reagents were from Promega (Madison, WI, USA). Lipofectamine, pcDNA3.1, Top 10F competent cells, and OPTIMEM were from Life Technologies (Paisley, UK). QuikChange mutagenesis kits were from Stratagene (La Jolla, CA), and fetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK).  $^3\text{H}$ -CGP12177 was from Amersham International (Buckinghamshire, UK), and Microscint 20 and Ultima Gold XR scintillation fluid were from PerkinElmer (Shelton, CT, USA). Xamoterol (0950), ICI89406 (0832), and betaxolol (0906) were from Tocris Life Sciences (Avonmouth, UK). Nebivolol (SRP035255n) was from Sequoia (Pangbourne, UK). Bisoprolol (B2185) and esmolol (E8031) and all other reagents were from Sigma Aldrich (Poole, Dorset, UK). A derivative of ICI89406 without the terminal phenyl moiety (ICI89406np) was from MolPort (MolPort-031-323-389). The chemical structure of the ligands studied are shown in Fig. 1.

### Molecular Biology

The wild-type human  $\beta$ 1-adrenoceptor ( $\beta$ 1-WT), wild-type human  $\beta$ 2-adrenoceptor ( $\beta$ 2-WT), and all transmembrane (TM) and extracellular region chimera constructs, as well as all stable cell lines, are as reported in Baker et al. (2014, 2015). The single point mutations used here (Tables 1 and 2) were generated using QuikChange mutagenesis and BioLine PolyMate Additive for GC-rich templates (as in Baker et al., 2014). After subcloning in Top 10F competent cells, each mutant cDNA was excised with 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 Life Sciences Sequencing Facility.

### Cell Culture

Chinese hamster ovary (CHO)-K1 cells (RIDD: CVCL\_0214) stably transfected with the wild-type human  $\beta$ 1 or  $\beta$ 2-adrenoceptor, or one of the full extracellular loop (EL) region or TM domain chimeric receptors (total 24 cell lines) were used (see Baker et al., 2014, 2015 for full details). For example,  $\beta$ 1-TM1 is where 6 point mutations in the  $\beta$ 1-AR DNA have been made to the 6 amino acids that differ between



**Fig. 1.** Chemical structures of xamoterol, nebivolol, ICI89406, ICI89406np, betaxolol, bisoprolol, and esmolol.

TABLE 1

Affinity (log  $K_i$  values) of  $\beta$ -adrenoceptor ligands for the  $\beta$ 1-WT and  $\beta$ 2-WT obtained from  $^3\text{H}$ -CGP12177 whole cell binding in cells stably transfected with whole TM or EL changes

The values are mean  $\pm$  S.E. mean for  $n$  separate experiments. The  $\beta$ 1 over  $\beta$ 2-selectivity for the wild-type receptors is also given, thus xamoterol had 20-fold higher affinity for  $\beta$ 1 than  $\beta$ 2.

	Log $K_i$ xamoterol	n	Log $K_i$ nebivolol	n
$\beta$ 1-WT	$-7.19 \pm 0.04$	25	$-8.33 \pm 0.05$	14
$\beta$ 1-N	$-7.10 \pm 0.11$	5	$-8.18 \pm 0.11$	10
$\beta$ 1-EL1	$-7.38 \pm 0.08$	5	$-8.35 \pm 0.07$	10
$\beta$ 1-EL2	$-7.01 \pm 0.11$	5	$-8.11 \pm 0.09$	10
$\beta$ 1-EL3	$-7.41 \pm 0.13$	5	$-8.10 \pm 0.09$	10
$\beta$ 1-TM1	$-7.31 \pm 0.08$	8	$-8.53 \pm 0.04$	6
$\beta$ 1-TM2	$-6.35 \pm 0.05^{****}$	8	$-7.32 \pm 0.07^{****}$	6
$\beta$ 1-TM3	$-7.17 \pm 0.08$	8	$-8.15 \pm 0.09$	6
$\beta$ 1-TM4	$-7.41 \pm 0.08$	8	$-7.91 \pm 0.09^*$	6
$\beta$ 1-TM5	$-7.24 \pm 0.08$	8	$-8.28 \pm 0.05$	6
$\beta$ 1-TM6	$-6.97 \pm 0.09$	7	$-7.93 \pm 0.05^*$	6
$\beta$ 1-TM7	$-6.89 \pm 0.10$	7	$-8.06 \pm 0.06$	6
$\beta$ 2-WT	$-5.89 \pm 0.03$	30	$-6.93 \pm 0.06$	12
$\beta$ 2-N	$-5.97 \pm 0.11$	8	$-6.89 \pm 0.08$	10
$\beta$ 2-EL1	$-5.85 \pm 0.04$	8	$-7.05 \pm 0.04$	10
$\beta$ 2-EL2	$-6.06 \pm 0.05$	8	$-6.82 \pm 0.05$	9
$\beta$ 2-EL3	$-5.91 \pm 0.06$	8	$-7.15 \pm 0.07$	10
$\beta$ 2-TM1	$-5.85 \pm 0.04$	8	$-7.05 \pm 0.06$	6
$\beta$ 2-TM2	$-6.84 \pm 0.07^{****}$	7	$-7.74 \pm 0.12^{****}$	6
$\beta$ 2-TM3	$-6.01 \pm 0.07$	8	$-6.76 \pm 0.03$	6
$\beta$ 2-TM4	$-5.68 \pm 0.04$	7	$-6.90 \pm 0.03$	6
$\beta$ 2-TM5	$-5.89 \pm 0.03$	6	$-6.95 \pm 0.02$	6
$\beta$ 2-TM6	$-6.09 \pm 0.05$	7	$-6.91 \pm 0.05$	6
$\beta$ 2-TM7	$-6.23 \pm 0.04^{***}$	9	$-6.97 \pm 0.08$	6
$\beta$ 1-selectivity	20.0		25.1	

\*\*\*\* $P < 0.000001$  One-way ANOVA with post hoc Tukey comparing values from the mutant receptors with those obtained from the  $\beta$ 1-WT or the  $\beta$ 2-WT.

\*\*\* $P < 0.00003$  and \* $P < 0.05$  compared with  $\beta$ 1-WT or the  $\beta$ 2-WT.

the  $\beta$ 1 and  $\beta$ 2-AR such that the TM1 region only now resembles that of the  $\beta$ 2-AR (see Supplementary Figure 3 for pictorial diagram). For transiently transfected cells the parent CHO-K1 cells were transfected in a T75 with 10 ng DNA in 100  $\mu$ l Lipofectamine and 8ml OP-TIMEM as per manufacturer's instructions on day 1, the transfection reagents removed and replaced with media on day 2, the cells plated into 96-well plates on day 3, and the experiments performed on day 4. All CHO cells were grown in Dulbecco's modified Eagle's medium nutrient mix F12, containing 10% fetal calf serum and 2 mM L-glutamine in a 37°C humidified 5% CO<sub>2</sub> 95% air atmosphere. Cells were always grown in the absence of any antibiotics. Mycoplasma contamination is intermittently monitored within the laboratory (negative) but cell lines were not tested routinely with each experiment.

### $^3\text{H}$ -CGP12177 Whole Cell Binding

Cells were grown to confluence in tissue-culture-treated white-sided 96-well view plates. The affinity ( $K_D$ , concentration required to bind half of the receptors) of  $^3\text{H}$ -CGP12177 was determined for each construct from saturation binding experiments. The affinity for competing ligands was determined by incubating the competing ligand in the presence of a fixed concentration of  $^3\text{H}$ -CGP12177 and, as determined from inhibition of radioligand, is thus referred to as  $K_i$  values.

Briefly, the affinity ( $K_D$ ) of  $^3\text{H}$ -CGP12177 was determined for each construct from saturation experiments, with concentrations of  $^3\text{H}$ -CGP12177 in the range of 0.005 to 42.8 nM and the  $K_i$  for competing ligands by incubating the competing ligand in the presence of a fixed concentration of  $^3\text{H}$ -CGP12177 as previously described (Baker, 2005) in 200  $\mu$ l total well volume for 2 hours at 37°C before being washed with  $2 \times 200$  ml cold (4°C) PBS. A volume of 100  $\mu$ l Microscint 20 was then added to each well, the plates left for

several hours before being counted on a Topcount for 2 minutes per well. Propranolol (10  $\mu$ M) was used to define nonspecific binding in all experiments.

### Data and Statistical Analysis

**Whole Cell Binding.** The affinity of  $^3\text{H}$ -CGP12177 for each mutant was determined from saturation binding, using 10  $\mu$ M propranolol to determine nonspecific binding, and all data points were performed in quadruplicate. Specific binding (Eq. 1) of  $^3\text{H}$ -CGP12177 at different concentrations of  $^3\text{H}$ -CGP12177 was fitted using the nonlinear regression program Prism 7 to the equation:

$$SB = \frac{(A \times B_{\max})}{(A + K_D)} \quad (1)$$

where A is the concentration of  $^3\text{H}$ -CGP12177,  $B_{\max}$  is the maximal specific binding, and  $K_D$  is the dissociation constant of  $^3\text{H}$ -CGP12177.

The affinity of the other ligands was determined from competition binding. All data points were recorded in triplicate, and each 96-well plate contained six determinations of total and nonspecific binding. A sigmoidal concentration-response curve was then fitted to the data using Graphpad Prism 7, and the IC<sub>50</sub> was determined as the concentration required to inhibit 50% of the specific binding using Eq. 2.

$$\% \text{ uninhibited binding} = 100 - \frac{(100 \times A)}{(A + IC_{50})} + NS \quad (2)$$

where A is the concentration of the competing ligand, IC<sub>50</sub> is the concentration at which half of the specific binding of  $^3\text{H}$ -CGP12177 has been inhibited, and NS is the nonspecific binding.

From the IC<sub>50</sub> value and the known concentration of  $^3\text{H}$ -CGP12177, a  $K_i$  value (concentration at which half the receptors are bound by the competing ligand) was calculated using Eq. 3:

$$K_i = \frac{IC_{50}}{1 + ([^3H - CGP12177]/K_D - ^3H - CGP12177)} \quad (3)$$

To explore whether a single receptor region (Table 1) or individual amino acid (Table 2) was statistically different from all of the others, a one-way ANOVA with post hoc Tukey analysis was conducted. The one-way ANOVA determines if there is a difference within the datasets as a whole, when comparing each mutant with each other and wild-type (e.g., xamoterol affinity at  $\beta$ 1-WT,  $\beta$ 1-TM1,  $\beta$ 1-TM2,  $\beta$ 1-TM3,  $\beta$ 1-TM4,  $\beta$ 1-TM5,  $\beta$ 1-TM6,  $\beta$ 1-TM7,  $\beta$ 1-N,  $\beta$ 1-EL1,  $\beta$ 1-EL2, and  $\beta$ 1-EL3) in a single statistical test. If the ANOVA does detect a difference, the post hoc analysis determines within the entire dataset, which mutants have caused a change that is statistically different from wild-type (i.e., from  $\beta$ 1-WT). Thus, for the affinity of xamoterol, the one-way ANOVA determined that there was a significant difference between the datasets in the different mutants and post hoc Tukey analysis that  $\beta$ 1-TM2 was different from  $\beta$ 1-WT with a  $P$  value of  $P < 0.000001$ .

### Modeling of Human $\beta$ 1-AR Structures

Active and inactive conformation structures of human  $\beta$ 1-AR were modeled using MODELER (Webb and Sali, 2014). The inactive state was modeled based upon the template with PDB ID 2VT4 and the active state based on the template with PDB ID 3SN6. The G<sub>zs</sub> subunit was added to the  $\beta$ 1-AR active-state model by superimposing this model with the ternary complex of the  $\beta$ 2-AR (PDB ID 3SN6) and removing the receptor portion and small-molecule ligand of 3SN6.

### $\beta$ 2-AR Crystal Structures

Crystal structures of the  $\beta$ 2-AR in an active or inactive conformation were obtained from the PDB, PDB IDs 3SN6, and 2RH1, respectively.

**TABLE 2**  
Affinity (log  $K_i$  values) of  $\beta$ -adrenoceptor ligands for the  $\beta 1$ -WT,  $\beta 2$ -WT and receptors containing single point mutations in TM2 obtained from  $^3\text{H}$ -CGP12177 whole cell binding in transiently transfected populations of cells  
The  $K_D$  values of  $^3\text{H}$ -CGP12177 and the receptor expression levels obtained from saturation studies in these transient populations are given. The values are mean  $\pm$  S.E. mean for  $n$  separate experiments. The  $\beta 1$  over  $\beta 2$ -selectivity for the wild-type receptors is also given.

	K <sub>D</sub> <sup>3</sup> H- CGP12177	fmol/mg protein	Log K <sub>i</sub> xamoterol	n	Log K <sub>i</sub> nebivolol	n	Log K <sub>i</sub> ICI89406	n	Log K <sub>i</sub> betaxolol	n	Log K <sub>i</sub> bisoprolol	n	Log K <sub>i</sub> esmolol	n	
β1-WT	0.28 ± 0.02 <sup>a</sup>	731 ± 96	27	-7.13 ± 0.04	12	-8.01 ± 0.09	11	-8.88 ± 0.07	7	-8.07 ± 0.06	6	-7.96 ± 0.05	11	-6.65 ± 0.08	7
β1-M98T	0.31 ± 0.03	620 ± 109	6	-7.30 ± 0.10	7	-7.85 ± 0.12	6	-9.01 ± 0.11	5	-8.10 ± 0.05	7	-8.01 ± 0.05	6	-6.69 ± 0.08	5
β1-S102C	0.25 ± 0.03	430 ± 68	6	-7.21 ± 0.07	7	-7.93 ± 0.12	7	-8.84 ± 0.06	7	-8.03 ± 0.05	7	-8.00 ± 0.05	7	-6.78 ± 0.04	5
β1-L110A	0.22 ± 0.02	334 ± 80	5	-7.31 ± 0.06	6	-7.94 ± 0.11	6	-8.93 ± 0.07	6	-8.22 ± 0.06	6	-8.12 ± 0.09	5	-6.74 ± 0.08	5
β1-T117A	0.26 ± 0.03	414 ± 61	6	-7.05 ± 0.08	6	-7.87 ± 0.10	8	-8.66 ± 0.04	6	-7.98 ± 0.08	6	-7.88 ± 0.07	6	-6.66 ± 0.03	5
β1-I118H	0.21 ± 0.03	292 ± 73	6	-6.33 ± 0.09***	8	-7.29 ± 0.09***	7	-7.64 ± 0.04***	6	-7.80 ± 0.04	8	-7.69 ± 0.04*	6	-6.33 ± 0.05	7
β1-V119I	0.30 ± 0.05	340 ± 76	6	-7.27 ± 0.06	8	-7.89 ± 0.10	7	-9.21 ± 0.06*	7	-8.07 ± 0.08	6	-7.95 ± 0.08	6	-6.65 ± 0.07	7
β1-V120L	0.28 ± 0.04	528 ± 129	6	-7.11 ± 0.08	6	-7.73 ± 0.09	7	-8.69 ± 0.07	7	-8.10 ± 0.10	6	-7.89 ± 0.05	5	-6.66 ± 0.15	6
β2-WT	0.18 ± 0.01 <sup>a</sup>	165 ± 13	30	-5.96 ± 0.06	7	-6.88 ± 0.12	7	-6.97 ± 0.08	7	-6.92 ± 0.07	6	-6.49 ± 0.07	7	-5.53 ± 0.12	5
β2-T73M	0.22 ± 0.03	59 ± 11	10	-5.76 ± 0.05	8	-6.94 ± 0.12	8	-6.90 ± 0.06	8	-6.83 ± 0.08	8	-6.51 ± 0.13	8	-5.69 ± 0.09	7
β2-C77S	0.22 ± 0.03	18 ± 3	10	-5.89 ± 0.13	10	-6.97 ± 0.07	11	-6.79 ± 0.12	9	-6.94 ± 0.13	8	-6.28 ± 0.11	10	-5.42 ± 0.15	8
β2-A85L	0.22 ± 0.05	38 ± 6	13	-5.75 ± 0.05	10	-6.85 ± 0.10	10	-6.77 ± 0.09	10	-6.88 ± 0.08	11	-6.22 ± 0.05	11	-5.67 ± 0.11	11
β2-A92T	0.25 ± 0.04	48 ± 6	12	-5.63 ± 0.10	8	-6.86 ± 0.14	7	-6.71 ± 0.08	9	-6.84 ± 0.07	8	-6.36 ± 0.07	8	-5.34 ± 0.18	8
β2-H93I	0.30 ± 0.05	20 ± 2	12	-6.82 ± 0.07***	9	-7.76 ± 0.11***	12	-7.59 ± 0.14**	11	-7.07 ± 0.09	11	-6.51 ± 0.11	8	-5.87 ± 0.09	11
β2-I94V	0.21 ± 0.03	35 ± 5	11	-5.99 ± 0.08	12	-6.90 ± 0.08	12	-6.83 ± 0.09	12	-7.02 ± 0.08	12	-6.48 ± 0.10	12	-5.32 ± 0.10	12
β2-L95V	0.20 ± 0.03	38 ± 5	10	-6.29 ± 0.09	11	-7.17 ± 0.09	11	-6.94 ± 0.10	9	-7.11 ± 0.06	11	-6.42 ± 0.11	11	-5.54 ± 0.09	11
β1-selectivity				14.8	13.5	81.3	14.1	29.5	13.2						

<sup>a</sup>Saturation data from Baker et al. (2015).

\*\*\* $P < 0.0001$  One-way ANOVA with post hoc Tukey comparing values from the mutant receptors with those obtained from the  $\beta 1$ -WT or the  $\beta 2$ -WT.

\*\* $P < 0.003$  and \* $P < 0.05$  compared with  $\beta 1$ -WT or the  $\beta 2$ -WT.

Given the partial agonist nature of xamoterol and ICI89406 (Mistry et al., 2013), both ligands were manually docked into both the active and inactive conformations of the receptors. Betaxolol, bisoprolol, and esmolol were docked into the inactive  $\beta 1$ - and  $\beta 2$ -AR structures only. Nebivolol was not docked or simulated due to the high number of chiral centers (four), which makes it impossible to determine the correct pose of each enantiomer and correlate the simulation results with the experimental evidence obtained for the racemate. Calculations were performed using Chemical Computing Group's Molecular Operating Environment (<https://www.chemcomp.com/Products.htm>).

## Molecular Dynamics Simulations

Molecular dynamics (MD) simulations of the  $\beta 1$ - and  $\beta 2$ -AR to investigate the structural basis of selectivity of the ligands tested were carried out in the NPT ensemble. Structures were embedded in a homogeneous POPC bilayer membrane with CHARMM-GUI's Membrane Builder (Lee et al., 2019) and solvated with TIP3P water in a 0.15 nM NaCl solution. The CHARMM36 force field and the CHARMM General Force Field were used in the simulations. The prepared structures were first equilibrated with NAMD (Phillips et al., 2020), using parameters provided by CHARMM-GUI, at 300 K. Following equilibration, ACEMD3 (Harvey et al., 2009) was used for production runs. Five replicate calculations of 500 ns were performed for each system (with detailed values calculated from all replicates provided in Supplemental Tables 1–4).

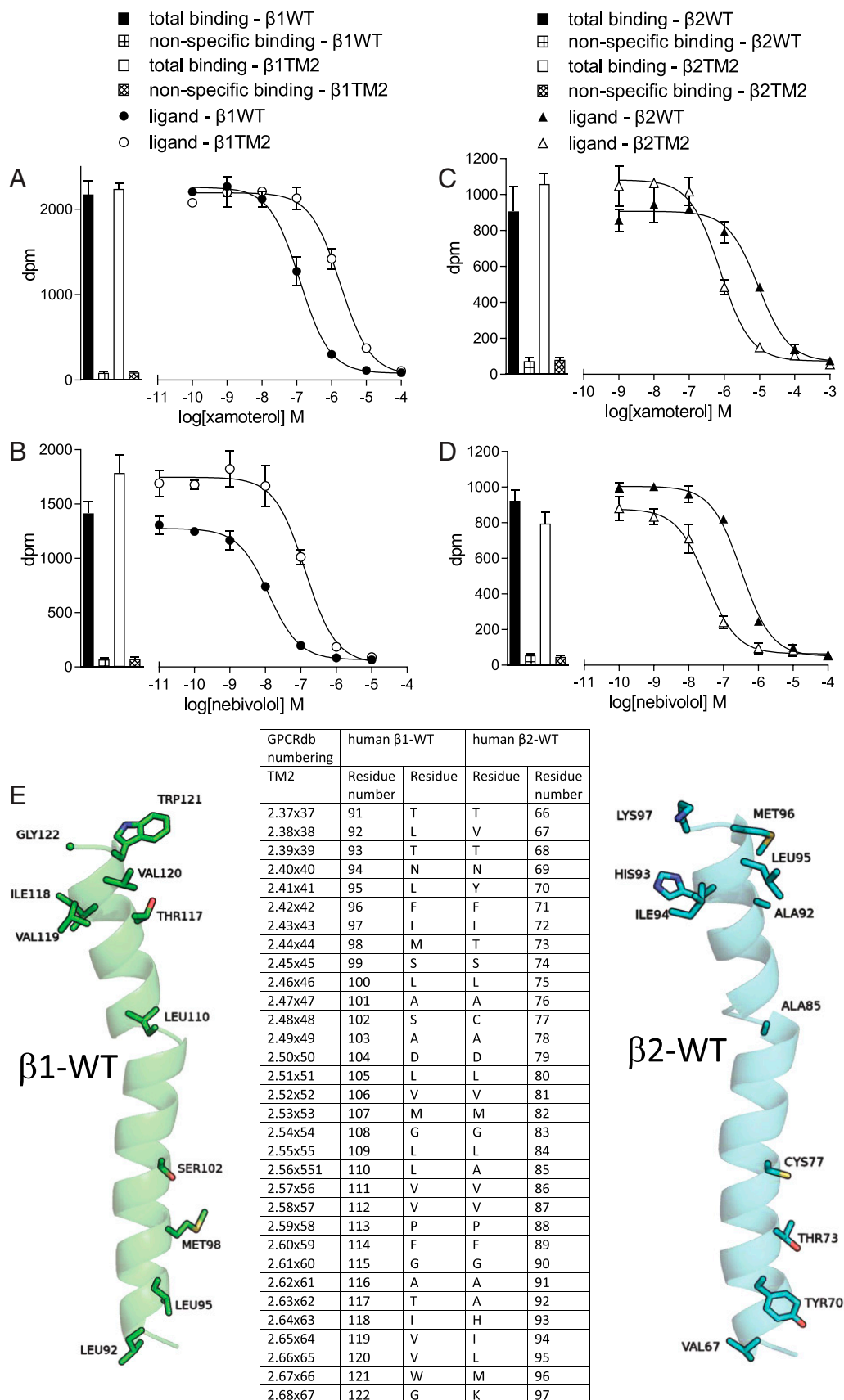
*Apo* (unbound) structures of both receptors and their states were simulated and used as control for the simulations of the *holo* (ligand-bound) proteins. Xamoterol and ICI89406 were simulated in complex with both active and inactive protein states because of their partial agonistic effect.

Interaction energy, hydrogen bond, solvation, and water bridge analyses of the trajectories were performed using CPPTRAJ (Roe et al., 2013). The interaction energy is calculated as the sum of the van der Waals (Lennard-Jones potential) and electrostatic (Coulombic potential) terms individually for each frame and averaged over all frames. A hydrogen bond is defined to occur whenever a positively polarized hydrogen bound to a heteroatom is within 3 Å and at an angle of 135° to a heteroatom with a lone electron pair. An atom is defined to be solvated whenever it forms at least one hydrogen bond with a water molecule. Finally, a water bridge connects two groups, where each is able to form hydrogen bonds, via a water molecule. The three latter descriptors are calculated as the percentage of frames in a trajectory in which the respective feature occurs. *Holo* simulations were compared with the *apo* simulations for differences in hydrogen bonding, solvation, and water bridge formation.

Where appropriate, unpaired *t* tests were performed, comparing the calculated values for all descriptors between unbound (*apo*) and bound (*holo*) structures and between ICI89406 and ICI89406np in the different receptor structures and conformations.

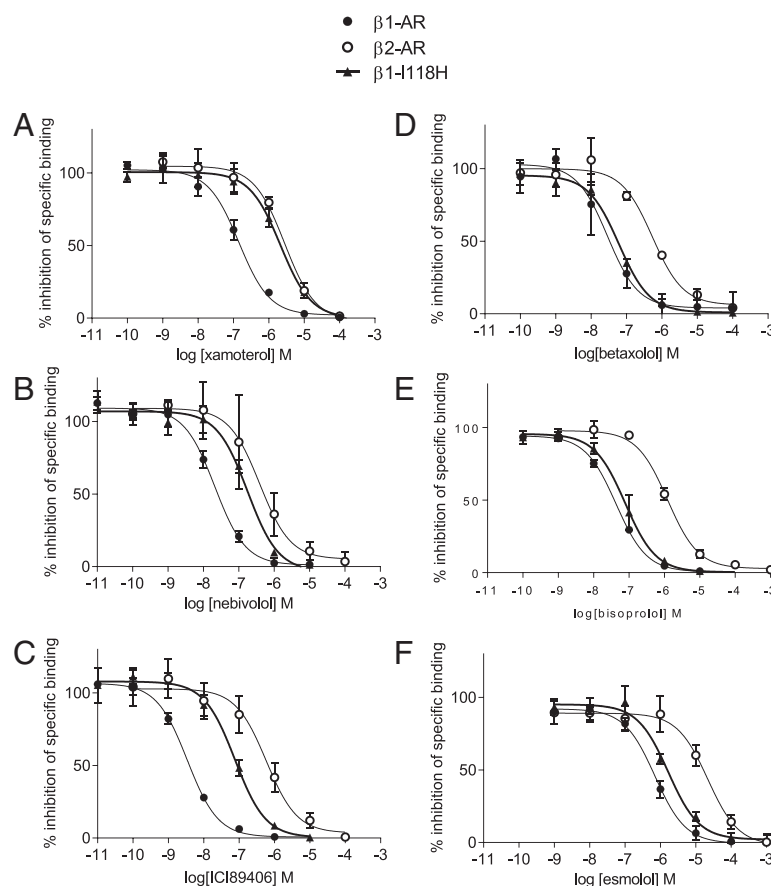
## Sourcing Analog Compounds

Following the docking calculations and MD simulations, and based on the observed ligand:receptor interactions in these calculations, analogs of the initially investigated ligands were searched in the small-molecule database ZINC (Sterling & Irwin, 2015), using a similarity threshold of at least 30%. We were specifically looking for compounds that would miss key interaction motifs in regions highlighted as important by the pharmacological experiments. Thus, we wished to test the hypotheses generated based on the MD simulation data. No commercially available xamoterol analogs that fulfilled all the criteria were identified, but we identified and sourced ICI89406np (ICI89406 without the terminal phenyl group) and evaluated it pharmacologically.



**Fig. 2.** (a-d) Inhibition of  $^3\text{H}$ -CGP12177 binding in (a) and (b) CHO- $\beta$ 1-WT and CHO- $\beta$ 1-TM2 cells and (c) and (d) CHO- $\beta$ 2-WT and CHO- $\beta$ 2-TM2 cells by xamoterol (a and c) and nebivolol (b and d). Nonspecific binding was determined by 10  $\mu\text{M}$  propranolol in all cases. These are raw data (dpm) from single experiments with the data from two different cell lines in each case plotted on the same graph. The concentration of  $^3\text{H}$ -CGP12177 present





**Fig. 3.** Inhibition of  $^3\text{H}$ -CGP12177 whole cell binding by (a) xamoterol, (b) neбиволol, (c) ICI89406, (d) betaxolol, (e) bisoprolol, and (f) esmolol in transiently transfected cells. As data from three different transiently transfected constructs are shown on each graph, for clarity data are normalized to that for total and nonspecific (as determined from an average of six wells on each plate) within each transfection. Nonspecific binding was determined by 10  $\mu\text{M}$  propranolol. The concentration of  $^3\text{H}$ -CGP12177 present in these experiments was (a) 0.57 nM, (b) 0.49 nM, (c) 0.49 nM, (d) 0.63 nM, (e) 0.57 nM, and (f) 0.68 nM and is representative of (a) seven, (b) seven, (c) six, (d) six, (e) six, and (f) five separate experiments. Data points are mean  $\pm$  SD of triplicate determinations.

## Results

### Identification of the Regions in the Human $\beta_1$ -AR Important for Xamoterol and Nebivolol Affinity: Stable Cell Lines

Xamoterol and neбиволol inhibited the specific binding of  $^3\text{H}$ -CGP12177 in CHO- $\beta_1$ -WT cells to yield log  $K_i$  values of  $-7.19$  and  $-8.33$ , respectively (Table 1). In the CHO- $\beta_2$ -WT cells the log  $K_i$  values were  $-5.89$  and  $-6.93$ , giving a  $\beta_1$  versus  $\beta_2$  selectivity for these compounds of 20 and 25-fold for xamoterol and neбиволol, respectively, in keeping with previous studies (Baker 2005, 2010).

When the affinity of these compounds was studied in cell lines expressing chimeric  $\beta_1$ -receptors, the affinity of xamoterol and neбиволol were reduced in the  $\beta_1$ -TM2 receptor (i.e., the  $\beta_1$ -AR but with mutations such that transmembrane helix 2 is that of the  $\beta_2$ -AR) compared with all other  $\beta_1$  receptors (WT and each other single-region chimeric receptor; Table 1, Fig. 2). This reduction in affinity, more closely resembling that of the affinity at the  $\beta_2$ -AR, suggests that this region contained one or more amino acids that form an important (direct or indirect) interaction with the ligands and therefore has a large impact on the binding selectivity of xamoterol and neбиволol. When the reciprocal receptors were studied (i.e.,  $\beta_2$ -chimeric receptors), the affinity of xamoterol and neбиволol was significantly increased in the CHO- $\beta_2$ -TM2 cells, compared with  $\beta_2$ -WT and all other  $\beta_2$ -chimeras,

again suggesting that this region is important for the selectivity of these two ligands (Table 1, Fig. 2).

### Identification of the Individual Amino Acids Involved in TM2 for Xamoterol and Nebivolol: Transiently Transfected Cells

From the stable cell line experiments described here, TM2 appeared to be the most important region for the selectivity of xamoterol and neбиволol. Single point mutations were therefore made at each of the seven sites in TM2 where amino acids between the two receptors are different. For example, at position 98 in the  $\beta_1$ -AR, a single point mutation was made such that this amino acid was mutated from a methionine M to a threonine T (i.e.,  $\beta_1$ -M98T, Table 2). The reciprocal  $\beta_2$  construct (e.g.,  $\beta_2$ -AR with a single point mutation to the amino acid of the  $\beta_1$ -AR, e.g.,  $\beta_2$ -T73M) was also made. Binding studies with the individual amino acid mutations revealed that of the  $\beta_1$ -TM2 constructs,  $\beta_1$ -I118H reduced the affinity of both xamoterol and neбиволol compared with  $\beta_1$ -WT (Table 2, Fig. 3). When the reciprocal  $\beta_2$ -TM2 individual amino acid constructs were examined, the corresponding construct ( $\beta_2$ -H93I) had higher affinity for xamoterol and neбиволol (Table 2, Fig. 3). Taken together, this suggests that this amino acid at position 118/93 is very important in determining the selective binding affinity of xamoterol and neбиволol.

in these experiments was (a) 0.73 nM, (b) 0.43 nM, (c) 0.89 nM, and (d) 0.49 nM and are representative of (a) eight, (b) six, (c) seven, and (d) six separate experiments. Data points are mean  $\pm$  SD of triplicate determinations. (e) The sequence alignment of the amino acid residues in TM2 of the  $\beta_1$ -WT and  $\beta_2$ -WT receptor.

### Determination of Whether $\beta$ 1-I118 Can Explain the $\beta$ 1-Selectivity of Other $\beta$ 1-Antagonists: Transiently Transfected Cells

Other  $\beta$ -ligands with moderate  $\beta$ 1-selectivity were then examined. ICI89406 (another  $\beta$ 1-partial agonist, Mistry et al., 2013), and betaxolol, bisoprolol, and esmolol ( $\beta$ -antagonists without efficacy in these cells; Baker et al., 2011; Baker et al., 2017; Baker et al., 2020; Mistry et al., 2013) had  $\beta$ 1-WT (versus  $\beta$ 2-WT) binding affinity selectivities of 81-, 14-, 30-, and 13-fold, respectively (Table 2, Fig. 3). When ICI89406 was examined in the TM2 constructs, it also had reduced affinity in the  $\beta$ 1-I118H, compared with  $\beta$ 1-WT. An increase in affinity at the reciprocal  $\beta$ 2-H93I construct was also seen (compared with  $\beta$ 2-WT). The affinities of betaxolol and esmolol were unchanged in any of the TM2 constructs, whereas the affinity of bisoprolol was reduced by twofold by the  $\beta$ 1-I118H construct (which is of uncertain pharmacological significance).

### In Silico Ligand:Receptor Interaction Analyses

To understand the role of the isoleucine at position 118 in the  $\beta$ 1, compounds were docked into models and X-ray structures of the human  $\beta$ 1 and  $\beta$ 2, respectively, and MD simulations of these complexes were analyzed. All docked and simulated compounds have one chiral center and are the (*S*)-enantiomer at the common hydroxy group. Computed interaction energies, as well as hydrogen bond, solvation, and water-bridge formation frequencies were determined along the simulation trajectories (Tables 3 and 4) and were compared with the values obtained for the unbound *apo* structures. Given the partial agonist nature of xamoterol and ICI89406, both compounds were examined in both the active and inactive structures of the human  $\beta$ 1 and  $\beta$ 2, while the other three compounds (known antagonists) were examined in the inactive structures only.

**Xamoterol.** In the simulations, xamoterol showed similar trends of solvation and interaction energies in both active and inactive  $\beta$ 1/ $\beta$ 2-ARs. In the  $\beta$ 2 simulations, xamoterol reduced the solvation of H93's  $\epsilon$  nitrogen compared with the *apo* simulation (34.8% in *apo* vs. 18.9% in the bound form for the active conformation, 41.2% in *apo* vs. 24.4% in the bound form for

the inactive conformation; Tables 3 and 4). However, calculated mean interaction energies showed that xamoterol interacts less favorably with I118 in  $\beta$ 1-AR than with H93 in  $\beta$ 2 ( $-0.4 \pm 0.1$  kcal/mol and  $-2.8 \pm 0.2$  kcal/mol, respectively, in the active state,  $-0.7 \pm 0.1$  kcal/mol and  $-2.9 \pm 0.3$  kcal/mol, respectively, in the inactive state; Tables 3 and 4).

**ICI89406.** ICI89406 affects H93 differently in active and inactive  $\beta$ 2 MD simulations. In the active state, ICI89406 has more favorable interaction energies with I118 in the  $\beta$ 1 ( $-2.5 \pm 0.2$  kcal/mol) compared with H93 in the  $\beta$ 2 ( $-0.9 \pm 0.1$  kcal/mol). However, the difference in interaction energies for the inactive state between  $\beta$ 1/ $\beta$ 2 was much smaller and not significant ( $-2.1 \pm 0.3$  kcal/mol and  $-1.9 \pm 0.7$  kcal/mol, respectively). In the inactive  $\beta$ 2, ICI89406 hinders the solvation of H93's  $\epsilon$  nitrogen (41.2% in *apo* vs. 11.0% bound; Table 4) and disrupts the water bridge between H93 and D192 (20.3% in *apo* vs. 2.1% bound; Table 4). However, this was not observed in the active state simulations of  $\beta$ 2.

**Betaxolol, Bisoprolol, and Esmolol.** Betaxolol, bisoprolol, and esmolol were also examined in the inactive  $\beta$ 1 and  $\beta$ 2 structures and the effects on H93/I118 were small or statistically insignificant (Supplemental Fig. 2 and Supplemental Tables 3 and 4 for docking poses and nitrogen solvation, water-bridge formation, and interaction energies). The only exception was for esmolol in  $\beta$ 2, where there was a significant improvement in the frequency of a water-bridge between H93 and D192 in  $\beta$ 2-AR (20.3% in *apo* vs. 31.0% bound).

**Analog ICI89406np.** No commercially available xamoterol analogs lacking key interaction motifs were found in ZINC. The derivative of ICI89406 lacking the terminal phenyl moiety (here termed ICI89406 no phenyl, abbreviated as ICI89406np) was docked and investigated further, as it seemed likely to be informative with respect to the investigation of the role of the isoleucine versus histidine at the far end of the binding site. MD simulations showed that ICI89406np improved the solvation of the  $\epsilon$  nitrogen of H93 (11.0% for ICI89406 vs. 27.9% for ICI89406np; Table 3; Fig. 4) and the water-bridge between H93 and D192 (2.1% for ICI89406 vs. 21.4% for ICI89406np; Table 3) compared with ICI89406 in the inactive conformation

TABLE 3

Table of interaction energies, solvation, and water-bridges of ligand and important residues calculated from MD simulations for ligands examined in the active structures

Numerical values are calculated as percentage of frames (for nitrogen solvation and water bridge formation) and the interaction energy is given in kcal/mol. Values are mean  $\pm$  S.E. mean of *n* separate determinations. Compounds are compared with values obtained in the unbound (*apo*) structure using an unpaired *t* test. The difference in the interaction energy to I118/H93 is also given between the compound binding to the  $\beta$ 1-WT and  $\beta$ 2-WT and compared using an unpaired *t* test. Full details of each replicate, and all *P* values are given in Supplemental Tables 1 and 2.

	Unbound <i>apo</i>	<i>n</i>	xamoterol	<i>n</i>	ICI89406	<i>n</i>	ICI89406np	<i>n</i>
$\beta$ 1-WT active								
$\beta$ 1-I118 LIG electrostatic energy			$0.8 \pm 0.1$	5	$-0.0 \pm 0.1$	5	$0.0 \pm 0.1$	5
$\beta$ 1-I118 LIG Van der Waals energy			$-1.2 \pm 0.1$	5	$-2.5 \pm 0.2^\dagger$	5	$-0.4 \pm 0.0^\dagger$	5
$\beta$ 1-I118 LIG Interaction energy			$-0.4 \pm 0.1$	5	$-2.5 \pm 0.2^\dagger$	5	$-0.4 \pm 0.1^\dagger$	5
$\beta$ 2-WT active								
$\beta$ 2-H93 $\epsilon$ Nitrogen Solvation	$34.8 \pm 3.0$	5	$18.9 \pm 2.4^*$	5	$29.9 \pm 2.4$	5	$33.5 \pm 3.0$	5
$\beta$ 2-H93 D192 Water-bridge	$23.5 \pm 3.1$	5	$18.7 \pm 2.1$	5	$21.8 \pm 1.1$	5	$26.6 \pm 2.7$	5
$\beta$ 2-H93 LIG Electrostatic energy			$-1.0 \pm 0.2$	5	$-0.1 \pm 0.2^\dagger$	5	$0.5 \pm 0.1^\dagger$	5
$\beta$ 2-H93 LIG Van der Waals energy			$-1.8 \pm 0.2$	5	$-0.8 \pm 0.1$	5	$-0.6 \pm 0.1$	5
$\beta$ 2-H93 LIG Interaction energy			$-2.8 \pm 0.2$	5	$-0.9 \pm 0.1^\dagger$	5	$-0.1 \pm 0.2^\dagger$	5
$\beta$ 1-WT active versus $\beta$ 2-WT active energy			2.4#		-1.6#		-0.3	

\**P* < 0.05 compared with the values obtained from the *apo* unbound structure using an unpaired *t* test.

#*P* < 0.05 comparing the interaction energy between  $\beta$ 1 and  $\beta$ 2.

†*P* < 0.05 comparing values between ICI89406 and ICI89406np for the  $\beta$ 1-WT or  $\beta$ 2-WT.

TABLE 4

Table of interaction energies, solvation, and water-bridges of ligand and important residues calculated from MD simulations for ligands examined in the inactive structures. Numerical values are calculated as percentage of frames (for nitrogen solvation and water bridge formation) and the interaction energy is given in kcal/mol. Values are mean  $\pm$  S.E. mean of  $n$  separate determination. Compounds are compared with values obtained in the unbound (*apo*) structure using an unpaired  $t$  test. The difference in the interaction energy to I118/H93 is also given between the compound binding to the  $\beta$ 1-WT and  $\beta$ 2-WT and compared using an unpaired  $t$  test. Full details of each replicate, and all  $P$  values are given in Supplemental Tables 1 to 4.

	Unbound <i>apo</i>	<i>n</i>	xamoterol	<i>n</i>	ICI89406	<i>n</i>	ICI89406np	<i>n</i>	betaxolol	<i>n</i>	bisoprolol	<i>n</i>	esmolol	<i>n</i>
<b><math>\beta</math>1-WT-inactive</b>														
$\beta$ 1-I118 LIG electrostatic energy			0.9 $\pm$ 0.1	5	0.3 $\pm$ 0.1 <sup>†</sup>	5	0.1 $\pm$ 0.0 <sup>†</sup>	5	0.7 $\pm$ 0.0	5	0.7 $\pm$ 0.1	5	0.6 $\pm$ 0.0	5
$\beta$ 1-I118 LIG Van der Waals energy			-1.6 $\pm$ 0.1	5	-2.5 $\pm$ 0.3 <sup>†</sup>	5	-0.6 $\pm$ 0.1 <sup>†</sup>	5	-0.06 $\pm$ 0.01	5	-0.09 $\pm$ 0.03	5	-0.05 $\pm$ 0.01	5
$\beta$ 1-I118 LIG Interaction energy			-0.7 $\pm$ 0.1	5	-2.1 $\pm$ 0.3 <sup>†</sup>	5	-0.5 $\pm$ 0.1 <sup>†</sup>	5	0.6 $\pm$ 0.0	5	0.7 $\pm$ 0.1	5	0.6 $\pm$ 0.0	5
<b><math>\beta</math>2-WT-inactive</b>														
$\beta$ 2-H93 $\epsilon$ Nitrogen Solvation	41.2 $\pm$ 1.2	5	24.4 $\pm$ 5.2 <sup>*</sup>	5	11.0 $\pm$ 3.2 <sup>*</sup>	5	27.9 $\pm$ 6.6	5	40.8 $\pm$ 4.1	5	36.8 $\pm$ 4.9	5	39.4 $\pm$ 2.0	5
$\beta$ 2-H93 D192 Water-bridge	20.3 $\pm$ 3.4	5	16.4 $\pm$ 4.1	5	2.1 $\pm$ 1.8 <sup>†</sup>	5	21.4 $\pm$ 6.3 <sup>†</sup>	5	15.3 $\pm$ 4.1	5	19.2 $\pm$ 5.2	5	31.0 $\pm$ 1.5 <sup>*</sup>	5
$\beta$ 2-H93 LIG Electrostatic energy			-1.3 $\pm$ 0.2	5	0.5 $\pm$ 0.3	5	0.3 $\pm$ 0.1	5	0.2 $\pm$ 0.2	5	0.3 $\pm$ 0.2	5	0.4 $\pm$ 0.1	5
$\beta$ 2-H93 LIG Van der Waals energy			-1.6 $\pm$ 0.1	5	-2.4 $\pm$ 0.4 <sup>†</sup>	5	-0.3 $\pm$ 0.1 <sup>†</sup>	5	-0.1 $\pm$ 0.0	5	-0.1 $\pm$ 0.0	5	-0.1 $\pm$ 0.0	5
$\beta$ 2-H93 LIG Interaction energy			-2.9 $\pm$ 0.3	5	-1.9 $\pm$ 0.7 <sup>†</sup>	5	0.0 $\pm$ 0.0 <sup>†</sup>	5	0.2 $\pm$ 0.2	5	0.2 $\pm$ 0.1	5	0.4 $\pm$ 0.1	5
$\beta$ 1-WT inactive vs. $\beta$ 2-WT energy inactive			2.1#		-0.2		-0.5#		0.4		0.4#		0.2	

\* $P < 0.05$  compared with the values obtained from the *apo* unbound structure using an unpaired  $t$  test.

# $P < 0.05$  comparing the interaction energy between  $\beta$ 1 and  $\beta$ 2.

† $P < 0.05$  comparing values between ICI89406 and ICI89406np for the  $\beta$ 1-WT/ $\beta$ 2-WT.

of the  $\beta$ 2. The same is not observed for active  $\beta$ 2. The interaction energies to I118 and H93 for ICI89406np were comparable for both active ( $-0.4 \pm 0.1$  kcal/mol to I118  $\beta$ 1,  $-0.1 \pm 0.2$  kcal/mol to H93  $\beta$ 2) and inactive state ( $-0.5 \pm 0.1$  kcal/mol to I118  $\beta$ 1,  $-0.0 \pm 0.0$  kcal/mol to H93  $\beta$ 2) simulations.

### <sup>3</sup>H-CGP12177 whole cell binding of ICI8940np

The experimental affinity of ICI89406np was then examined in the stable CHO- $\beta$ 1-WT and CHO- $\beta$ 2-WT cell lines. While ICI89406 was 170-fold  $\beta$ 1-selective (log  $K_i$  of  $-9.09 \pm 0.07$  and  $-6.86 \pm 0.06$ ,  $n = 6$  for  $\beta$ 1 and  $\beta$ 2, respectively, in keeping with previous studies (Mistry et al., 2013), ICI89406np had far lower affinity for the  $\beta$ 1-WT, while that at the  $\beta$ 2-AR was unchanged (log  $K_i$   $-6.97 \pm 0.06$  and  $-7.04 \pm 0.05$ ,  $n = 7$  for  $\beta$ 1 and  $\beta$ 2, respectively; Fig. 5). Thus, all  $\beta$ 1 selectivity was lost with removal of the phenyl group.

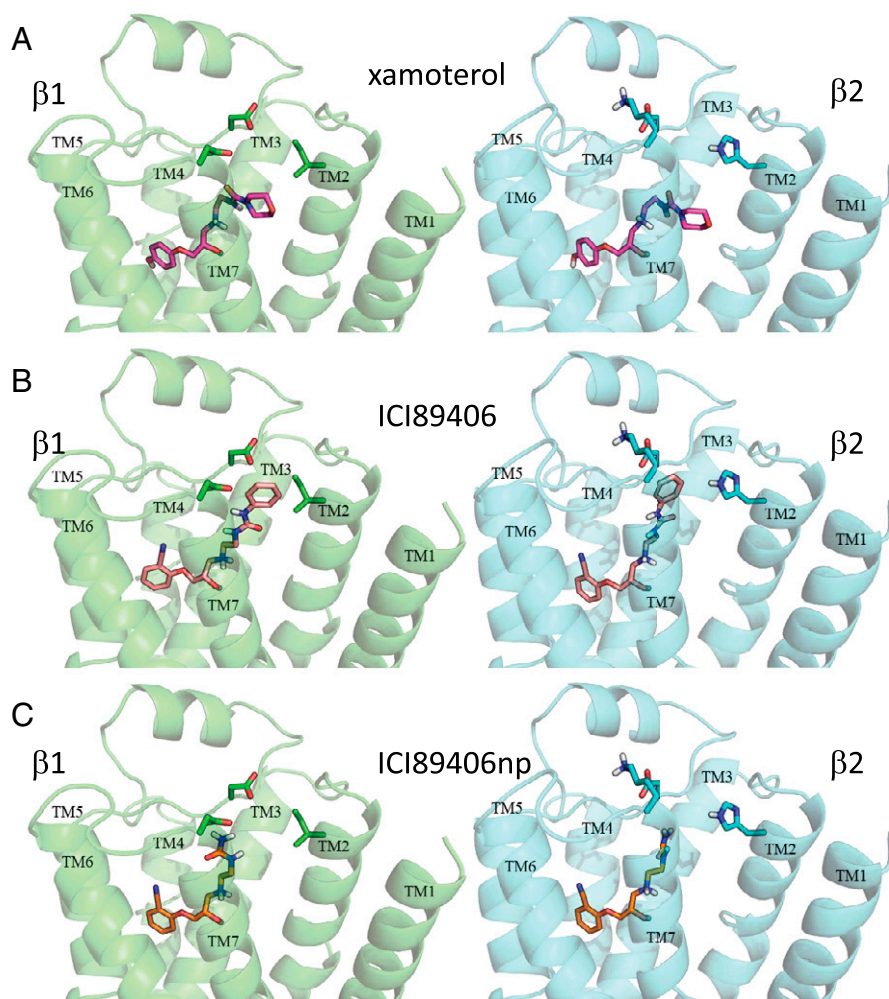
## Discussion

Computer-aided drug design offers a method for speeding up the drug discovery process, but to be accurate it requires very high-quality computer models, which in turn require large volumes of high-quality data, be that pharmacological, structural, or—better—both. A good start is to understand the structural basis of current drug selectivity, beginning with the pharmacologically most simple parameter, affinity. To understand the structural reasons for receptor subtype selectivity, crystal structures of each ligand-receptor subtype are required, a process that is difficult, time consuming and costly, and often involves heavily mutated receptors (truncated and stabilized with additions or multiple mutations to reduce flexibility; Cherezov et al., 2007; Rasmussen et al., 2007; Warne et al., 2008). Even then, the structural data obtained may not offer sufficient information to understand ligand selectivity (e.g., structure PDB ID 3NY8 with the  $\beta$ 2-selective antagonist ICI118551), as X-ray structures are, by definition, static snapshots.

Other methods to understand selectivity use pharmacological techniques. These have the advantage of acquiring data from native (nontagged, not stabilized) receptors expressed in living mammalian cells. The pharmacological outcome of single amino acid changes can be examined with far greater throughput than could be achieved with crystal structures. Importantly,  $\beta$ -AR pharmacological methods and crystal structures have been in agreement, highlighting the same important ligand-amino acid interactions (salmeterol, Baker et al., 2015; Masureel et al., 2018). Finally, although pharmacological techniques are able to determine the sites of interaction and selectivity, they do not give the structural explanation. When this is combined with detailed receptor modeling studies, however, first explanations for the observed selectivity can be suggested but second the existence of the pharmacological data increases the quality of the computer models, thus improving accuracy of future predictions for novel drugs. At their most powerful, such investigations will make predictions, which can then be tested pharmacologically, thereby providing more than just post hoc hypotheses.

This study used a chimeric mutagenesis approach to understand the  $\beta$ 1- over  $\beta$ 2-selectivity for xamoterol and nebivolol. Initially, the TM2 region was found to be crucial, then with single amino acid mutations highlighting that when the





**Fig. 4.** Docking pose of (a) xamoterol (b) ICI89406 and (c) ICI89406np in inactive  $\beta$ 1- (green) and  $\beta$ 2-AR (blue) receptors. The docking poses of the three compounds are similar in the active receptors.

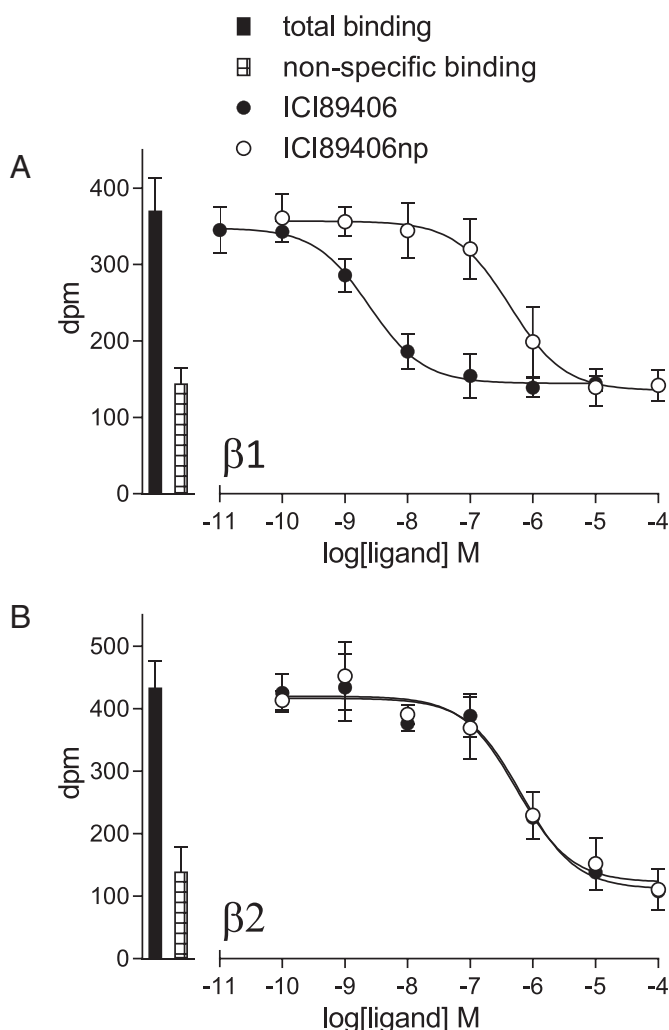
$\beta$ 1 amino acid I118 was mutated to an H (as in the  $\beta$ 2), the affinity of xamoterol and nebivolol was reduced to within twofold of that of the  $\beta$ 2. Equally importantly, when the reciprocal receptors were studied ( $\beta$ 2-AR where the equivalent amino acid was mutated from an H to an I at position 93), a gain in affinity was seen (in both TM2 region receptor and single point mutation) such that the affinity of xamoterol and nebivolol for  $\beta$ 2-H93I was within twofold of that for the  $\beta$ 1. This independently highlighted this amino acid's important role in the selectivity of these compounds.

To determine whether this single amino acid is important for all  $\beta$ 1-selective ligands, four other similarly moderately selective  $\beta$ 1-ligands were examined. ICI89406 (a  $\beta$ 1-partial agonist Mistry et al., 2013) was found to be greatly affected by this mutation (affinity reduced by 17-fold; Table 2).

MD simulations of xamoterol showed that, in the  $\beta$ 2, xamoterol hinders the  $\epsilon$  nitrogen of H93 forming hydrogen bonds with water when compared with the *apo* (unbound) receptor (Tables 3 and 4; we note that many other properties investigated did not show significant differences in both active and inactive states). This observation is consistent with the experimentally determined selectivity, as *in silico* binding of xamoterol to  $\beta$ 2 is less favorable because the ligand displaces water molecules that would otherwise interact with the  $\epsilon$  nitrogen of H93 in the *apo* receptor. The more favorable interaction with H93 in  $\beta$ 2 ( $-2.8 \pm 0.2$  kcal/mol compared with

$-0.4 \pm 0.1$  kcal/mol in  $\beta$ 1-active state,  $-2.9 \pm 0.3$  kcal/mol compared with  $-0.7 \pm 0.1$  kcal/mol in  $\beta$ 1-inactive state) might not be sufficient to compensate for the difference arising from the solvation pattern. Solvation of the  $\delta$  nitrogen of H93 was not considered, as it is pointing away from the ligand.

For ICI89406, a possible explanation is not as straightforward across the different activation states. In the  $\beta$ 2-inactive, ICI89406 also disrupts the solvation (41.2% in *apo* vs. 11.0% bound) and water-bridge formation (20.3% in *apo* vs. 2.1% bound) of H93 in  $\beta$ 2-AR compared with the *apo* structure. However, this is not observed in the  $\beta$ 2-active state. In the active state, ICI89406 displays more favorable interaction energies to  $\beta$ 1-I118 than  $\beta$ 2-H93 ( $-2.2 \pm 0.2$  kcal/mol to  $\beta$ 1-I118 vs.  $-0.9 \pm 0.1$  kcal/mol to  $\beta$ 2-H93). This is likely due to the nonpolar terminal phenyl group ability to form more favorable hydrophobic interactions with the nonpolar  $\beta$ 1-I118 than with the polar  $\beta$ 2-H93. This can be garnered from the different van der Waals interaction energies compared with  $\beta$ 2-inactive. The difference between the active and inactive state interactions for ICI89406 is likely due to the size and shape of the pocket. In  $\beta$ 2-active, ICI89406 adopts a different position (Supplemental Fig. 1) that causes it to be further away from H93, resulting in lower van der Waals interaction energies compared with the inactive state ( $-0.9 \pm 0.1$  kcal/mol in  $\beta$ 2-active versus  $-2.4 \pm 0.4$  kcal/mol in  $\beta$ 2-inactive). In contrast,  $\beta$ 2-active, ICI89406's phenyl group is



**Fig. 5.** Inhibition of  $^3\text{H}$ -CGP12177 whole cell binding by ICI89406 and ICI89406np in (a) CHO- $\beta_1$ -WT and (b) CHO- $\beta_2$ -WT cells. Nonspecific binding was determined by 10  $\mu\text{M}$  propranolol. The concentration of  $^3\text{H}$ -CGP12177 present in these experiments was 0.75 nM, and they are representative of six separate experiments in each case. Data points are mean  $\pm$  SD of triplicate determinations.

closer to H93, thereby disrupting the solvation despite a more favorable overall interaction energy. Both the poses in the active and the inactive state support  $\beta_1$ -selectivity, albeit for different reasons, indicating that the mechanism behind selectivity for these ligands is not as simple as one might envision. By considering the different states of the protein, it was possible to suggest a more complete picture of the potential molecular explanations behind selectivity.

When ICI89406np (ICI89406 lacking the terminal phenyl group), was examined experimentally in  $\beta_1$ -WT and  $\beta_2$ -WT receptors, the loss of the phenyl group had no effect on  $\beta_2$ -AR affinity. However, the  $\beta_1$ -WT affinity was dramatically reduced to that of the  $\beta_2$ -WT. This suggests that ICI89406's selectivity can be partially attributed to the interaction of the phenyl group with I118/H93. MD simulations of ICI89406np suggest that without the phenyl group, it is not able to disrupt the solvation of H93's  $\epsilon$  nitrogen or the water-bridge between H93 and D192 in  $\beta_2$  in both  $\beta_2$ -active and  $\beta_2$ -inactive states (Tables 2 and 3). The interaction energies to  $\beta_1$ -I118 and  $\beta_2$ -

H93 are comparable in both states (Tables 2 and 3). The low interaction energies suggest that the phenyl group plays an important role interacting with I118 in  $\beta_1$ -AR and H93 in  $\beta_2$ . Given similar moieties on the right part of xamoterol (Fig. 1), the morpholine group might play a similar role.

For the other three  $\beta_1$ -AR compounds, betaxolol, bisoprolol, and esmolol (which are neutral antagonists in these cells; Baker et al., 2011; Baker et al., 2017; Baker et al., 2020; Mistry et al., 2013), their moderate  $\beta_1$ -selectivity was not affected at the  $\beta_1$ -I118H or the  $\beta_2$ -H93I receptor (or indeed any other amino acid investigated in TM2). While statistically significant, the result for bisoprolol (a reduction in affinity from log  $K_i$   $-7.96$   $\beta_1$ -AR to  $-7.69$  in  $\beta_2$ -I118H) corresponds to a less than twofold decrease in affinity so is of uncertain significance. The ligand-amino acid interactions that are important for these compounds' selectivity must therefore lie elsewhere. This is in keeping with Marullo et al. (1990) who already hinted that different ligands may involve different amino acid interactions.

Docking of betaxolol, bisoprolol, and esmolol to  $\beta_1$  and  $\beta_2$ -inactive structures showed a distinct similarity between the poses of all three compounds—the isopropyl group next to the  $\beta$ -hydroxylamine was positioned in a similar location (Supplemental Fig. 2). The isopropyl group of these compounds is a considerable distance away from I118 (6.3 Å between the closest atoms). Therefore, this group is unlikely to have any direct interaction with I118, nor can it interfere with solvation of this residue, and thus the mutation I118H does not affect these compounds. MD simulations also showed little to no difference in solvation and interaction energies to these residues (Table 4).

As xamoterol and ICI89406 are partial agonists at both the human  $\beta_1$  and  $\beta_2$ -adrenoceptor, their partial agonist nature is not dependent on whether a I or H is present in TM2 (Mistry et al., 2013). Nebivolol stimulates such a poor partial agonist response it is barely measurable despite a high receptor expression level in these cells (Baker 2010). Ligand-amino acid interaction elsewhere on the receptor must therefore be important for determining the efficacy of compounds, and this TM2 residue identified is only responsible for determining the  $\beta_1$ -selective affinity interaction of certain  $\beta_1$ -selective compounds.

In conclusion, the isoleucine at position 118 in TM2 of the human  $\beta_1$ -adrenoceptor is an important interaction site for explaining the  $\beta_1$  versus  $\beta_2$  selectivity affinity of xamoterol, nebivolol, and ICI89406. The most likely structural explanation is based upon the computational observation that those moieties on the affected ligands close to I118 can interact and interfere with the solvation of this residue. This plays a more important role for the polar H of the  $\beta_2$ -AR but is less influential with the apolar I. Importantly, however, this amino acid does not explain the selectivity of other  $\beta_1$ -selective ligands, such as betaxolol, bisoprolol, and esmolol, despite similar  $\beta_1$ -selective affinities compared with xamoterol, nebivolol, and ICI89406. Thus betaxolol, bisoprolol, and esmolol must also be interacting with amino acids in different parts of the receptor to achieve their selective affinity. Overall, it therefore appears that different ligands, even with similar pharmacological characteristics, interact with different amino acids to achieve their pharmacological outcome. However, if novel  $\beta$ -compounds with  $\beta_1$ -affinity selectivity were to be designed, compounds designed to interact with I118 in TM2 may well have higher selective affinity, and therefore less potential for causing bronchospasm, than those that do not.

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## Authorship Contributions

*Participated in research design:* Lim, Kolb, Baker.

*Conducted experiments:* Lim, Proudman, Monteleone, Baker.

*Contributed new reagents or analytical tools:* Proudman.

*Performed data analysis:* Lim, Kolb, Baker.

*Wrote or contributed to the writing of the manuscript:* Lim, Kolb, Baker.

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