The isoleucine at position 118 in transmembrane 2 is responsible for the selectivity of xamoterol, nebivolol and ICI89406 for the human β1-adrenoceptor.

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Running title: β1-selectivity of xamoterol and nebivolol

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Text pages: 43
Tables: 4
Figures: 5
References: 34
Abstract: 240 words
Introduction: 701 words
Discussion: 1585 words

Abbreviations
CHO: Chinese hamster ovary
EL: extracellular loop
PBS: phosphate buffered saline
sfm: serum free media
TM: transmembrane
WT: wildtype
MD: Molecular Dynamics
Abstract
Known off-target interactions frequently cause predictable drug side-effects, e.g. β1-antagonists (used for heart disease) risk β2-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 β1-selective affinity of xamoterol and nebivolol, followed by computer-based modelling to provide possible structural explanations. 3H-CGP12177 whole cell binding was conducted in CHO cells stably expressing human β1, β2 and chimeric β1/β2-adrenoceptors (ARs). Single point mutations were investigated in transiently transfected cells. Modelling studies involved docking ligands into three-dimensional receptor structures and performing Molecular Dynamics simulations, comparing interaction frequencies between apo and holo structures of β1 and β2-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 β1-selective affinity of xamoterol and nebivolol. Single point mutations determined that the β1-AR isoleucine (I118) rather than the β2 histidine (H93) explained selectivity. Studies of other β1-ligands found I118 was important for ICI89406 selective affinity but not that for betaxolol, bisoprolol or esmolol. Modelling studies suggested that the interaction energies and solvation of β1-I118 and β2-H93 are factors determining selectivity of xamoterol and ICI89406. ICI89406 without its phenyl group loses its high β1-AR affinity, resulting in the same affinity as for the β2-AR. The human β1-AR residue I118 is crucial for the β1-selective affinity of xamoterol, nebivolol and ICI89406, but not all β1-selective compounds.
Significance statement – 79 words

Some ligands have selective binding affinity for the human β1 versus the β2-adrenoceptor however the molecular / structural reason for this is not known. The transmembrane 2 residue isoleucine I118 is responsible for the selective β1-binding of xamoterol, nebivolol and ICI89406, but does not explain the selective β1-binding of betaxolol, bisoprolol or esmolol. Understanding the structural basis of selectivity is important to improve computer aided ligand design and targeting I118 in β1-adrenoceptors is likely to increase β1-selectivity of drugs.
**Introduction.**

Predictable side-effects from known off-target interactions frequently cause adverse drug effects. Thus, drug discovery efforts are increasingly centred around creating compounds that are highly selective for only the clinical target, in the expectation of maximising clinical effectiveness whilst minimising harm (Clarke and Bond, 1998). However, the precise structural basis of selectivity for most ligands and most GPCRs is poorly understood, including the long studied prototypical β-adrenoceptors (β-AR).

A clinical example of where receptor selectivity matters, and indeed careful control of all pharmacological characteristics is required for minimum harm, are β-blockers for cardiovascular disease. β-blockers are important in the management of heart failure and ischaemic heart disease, where blockade of the cardiac β1-AR is thought to be the main therapeutic factor (Cruickshank, 2007). In those with heart failure, β-blockers lower mortality by ~35% and several different compounds have been shown to be beneficial (metoprolol, MERIT-HF, 1999; bisoprolol, CIBIS-II, 1999; carvedilol, COPERNICUS, Packer et al., 2002; nebivolol, SENIORS, Flather et al., 2005). A similar reduction in mortality is seen in ischaemic heart disease with a wider range of β-blockers (Baker and Wilcox 2017 and references therein). However, receptor selectivity is a concern: drugs with concomitant β2-AR antagonism can cause bronchospasm in those with asthma and impair the efficacy of β2-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 β1-AR ligand which has a similar degree of β1-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 synthesising further analogues, until the required characteristics have sufficiently been optimised. This basic medicinal chemistry technique was used in the original design of β-blockers (Black et al., 1965) and is still used to develop novel β-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 β-AR, remains largely unknown (e.g. β2-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). From our previous work, the precise amino
acid interactions required for the highly β2-selective affinity of salmeterol determined from chimeric mutagenesis studies (Baker et al., 2015) have been confirmed in the β2-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 β1- vs β2-selectivity of two moderately selective β1-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 β1-AR over the β2-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 foetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK). \(^3\)H-CGP12177 was from Amersham International (Buckinghamshire, UK) and Microscint 20 and Ultima Gold XR scintillation fluid 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 drugs studied are shown of Figure 1.

Molecular biology

The wildtype human β1-adrenoceptor (β1-WT), wild type human β2-adrenoceptor (β2-WT) and all transmembrane and extracellular region chimera constructs, as well as all stable cell lines, are as reported in Baker et al., 2014 and 2015. The single point mutations used here in (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

CHO-K1 cells (RIDD: CVCL_0214) stably transfected with the wildtype human β1 or β2-adrenoceptor, or one of the full EL region or TM domain chimeric receptors (total 24 cell lines) were used (see Baker et al., 2014 and 2015 for full details). For transiently transfected cells the parent CHO-K1 cells were transfected in a T75 with 10 ng DNA in 100 μl Lipofectamine and 8ml OPTIMEM 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 (DMEM/F12), containing 10% foetal calf serum and 2 mM L-glutamine in a 37°C humidified 5% CO₂: 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.

3H-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 3H-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 3H-CGP12177, and as determined from inhibition of radioligand, is thus referred to as K_i values.

Briefly, the affinity (K_D) of 3H-CGP12177 was determined for each construct from saturation experiments, with concentrations of 3H-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 $^3$H-CGP12177 as previously described (Baker, 2005) in 200 μl total well volume for 2 hrs at 37°C before being washed with 2 x 200 ml cold (4°C) PBS. A volume of 100 μl Microscint 20 was then added to each well, the plates left for several hours before being counted on a Topcount for 2 min per well. Propranolol (10 μM) was used to define non-specific binding in all experiments.

Data and statistical analysis

Whole cell binding

The affinity of $^3$H-CGP12177 for each mutant was determined from saturation binding, using 10 μM propranolol to determine non-specific binding and all data points were performed in quadruplicate. Specific binding (SB, equation 1) of $^3$H-CGP12177 at different concentrations of $^3$H-CGP12177 was fitted using the non-linear regression program Prism 7 to the equation:

Equation 1: \[ SB = \frac{(A \times B_{\text{max}})}{(A + K_D)} \]

where A is the concentration of $^3$H-CGP12177, $B_{\text{max}}$ is the maximal specific binding and $K_D$ is the dissociation constant of $^3$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 6 determinations of total and non-specific binding. A sigmoidal concentration-response curve was then fitted to the data using Graphpad Prism 7 and the IC$_{50}$ was determined as the concentration required to inhibit 50% of the specific binding using equation 2.
Equation 2: \[
\% \text{ uninhibited binding} = 100 - \frac{(100 \times A)}{A + IC_{50}} + \text{NS}
\]

where A in the concentration of the competing ligand, IC$_{50}$ is the concentration at which half of the specific binding of $^3$H-CGP12177 has been inhibited, and NS is the non-specific binding.

From the IC$_{50}$ value and the known concentration of $^3$H-CGP12177, a K$_i$ value (concentration at which half the receptors are bound by the competing ligand) was calculated using equation 3:

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

In order 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 wildtype (e.g. xamoterol affinity at β1-WT, β1-TM1, β1-TM2, β1-TM3, β1-TM4, β1-TM5, β1-TM6, β1-TM7, β1-N, β1-EL1, β1-EL2 and β1-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 wildtype, i.e. from β1-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 β1-TM2 was different from β1-WT with a p value of p<0.000001.
**Modelling of Human β1-AR Structures.**

Active and inactive conformation structures of human β1-AR were modelled using MODELLER (Webb and Sali, 2014). The inactive state was modelled based upon the template with PDB ID 2VT4 and the active state based on the template with PDB ID 3SN6. The G_{as} subunit was added to the β1-AR active-state-model by superimposing this model with the ternary complex of the β2-AR (PDB ID 3SN6) and removing the receptor portion and small-molecule ligand of 3SN6.

**β2-AR Crystal Structures.**

Crystal structures of the β2-AR in an active or inactive conformation were obtained from the PDB, PDB IDs 3SN6 and 2RH1, respectively.

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 β1- and β2-AR structures only. Nebivolol was not docked or simulated due to the high number of chiral centres (4), 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 2019 (MOE; Chemical Computing Group ULC 1010 Sherbooke St. West Suite 910, Montreal QC, 2018).

**Molecular Dynamics Simulations.**

Molecular dynamics (MD) simulations of the β1- and β2-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 bi-layer membrane with CHARMM-GUI’s Membrane Builder (Lee, Patel et al., 2018) and solvated with TIP3P water in a 0.15 nM NaCl solution. The CHARMM36 force field and the CHARMM General Force Field (CGenFF) 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 Supp. 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 polarised 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 to 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 analogue compounds

Following the docking calculations and MD simulations, and based on the observed ligand:receptor interactions in these calculations, analogues 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 analogues that fulfilled all the criteria were identified, but we identified and sourced ICI89406np (ICI89406 without the terminal phenyl group), and evaluated it pharmacologically.
Results

Identification of the regions in the human β1-AR important for xamoterol and nebivolol affinity - stable cell lines.

Xamoterol and nebivolol inhibited the specific binding of $^3$H-CGP12177 in CHO-β1-WT cells to yield log $K_i$ values of -7.19 and -8.33 respectively (Table 1). In the β2-WT cells the log $K_i$ values were -5.89 and -6.93, giving a β1 vs β2 selectivity for these compounds of 20 and 25-fold for xamoterol and nebivolol respectively, in keeping with previous studies (Baker 2005, 2010).

When the affinity of these compounds was studied in cell lines expressing chimeric β1-receptors, the affinity of xamoterol and nebivolol were reduced in the β1-TM2 receptor (i.e. the β1-AR but with mutations such that transmembrane helix 2 [TM2] is that of the β2-AR) compared with all other β1 receptors (WT and each single-region chimeric receptor; Table 1, Figure 2). This reduction in affinity, more closely resembling that of the affinity at the β2-AR, suggests that this region contained one or more amino acids which form an important (direct or indirect) interaction with the ligands and therefore has a large impact on the binding selectivity of xamoterol and nebivolol. When the reciprocal receptors were studied (i.e. β2-chimeric receptors), the affinity of xamoterol and nebivolol was significantly increased in the CHO-β2-TM2 cells, compared to β2-WT and all other β2-chimeras, again suggesting that this region is important for the selectivity of these two ligands (Table 1, Figure 2).

Identification of the individual amino acids involved in TM2 for xamoterol and nebivolol - transiently transfected cells.

From the stable cell line experiments above, TM2 appeared to be the most important region for the selectivity of xamoterol and nebivolol. Single point mutations were therefore made at
each of the 7 sites in TM2 where amino acids between the two receptors are different. For example, at position 98 in the β1-AR, a single point mutation was made such that this amino acid was mutated from a methionine M to a threonine T (i.e. β1-M98T, Table 2). The reciprocal β2 construct (e.g. β2-AR with a single point mutation to the amino acid of the β1-AR, e.g. β2-T73M) was also made. Binding studies with the individual amino acid mutations revealed that of the β1-TM2 constructs, β1-I118H reduced the affinity of both xamoterol and nebivolol compared with β1-WT (Table 2, Figure 3). When the reciprocal β2-TM2 individual amino acid constructs were examined, the corresponding construct (β2-H93I) had higher affinity for xamoterol and nebivolol (Table 2, Figure 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 nebivolol.

_Determination of whether β1-M98 can explain the β1-selectivity of other β1-antagonists – transiently transfected cells._

Other β-ligands with moderate β1-selectivity were then examined. ICI89406 (another β1-partial agonist, Mistry et al., 2013), and betaxolol, bisoprolol and esmolol (β-antagonists without efficacy in these cells; Baker et al., 2011; Baker et al., 2017; Baker et al., 2020; Mistry et al., 2013) had β1-WT (vs β2-WT) binding affinity selectivities of 81-, 14-, 30- and 13-fold, respectively (Table 2, Figure 3). When ICI89406 was examined in the TM2 constructs, it also had reduced affinity in the β1-I118H, compared to β1-WT. An increase in affinity at the reciprocal β2-H93I construct was also seen (compared with β2-WT). The affinities of betaxolol and esmolol were unchanged in any of the TM2 constructs, whereas the affinity of bisoprolol was reduced by 2-fold by the β1-I118H construct (which is of uncertain pharmacological significance).
In silico ligand:receptor interaction analyses

In order to understand the role of the isoleucine at position 118 in the β1, compounds were docked into models and X-ray structures of the human β1 and β2, respectively, and molecular dynamics simulations of these complexes were analysed. All docked and simulated compounds have one chiral centre 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 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 β1 and β2, whilst the other 3 compounds (known antagonists) were examined in the inactive structures only.

Xamoterol

In our simulations, xamoterol showed similar trends of solvation and interaction energies in both active and inactive β1/β2-ARs. In the β2 simulations, xamoterol reduced the solvation of H93’s ε nitrogen compared to 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 favourably with I118 in β1-AR than with H93 in β2 (-0.4 ± 0.1 kcal/mol and -2.8 ± 0.2 kcal/mol, respectively, in the active state, -0.7 ± 0.1 kcal/mol and -2.9 ± 0.3 kcal/mol, respectively, in the inactive state; Tables 3 and 4).

ICI89406

ICI89406 affects H93 differently in active and inactive β2 MD simulations. In the active state, ICI89406 has more favourable interaction energies with I118 in the β1 (-2.5 ± 0.2
kcal/mol) compared to H93 in the β2 (-0.9 ± 0.1 kcal/mol). However, the difference in interaction energies for the inactive state between β1/β2 was much smaller and not significant (-2.1 ± 0.3 kcal/mol and -1.9 ± 0.7 kcal/mol respectively). In the inactive β2, ICI89406 hinders the solvation of H93’s ε 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 β2.

**Analogue ICI89406np**

No commercially available xamoterol analogues 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 vs. histidine at the far end of the binding site. MD simulations showed that ICI89406np improved the solvation of the ε nitrogen of H93 (11.0% for ICI89406 vs. 27.9% for ICI89406np; Table 3; Figure 4) and the water-bridge between H93 and D192 (2.1% for ICI89406 vs. 21.4% for ICI89406np; Table 4) compared to ICI89406 in the inactive conformation of the β2. The same is not observed for active β2. The interaction energies to I118 and H93 for ICI89406np were comparable for both active (-0.4 ± 0.1 kcal/mol to I118 β1, -0.1 ± 0.2 kcal/mol to H93 β2) and inactive state (-0.5 ± 0.1 kcal/mol to I118 β1, -0.0 ± 0.0 kcal/mol to H93 β2) simulations.

The experimental affinity of ICI89406np was then examined in the stable CHO-β1-WT and CHO-β2-WT cell lines. Whilst ICI89406 was 170-fold β1-selective (log Kᵢ of -9.09 ± 0.07 and -6.86 ± 0.06, n=6 for β1 and β2 respectively, in keeping with previous studies (Mistry et al., 2013), ICI89406np had far lower affinity for the β1-WT, whilst that at the β2-AR was
unchanged (log K, -6.97 ± 0.06 and -7.04 ± 0.05, n=7 for β1 and β2 respectively; Figure 5).
Thus, all β1 selectivity was lost with removal of the phenyl group.

**Betaxolol, bisoprolol and esmolol**

Betaxolol, bisoprolol and esmolol were also examined in the inactive β1 and β2 structures and the effects on H93/I118 were small or statistically insignificant (Supp. Fig 2, and Supp Tables 3 and 4 for docking poses and nitrogen solvation, water-bridge formation and interaction energies). The only exception was for esmolol in β2, where there was a significant improvement in the frequency of a water-bridge between H93 and D192 in β2-AR (20.3% in apo vs 31.0% bound).


**Discussion**

Computer-aided drug design offers a method for speeding up the drug discovery process, but to be accurate requires very high-quality computer models, which in turn require large volumes of high-quality data, be that pharmacological, or 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 which is difficult, time consuming and costly, and often involves heavily mutated receptors (truncated and stabilised 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 β2-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 (non-tagged, not stabilised) 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, β-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 modelling studies, however, firstly explanations for the observed selectivity can be suggested, but secondly 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 \textit{post hoc} hypotheses.

This study used a chimeric mutagenesis approach to understand the β1- over β2-selectivity for xamoterol and nebivolol. Initially, the TM2 region was found to be crucial, with single amino acid mutations highlighting that when the β1 amino acid I118 was mutated to an H (as in the β2), the affinity of xamoterol and nebivolol was reduced to within 2-fold of that of the β2. Equally importantly, when the reciprocal receptors were studied, (β2-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 β2-H93I was within 2-fold of that for the β1. 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 β1-selective ligands, four other moderately selective β1-ligands were examined. ICI89406 (a β1-partial agonist Mistry et al., 2013) was found to be greatly affected by this mutation (reduced by 17-fold; Table 2).

MD simulations of xamoterol showed that in the β2, xamoterol hinders the ε nitrogen of H93 forming hydrogen bonds with water when compared to the \textit{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 selectivity, as \textit{in silico} binding of xamoterol to β2 is less favourable because the ligand displaces water molecules that would otherwise interact with the ε nitrogen of H93 in the \textit{apo} receptor. The more favourable interaction with H93 in β2 (-2.8 ± 0.2 kcal/mol compared to -0.4 ± 0.1 kcal/mol in
β1-active state, -2.9 ± 0.3 kcal/mol compared to -0.7 ± 0.1 kcal/mol in β1-inactive state) might not be sufficient to compensate for the difference arising from the solvation pattern. Solvation of the δ 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 β2-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 β2-AR compared to the apo structure. However, this is not observed in the β2-active state. In the active state, ICI89406 displays more favourable interaction energies to β1-I118 than β2-H93 (-2.2 ± 0.2 kcal/mol to β1-I118 vs. -0.9 ± 0.1 kcal/mol to β2-H93). This is likely due to the non-polar terminal phenyl group ability to form more favourable hydrophobic interactions with the non-polar β1-I118 than with the polar β2-H93. This can be garnered from the different van der Waals interaction energies compared to β2-inactive. The difference between the active and inactive state interactions for ICI89406 is likely due to the size and shape of the pocket. In β2-active, ICI89406 adopts a different position (Supp. Fig. 1) that causes it to be further away from H93, resulting in lower van der Waals interaction energies compared to the inactive state (-0.9 ± 0.1 kcal/mol in β2-active vs. -2.4 ± 0.4 kcal/mol in β2-inactive). In contrast, β2-active, ICI89406’s phenyl group is closer to H93, thereby disrupting the solvation despite a more favourable overall interaction energy. Both the poses in the active and the inactive state support β1-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, we were able 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 β1-WT and β2-WT receptors, the loss of the phenyl group had no effect on β2-AR affinity. However, the β1-WT affinity was dramatically reduced, to that of the β2-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 ε nitrogen or the water-bridge between H93 and D192 in both β2-active and β2-inactive states (Tables 2 and 3). The interaction energies to β1-I118 and β2-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 β1-AR and H93 in β2. Given similar moieties on the right part of xamoterol (Figure 1), the morpholine group might play a similar role.

For the other three β1-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 β1-selectivity was not affected at the β1-I118H, nor the β2-H93I receptor (nor indeed any other amino acid in TM2). While statistically significant, the result for bisoprolol (a reduction in affinity from log Ki -7.96 β1-AR to -7.69 in β2-I118H) corresponds to a less than 2-fold 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 β1 and β2-inactive structures showed a distinct similarity between the poses of all three compounds - the isopropyl group next to the β-hydroxylamine was positioned in a similar location (Supp. 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 β1 and β2-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 β1-selective affinity interaction of certain β1-selective compounds.

In conclusion, the isoleucine at position 118 in TM2 of the human β1-adrenoceptor is an important interaction site for explaining the β1 vs β2 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 β2-AR, but is less influential with the apolar I. Importantly, however, this amino acid does not explain the selectivity of other β1-selective ligands, such as betaxolol, bisoprolol and esmolol, despite similar β1-selective affinities compared to xamoterol, nebivolol and ICI89406. Thus betaxolol, bisoprolol and esmolol must also be interacting with amino acids in different parts of the receptor in order to achieve their selectivity. 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 β-compounds with β1-affinity selectivity were to be designed, compounds designed to interact with I118 in TM2 may well have higher selectivity, and therefore less potential for causing bronchospasm, than those that do not.
Acknowledgements

We thank June McCulloch for technical assistance with tissue culture and washing the binding experiments and Frank Balzer for system maintenance of the computer infrastructure used in the computational experiments.

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

Datasharing / Availability of data

Data will be available on request from the corresponding authors.
References


**Funding**

This research was funded in whole, or in part, by the Wellcome Trust [Grant number 086039/Z/08/Z]. For the purpose of open access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission. P.K. thanks the German Research Foundation DFG for Heisenberg professorship grants KO4095/4-1 and KO4095/5-1.

**Conflicts of interest**

JGB has been on the Scientific Advisory Board for CuraSen Therapeutics since 2019. Most of the pharmacology work presented here pre-dates that appointment.
**Figure legends**

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

Figure 2
a) Xamoterol inhibition of $^{3}$H-CGP12177 binding in a) and b) CHO-β1-WT and CHO-β1-TM2 cells and c) and d) CHO-β2-WT and CHO-β2-TM2 cells by xamoterol (a and c) and nebivolol (b and d). Non-specific binding was determined by 10 μM propranolol in all cases. These are raw data (dpm) from single experiments with data from 2 different cell lines plotted on the same graph. The concentration of $^{3}$H-CGP12177 present in these experiments was a) 0.73 nM, b) 0.43nM c) 0.89 nM. and d) 0.49 nM and are representative of a) 8, b) 6, c) 7 and d) 6 separate experiments. Data points are mean ± SD of triplicate determinations.
e) the sequence alignment of the amino acid residues in TM2 of the β1-WT and β2-WT receptor

Figure 3
Inhibition of $^{3}$H-CGP12177 whole cell binding by a) xamoterol, b) nebivolol, 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 normalised to that for total and non-specific (as determined from an average of 6 wells on each plate) within each transfection. Non-specific binding was determined by 10 μM propranolol. The concentration of $^{3}$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) 7, b) 7, c) 6, d) 6, e) 6 and f) 5 separate experiments. Data points are
mean ± SD of triplicate determinations.

Figure 4
Docking pose of (a) xamoterol in active β1-(Green) and β2-AR (Blue), and (b) ICI89406 and
(c) ICI89406np in inactive β1-(Green) and β2-AR (Blue).

Figure 5
Inhibition of $^3$H-CGP12177 whole cell binding by ICI89406 and ICI89406np in a) CHO-β1-
WT and b) CHO-β2-WT cells. Non-specific binding was determined by 10 μM propranolol.
The concentration of $^3$H-CGP12177 present in these experiments was 0.75 nM and they are
representative of 6 separate experiments in each case. Data points are mean ± SD of triplicate
determinations.
Table 1

Affinity (log $K_i$ values) of $\beta$-adrenoceptor ligands for the $\beta_1$-WT and $\beta_2$-WT obtained from $^3H$-CGP12177 whole cell binding in cells stably transfected with whole TM or EL changes. The values are mean ± s.e.mean for n separate experiments. The $\beta_1$ over $\beta_2$-selectivity for the wildtype receptors is also given, thus xamoterol had 20-fold higher affinity for $\beta_1$ than $\beta_2$.

<table>
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<th>Log $K_i$ xamoterol</th>
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<td>-8.33 ± 0.05</td>
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<td>$\beta_1$-N</td>
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<td>-8.18 ± 0.11</td>
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<tr>
<td>$\beta_1$-EL1</td>
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<td>-8.35 ± 0.07</td>
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<tr>
<td>$\beta_1$-EL2</td>
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<td>-8.11 ± 0.09</td>
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<tr>
<td>$\beta_1$-EL3</td>
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<td>-8.10 ± 0.09</td>
<td>10</td>
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<tr>
<td>$\beta_1$-TM1</td>
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<td>-8.53 ± 0.04</td>
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<td>-7.32 ± 0.07****</td>
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<td>-8.15 ± 0.09</td>
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<td>-6.93 ± 0.06</td>
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<td>$\beta_2$-N</td>
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<td>-6.95 ± 0.02</td>
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<tr>
<td>β2-TM7</td>
<td>-6.23 ± 0.04***</td>
<td>9</td>
<td>-6.97 ± 0.08</td>
<td>6</td>
</tr>
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</table>

β1-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 β1-WT or the β2-WT.

***p<0.0003 and *p<0.05 compared with β1-WT or the β2-WT
Table 2

Affinity (log $K_i$ values) of β-adrenoceptor ligands for the β1-WT, β2-WT and receptors containing single point mutations in TM2 obtained from $^3$H-CGP12177 whole cell binding in transiently transfected populations of cells. The $K_D$ values of $^3$H-CGP12177 and the receptor expression levels obtained from saturation studies in these transient populations are given. The values are mean ± s.e.mean for n separate experiments. The β1 over β2-selectivity for the wildtype receptors is also given.

<table>
<thead>
<tr>
<th></th>
<th>$K_D$ $^3$H-CGP12177 fmol/mg protein</th>
<th>Log $K_i$ xamoterol n</th>
<th>Log $K_i$ nebivolol n</th>
<th>Log $K_i$ ICI89406 n</th>
<th>Log $K_i$ betaxolol n</th>
<th>Log $K_i$ bisoprolol n</th>
<th>Log $K_i$ esmolol n</th>
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<td>β1-WT</td>
<td>0.28 ± 0.02#</td>
<td>731 ± 96 27</td>
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<td>334 ± 80 5</td>
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<td>-8.66 ± 0.04</td>
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<td>-6.90 ± 0.06</td>
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<td>β2-L95V</td>
<td>0.20 ± 0.03</td>
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<td>10</td>
<td>-6.29 ± 0.09</td>
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<td>-7.17 ± 0.09</td>
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</table>

β1-selectivity | 14.8 | 13.5 | 81.3 | 14.1 | 29.5 | 13.2 |

#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 β1-WT or the β2-WT.

**p<0.003 and *p<0.05 compared with β1-WT or the β2-WT
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 ± sem 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 β1-WT and β2-WT and compared using an unpaired t-test. Full details of each replicate, and all p values are given in Supp. Table 1 and 2.

<table>
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<tr>
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<th>xamoterol n</th>
<th>ICI89406 n</th>
<th>ICI89406np n</th>
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<td></td>
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<td>-0.0 ± 0.1 5</td>
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<td>β1-I118 LIG Van der Waals energy</td>
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<td>β2-H93 ε Nitrogen Solvation</td>
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<td>5</td>
<td>-0.9 ± 0.1†</td>
<td>5</td>
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<tr>
<td>β1-WT active vs β2-WT active energy</td>
<td>2.4#</td>
<td></td>
<td>-1.6#</td>
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</table>

*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 β1 and β2

†p<0.05 comparing values between ICI89406 and ICI89406np for the β1-WT or β2-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 ± sem 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 β1-WT and β2-WT and compared using an unpaired t-test. Full details of each replicate, and all p values are given in Supp. Table 1-4.

<table>
<thead>
<tr>
<th></th>
<th>Unbound apo</th>
<th>n</th>
<th>xamoterol</th>
<th>n</th>
<th>ICI89406</th>
<th>n</th>
<th>ICI89406np</th>
<th>n</th>
<th>betaxolol</th>
<th>n</th>
<th>bisoprolol</th>
<th>n</th>
<th>esmolol</th>
<th>n</th>
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<tbody>
<tr>
<td>β1-WT-inactive</td>
<td></td>
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<tr>
<td>β1-I118 LIG electrostatic energy</td>
<td>0.9 ± 0.1</td>
<td>5</td>
<td>0.3 ± 0.1†</td>
<td>5</td>
<td>0.1 ± 0.0†</td>
<td>5</td>
<td>0.7 ± 0.0</td>
<td>5</td>
<td>0.7 ± 0.1</td>
<td>5</td>
<td>0.6 ± 0.0</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β1-I118 LIG Van der Waals energy</td>
<td>-1.6 ± 0.1</td>
<td>5</td>
<td>-2.5 ± 0.3†</td>
<td>5</td>
<td>-0.6 ± 0.1†</td>
<td>5</td>
<td>-0.06 ± 0.01</td>
<td>5</td>
<td>-0.09 ± 0.03</td>
<td>5</td>
<td>-0.05 ± 0.01</td>
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<tr>
<td>β1-I118 LIG Interaction energy</td>
<td>-0.7 ± 0.1</td>
<td>5</td>
<td>-2.1 ± 0.3†</td>
<td>5</td>
<td>-0.5 ± 0.1†</td>
<td>5</td>
<td>0.6 ± 0.0</td>
<td>5</td>
<td>0.7 ± 0.1</td>
<td>5</td>
<td>0.6 ± 0.0</td>
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<tr>
<td>β2-WT-inactive</td>
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<tr>
<td>β2-H93 ε Nitrogen Solvation</td>
<td>41.2 ± 1.2</td>
<td>5</td>
<td>24.4 ± 5.2*</td>
<td>5</td>
<td>11.0 ± 3.2*</td>
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<td>27.9 ± 6.6</td>
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<td>40.8 ± 4.1</td>
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<td>36.8 ± 4.9</td>
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<td>39.4 ± 2.0</td>
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<tr>
<td>β2-H93 D192 Water-bridge</td>
<td>20.3 ± 3.4</td>
<td>5</td>
<td>16.4 ± 4.1</td>
<td>5</td>
<td>2.1 ± 1.8*†</td>
<td>5</td>
<td>21.4 ± 6.3†</td>
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<td>15.3 ± 4.1</td>
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<td>19.2 ± 5.2</td>
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<td>31.0 ± 1.5*</td>
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<tr>
<td>β2-H93 LIG Electrostatic energy</td>
<td>-1.3 ± 0.2</td>
<td>5</td>
<td>0.5 ± 0.3</td>
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<td>0.3 ± 0.1</td>
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<td>0.2 ± 0.2</td>
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<td>0.3 ± 0.2</td>
<td>5</td>
<td>0.4 ± 0.1</td>
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<td></td>
</tr>
<tr>
<td>β2-H93 LIG Van der Waals energy</td>
<td>-1.6 ± 0.1</td>
<td>5</td>
<td>-2.4 ± 0.4†</td>
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<td>-0.3 ± 0.1†</td>
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<td>-0.1 ± 0.0</td>
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<td>-0.1 ± 0.0</td>
<td>5</td>
<td>-0.1 ± 0.0</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2-H93 LIG Interaction energy</td>
<td>-2.9 ± 0.3</td>
<td>5</td>
<td>-1.9 ± 0.7†</td>
<td>5</td>
<td>0.0 ± 0.0†</td>
<td>5</td>
<td>0.2 ± 0.2</td>
<td>5</td>
<td>0.2 ± 0.1</td>
<td>5</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>β1-WT inactive vs β2-WT energy inactive</td>
<td>2.1#</td>
<td>-0.2</td>
<td>-0.5#</td>
<td>0.4</td>
<td>0.4#</td>
<td>0.2</td>
<td></td>
<td></td>
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</tbody>
</table>

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*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 β1 and β2

†p<0.05 comparing values between ICI89406 and ICI89406np for the β1-WT / β2-WT
Figure 1

nebivolol

betaxolol

xamoterol

bisoprolol

ICI89406

esmolol

ICI89406np
Figure 2

Figure 2 shows the total binding and non-specific binding of ligands to β1 and β2 receptors. The graphs compare the binding of β1 WT and β1 TM2 receptors, and β2 WT and β2 TM2 receptors. The x-axis represents the concentration of the ligand in logarithmic scale (M), while the y-axis shows the dpm (disintegrations per minute).

The table provides GPCRdb numbering and residue numbers for human β1-WT and human β2-WT receptors. The residues are shown for TM2 and include:

- **β1-WT:**
  - Gly122, Val120, Thr117, Ser102, Leu95
  - Residues: 2.37x37, 91 T, 2.38x38, 92 L, 2.39x39, 93 T, 2.40x40, 94 N, 2.41x41, 95 L, 2.42x42, 96 F, 2.43x43, 97 I, 2.44x44, 98 M, 2.45x45, 99 S, 2.46x46, 100 L, 2.47x47, 101 A, 2.48x48, 102 S, 2.49x49, 103 A, 2.50x50, 104 D, 2.51x51, 105 L, 2.52x52, 106 V, 2.53x53, 107 M, 2.54x54, 108 G, 2.55x55, 109 L, 2.56x55, 110 L, 2.57x56, 111 V, 2.58x57, 112 V, 2.59x58, 113 P, 2.60x59, 114 F, 2.61x60, 115 G, 2.62x61, 116 A, 2.63x62, 117 T, 2.64x63, 118 I, 2.65x64, 119 V, 2.66x65, 120 V, 2.67x66, 121 W, 2.68x67, 122 G

- **β2-WT:**
  - Gln597, Met96, Thr73, Tyr70, Val67
  - Residues: 2.37x37, 91 T, 2.38x38, 92 L, 2.39x39, 93 T, 2.40x40, 94 N, 2.41x41, 95 L, 2.42x42, 96 F, 2.43x43, 97 I, 2.44x44, 98 M, 2.45x45, 99 S, 2.46x46, 100 L, 2.47x47, 101 A, 2.48x48, 102 S, 2.49x49, 103 A, 2.50x50, 104 D, 2.51x51, 105 L, 2.52x52, 106 V, 2.53x53, 107 M, 2.54x54, 108 G, 2.55x55, 109 L, 2.56x55, 110 L, 2.57x56, 111 V, 2.58x57, 112 V, 2.59x58, 113 P, 2.60x59, 114 F, 2.61x60, 115 G, 2.62x61, 116 A, 2.63x62, 117 T, 2.64x63, 118 I, 2.65x64, 119 V, 2.66x65, 120 V, 2.67x66, 121 W, 2.68x67, 122 G
Figure 3

- **a)** % inhibition of specific binding vs. log [xamoterol] M
- **b)** % inhibition of specific binding vs. log [nebivolol] M
- **c)** % inhibition of specific binding vs. log [Cl89406] M
- **d)** % inhibition of specific binding vs. log [betaxolol] M
- **e)** % inhibition of specific binding vs. log [bisoprolol] M
- **f)** % inhibition of specific binding vs. log [esmolol] M

- **β1-AR**
- **β2-AR**
- **β1-I118H**
Figure 4

(a) 

β1 

xamoterol

β2

(b) 

β1 

ICI89406

β2

(c) 

β1 

ICI89406np

β2
Figure 5

![Graphs showing binding data for beta 1 (β1) and beta 2 (β2) receptors. The graph for β1 shows total binding, non-specific binding, and binding of ICI89406 and ICI89406np at different concentrations of the ligand. The graph for β2 shows a similar trend but with different data points.](image-url)