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Monoclonal anti-β_1-adrenergic receptor antibodies activate G protein signaling in the absence of β-arrestin recruitment

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Abbreviations: StaR, Stabilized receptor; GPCR, G protein coupled receptor; β_1AR, Beta 1 adrenergic receptor; mAb, monoclonal antibody, ECD, extracellular domain; ECL, extracellular loop

Thermostabilized G protein-coupled receptors used as antigens for in vivo immunization have resulted in the generation of functional agonistic anti-β_1-adrenergic (β_1AR) receptor monoclonal antibodies (mAbs). The focus of this study was to examine the pharmacology of these antibodies to evaluate their mechanistic activity at β_1AR. Immunization with the β_1AR stabilized receptor yielded five stable hybridoma clones, four of which expressed functional IgG, as determined in cell-based assays used to evaluate cAMP stimulation. The antibodies bind diverse epitopes associated with low nanomolar agonist activity at β_1AR, and they appeared to show some degree of biased signaling as they were inactive in an assay measuring signaling through β-arrestin. In vitro characterization also verified different antibody-receptor interactions reflecting the different epitopes on the extracellular surface of β_1AR to which the mAbs bind. The anti-β_1AR mAbs only demonstrated agonist activity when in dimeric antibody format, but not as the monomeric Fab format, suggesting that agonist activation may be mediated through promoting receptor dimerization. Finally, we have also shown that at least one of these antibodies exhibits in vivo functional activity at a therapeutically-relevant dose producing an increase in heart rate consistent with β_1AR agonism.

Introduction

G protein-coupled receptors (GPCRs) represent one of the largest protein superfamilies and are the site of action for over 30% of drugs on the market. Traditionally GPCRs have been considered targets for small molecule drugs, however their development has been hampered by difficulties identifying molecules with suitable selectivity and drug-like properties.[1] Many GPCRs belong to subfamilies with conserved ligand binding sites such that the identification of highly selective molecules can be difficult. Many small-molecule compounds derived from high throughput screening approaches have high lipophilicity and molecular weight leading to an increased probability of off-target toxicity.[2] An alternative approach to GPCR drug discovery is to identify functional antibodies that selectively activate or inhibit GPCRs of interest. There are increasing efforts to discover and develop anti-GPCR antibodies as therapeutics to exploit the wide range of disease areas covered by this receptor class.[3,4] Antibodies not only offer the desired selectivity, but good affinity and improved serum half-life. Global sales of therapeutic antibody products were in excess of $US 64 billion in 2012 (http://www.pipelinereview.com/index.php/2013050850905/FREE-Reports/Blockbuster-Biologics-2012.html), with over 35 of these drugs approved by the FDA, and ~350 monoclonal antibodies (mAbs) under evaluation in the clinical pipeline.[5] Despite a growing interest in the use of antibodies as therapeutics, as exemplified by the success of mAbs targeting growth factors and receptor tyrosine kinases,[6] few antibodies directed at GPCRs have progressed as therapeutic agents.

Historically, the generation of antibodies against GPCRs has been associated with technical hurdles arising from the quality and quantity of available antigen,[7] e.g., maintaining a pure, homogeneous form relevant to the native receptor structure, epitope exposure, low receptor cell surface density, detergent solubilisation, maintaining epitopes and functional activity. Antibodies are able to recognize, bind to and therefore stabilize different conformations, as demonstrated by a panel of

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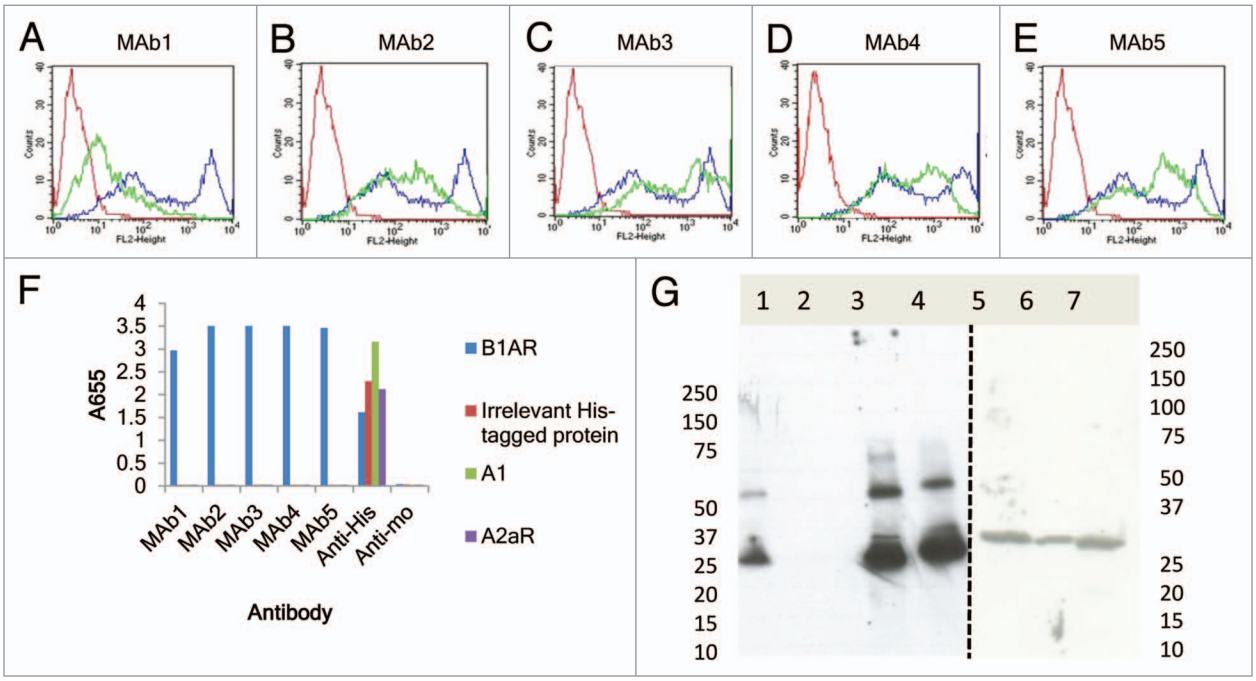

Figure 1. FACS histogram profiles of mAbs where each hybridoma supernatant binding profile (**A–E**) was confirmed by detection with anti-mouse PE conjugate where binding to HEK293 cells overexpressing β_1AR StaR is depicted by the green trace, background binding to untransfected cells is shown by the red trace and detection of the Nt tag confirming expression is shown by the blue trace. (**F**) Specific binding of mAbs to β_1AR Star protein was confirmed by ELISA where Nunc Immobiliser plates were coated with 5µg/well β_1AR StaR protein and incubated with 1µg/ml antibody. This was compared with the binding of the mAb panel against closely related stabilized receptors, such as the adenosine A_1 StaR and adenosine A_{2A} StaR vs. an irrelevant His-tagged receptor StaR (Orexin 1). Bound IgG was detected with anti-mouse secondary HRP conjugate and TMB substrate. Absorbance was read at 655 nm. The mean of triplicate data points was plotted. (**G**) western blot analysis of mAbs binding to denatured β_1AR to determine conformational sensitivity. β_1AR StaR protein was electrophoresed under reducing conditions prior to western blot transfer. Two independent blots were prepared as indicated by the vertical dotted line. Lanes were probed as follows: Lane 1) MAb3; Lane 2) MAb1; Lane 3) MAb2; Lane 4) MAb5; Lane 5) MAb4; Lane 6) MAb5 and Lane 7) Anti-His. Bound antibody was detected by using an anti-mouse HRP conjugate and the ECL detection system (Pierce). Sizes in kDa are indicated on the either side of the figure.

anti-CXCR4 antibodies that seem to recognize different conformations of receptor populations dependent on the type of host cell.[8,9]

Immunogens generated to represent GPCRs have covered a range of formats and sources, such as peptides corresponding to extracellular domains (ECDs), but the majority of antibodies obtained via this route react only with the immunizing peptides and not with native cell-expressed receptor. Where success has been achieved, the antibody has tended to be against a peptide receptor and acts by blocking the ligand/receptor interaction. Whole cell antigens and membrane preparations have also been used for in vivo in immunization as well as in vitro approaches, such as phage display (US2006/0275288). Virus-like particles,[10] liposomes,[11] nanodiscs,[12] exosomes[13] and dendritic cell immunization[14] are examples of other emerging routes for producing physiologically-relevant antigen.

Thermostabilization of GPCRs involves the introduction of point mutations that stabilize the receptor in a selected conformation.[15] This method allows extraction of correctly folded protein from the cell membrane and purification in detergent. The approach was initially developed to assist in the crystallization of GPCRs to generate structures of the β_1AR and the adenosine A_{2A} receptor.[16,17] The development of this technique to produce stabilized receptors (StaR proteins) allows large scale purification of stable protein that can be used for antibody generation. Stabilizing mutations are chosen such that they avoid the extracellular domains that may contribute to antibody binding sites.

At least 12 GPCRs are known to involve anti-receptor autoantibodies in immune-mediated disease.[18] For example, the hypoparathyroidism found in both autoimmune polyendocrine syndrome type 1[19] and Sjögren's syndrome[20] is caused by autoantibodies directed to the calcium-sensing receptor, and autoantibody activation of the thyroid stimulating hormone receptor occurs in Graves' disease.[21] It is thought that a number of these autoantibodies recognize an immunodominant epitope located on the second extracellular loop (ECL2). Consequently, substantial interest is growing in the potential of autoantibodies for their use in biomarker research and diagnostics, as exemplified by the presence of agonist autoantibodies against type 1 angiotensin II receptor in ovarian cancer, as well as their possible role in angiogenesis and metastasis.[22] A review of agonist autoantibodies to β-adrenergic receptors and muscarinic M2 receptors[23] suggests altered receptor conformation and function, allosteric agonism, and stabilization of oligomerization as potential mechanisms of action.

To investigate how stabilized receptors could be used to generate antibodies, we selected the β_1AR StaR because this presents the challenge of a non-peptide receptor with a limited extracellular surface and it is a well-characterized GPCR with a published crystal structure and established assays. The purpose of this study was 2-fold: first, to demonstrate that stabilized receptors are amenable to the generation of functional antibodies, and second to examine the physical and pharmacological properties of the resulting antibodies to gain further insight as to their mechanistic activity and interactions involved in β_1AR signaling.

Results

Antibody generation and characterization

cDNA immunization followed by protein boosting was the only immunization strategy advanced to in-depth characterization beyond analysis of the binding properties of the resulting stable hybridomas; we did evaluate hybridomas generated by protein immunization in the cAMP assay, but could not detect any agonist mAbs that were of particular interest for studying receptor signaling. In addition, we previously tried immunizing with wild type (WT) protein, but no immune response was detected. Historically, as described in the literature, DNA immunization with WT (GPCR) receptor DNA has raised a polyclonal response, but this has not been sufficiently robust to generate stable hybridomas[24] and the authors subsequently went on to evaluate the effect of an adjuvant on the genetic immunization process to increase the success of identifying stable antibody-secreting hybridomas.[25] Hence, our strategy was to prioritise the DNA prime + protein boost protocol and characterize the antibodies generated from this approach to gain further understanding of β_1AR signaling and function.

The scale of effort implemented to generate antibodies to β_1AR was that of a pilot study rather than that of a full therapeutic campaign. The immunization strategy implemented in this study identified five stable IgG-producing hybridoma clones (MAb1–5) from 1000 clones that were generated from one fusion using standard hybridoma methodology. Flow cytometry analysis using labeled antibodies demonstrated that all five mAbs were able to bind specifically to HEK293T cells transiently transfected with the β_1AR StaR (**Fig. 1A–E**), which demonstrated good levels of expression, and no background binding of the mAbs was detected on HEK293T cells transfected with an irrelevant cDNA.

Overexpression of WT receptor is difficult to achieve for many GPCRs as they can internalise or result in cell toxicity (the same is seen with ion channels). We have often observed that stabilized receptors are better tolerated when overexpressed, hence we evaluated binding to cells expressing the StaR and used a functional assay for the next step in the screening cascade. This can be considered an alternative method to assess antibody binding to WT receptor as functional binding assays utilize the WT receptor, and this approach is similar to that conducted for small molecule discovery. This is because the StaR platform provides a means to alter the equilibrium of conformations for a given GPCR, therefore an antibody will still bind to the corresponding WT receptor because the StaR conformation is identical to one of the WT conformations presented at the surface of the cell. In addition, all mAbs demonstrated specific binding to the purified turkey β_1AR StaR as displayed in the specificity ELISA (**Fig. 1F**) compared with StaR proteins of closely related receptors (adenosine A_1 and A_{2A} receptors), as well as a more distantly related peptide receptor (Orexin, OX1). This data also showed that the mAbs were specific for β_1AR receptor protein and not the tag (which was detectable on all StaR protein preparations by using an anti-HIS antibody).

Antibody binding was also interrogated using western blot analysis to determine whether the antibodies bound to linear epitopes since the receptor protein is presented in a denatured format in this assay. At least three of the antibodies tested were positive in the western blotting assay, indicating that they bound to a linear sequence not altered by detergent treatment of the protein (**Fig. 1G**). The apparent molecular weight of the major band observed was ~25kDa, which correctly corresponds to the size of the C-terminally truncated β_1AR StaR.[15] In contrast, MAb1 did not bind to the denatured protein, suggesting that it may be conformationally sensitive. Although MAb2 and MAb5 were not our focus in the study, the data indicates these antibodies do not bind to a linear epitope presented on any of the ECL peptides. However, neither antibody appears to be conformationally sensitive (positive in Western analysis), so they most likely bind to a discontinuous linear epitope that is distinct from that recognized by MAb1. This discontinuous epitope could represent a close association of two or more ECLs that is disrupted on denaturation.

The antibody isotype of the mAbs was determined by ELISA (at Aldevron Freiburg) revealing that 4 (MAbs1, 3, 4 and 5) were the murine IgG_1 isotype, whereas MAb2 was the IgG_{2b} isotype. These isotypes are characteristically produced as the predominant isotype in intradermal challenges and are indicative of a Th2 response, i.e., where Th2 cells have activated B cells in developing an antibody-mediated immune response.

Assessment of diversity

Epitope binning using surface plasmon resonance

Epitope binning using competition pair-wise analysis by Biacore was used to determine the number of different epitopes to which the mAb panel bound, thus providing an indication of diversity. All antibody permutations were analyzed, including the injection of two identical antibodies as a control. When the second antibody produced a response, we concluded that the two antibodies targeted different epitopes, and vice versa. **Figure 2A**, which shows a typical sensorgram where antibodies target different epitopes, presents this principle. Results obtained for MAbs2 to 4 are shown in **Figure 2B** and indicate that MAb3 and MAb4 target the same epitope that is different from that targeted by MAb2. Subsequent analysis also revealed that MAb1 binds to an epitope distinct from the previous two (data not shown), indicating that three different epitopes were targeted by the four mAbs.

Antigen-binding fragments (Fabs) were generated from MAb3 to investigate if MAb3 and MAb4 could sterically impede the other from binding or if they were competing for the same

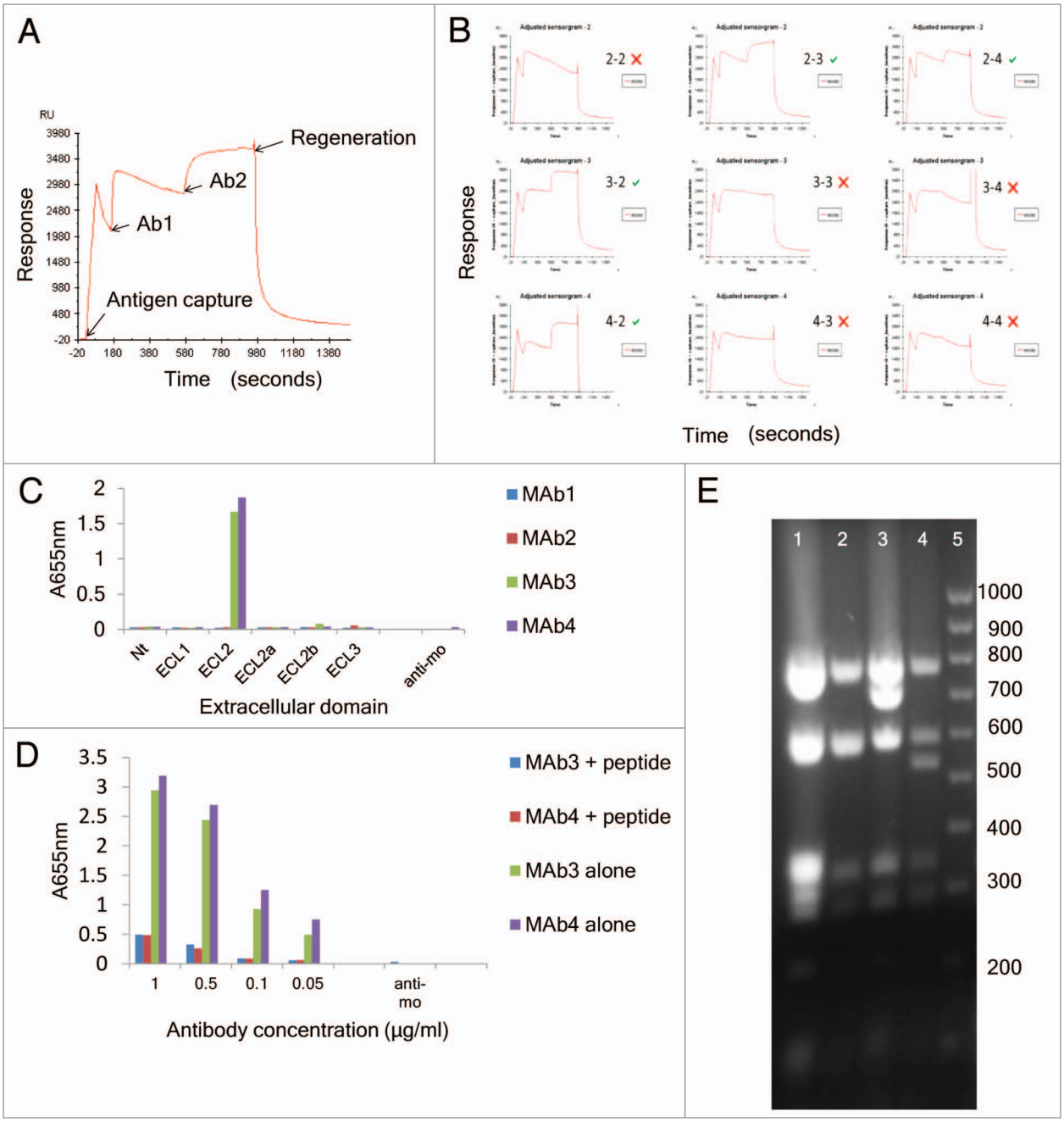

Figure 2. (**A**) Surface plasmon resonance and the the principle of epitope binning. This depicts a typical sensorgram (in red) showing two antibodies targeting different epitopes. The first increase in RU equates to immobilisation of the antigen to the Biacore chip. The first antibody (Ab1) is then bound resulting in a further increase in RU. This is followed by flowing the second antibody (Ab2) through as analyte. An increase in RU indicates that the two antibodies bind to two different epitopes. (**B**) Competition pair-wise analysis: epitope mapping sensorgrams for antibody pairs 2–3, 2–4 and 3–4 by using SPR analysis. An increase in RU, as seen in **Figure 3A**, indicates that the two antibodies bind to two different epitopes (green check), for example, MAb2-MAb3, MAb2-MAb4, MAb4-MAb2. Whereas no increase (red x) shows the two antibodies bind to the same epitope, for example, MAb3-MAb4. As expected, repeat injection of the same antibody did not produce any additional response, for example, MAb2-MAb2. (**C**) Epitope mapping using a crude form of peptide mapping was performed to interrogate the diversity of these mAbs further. Peptides corresponding to each extracellular domain (ECL1, ECL2, ECL3 and Nt) were synthesized and immobilised to a maleimide-activated surface via a C-terminal cysteine residue. Binding profiles were elucidated by ELISA revealing that MAb3 and MAb4 both map to ECL2 and verified our observations from the epitope binning, where both appear to bind to the same epitope on β_1AR. Specific binding was detected using an anti-mouse HRP conjugate which yielded a minimal amount of background binding (anti-mo). (**D**) β_1AR ELISA to demonstrate that binding of MAb3 and MAb4 to recombinant β_1AR StaR protein can be blocked by pre-incubation of each antibody with the ECL2 peptide in a concentration-dependent manner. (**E**) Restriction fragmentation with NlaIV to demonstrate antibody gene diversity in mAb V_H region where each mAb is shown in Lanes 1–4 alongside a 100bp DNA marker in Lane 5.

epitope (or overlapping epitope). No increase in response was detected, indicating that these antibodies compete for the same epitope (data not shown) rather than one impeding the other from binding because of steric hindrance due to the size of the dimeric molecule. This could be because these two antibodies are identical clones.

Peptide mapping

MAb3 and MAb4 map to the same epitope as determined by the Biacore-based epitope binning, and the data obtained by peptide ELISA (**Fig. 2C**) suggests that this epitope is an amino sequence presented on ECL2. However, it has not been determined from this analysis if the epitope is positioned in the proximal (ECL2a) or distal (ECL2b) domain of this receptor loop, where the first Cys residue in ECL2 is thought to form a disulfide bridge with the second Cys residue in this sequence and the third Cys residue forming a disulfide bridge with a Cys residue close to the membrane in TM3 in the expressed receptor. MAb2 does not appear to recognize a linear epitope represented on any of the peptide sequences for the ECLs

No other interactions with the extracellular loops or N terminus were detected. When the same peptides are pre-bound to these mAbs, they block MAb3 and MAb4 from binding to recombinant β_1AR protein (**Fig. 2D**) in a concentration-dependent manner. Thus, another indication of the diversity of functional anti-β_1AR mAbs is provided by the number of different epitopes identified from the epitope binning and peptide analysis.

Restriction digestion of V_H regions to evaluate diversity

Due to commercial reasons, we are unable to disclose the V_H and V_L region sequences. However, it was possible to demonstrate diversity by the alternative method of DNA fragmentation using a restriction enzyme that digests with high frequency, such as NlaIV. Separation of the resulting restriction fragments for each clone can be observed in **Figure 2E**. The pattern for MAb1 and MAb2 is very similar, although MAb1 appears to have a number of smaller fragments, but this could be due to a loading difference between samples. Nevertheless, as MAb1 and MAb2 bind to different epitopes, it can be concluded that these represent different clones. On the other hand, MAb3 and MAb4 bind to the same epitope and could therefore be identical, however their restriction profiles are very different. By combining the epitope mapping with this analysis, it can be concluded that all four mAbs represent different clones.

Effects of the β_1AR mAbs on G protein mediated signaling

A preliminary assessment for pharmacological properties revealed all four mAbs stimulated cAMP production with EC_{50} values in the range 0.1–10 nM compared with the isoprenaline EC_{50}, which was 40 nM ± 22 (**Fig. 3A**). MAb1 (EC_{50} = 0.1 nM ± 0.07), MAb3 (EC_{50} = 0.41nM ± 0.25) and MAb4 (EC_{50} = 0.03nM ± 0.018) demonstrated agonist activity, although this was only 86%, 73% and 48% (respectively) of the maximal response obtained with isoprenaline. MAb2 (EC_{50} = 9.9 nM ± 0.44 nM) stimulated cAMP production at a lower efficacy than the other mAbs. One mAb (MAb5), which showed potent binding in the ELISA and FACS assays, had no effect in the functional assays. Repeat experiments gave EC_{50} values in the range of 0.5–1.5 nM for MAbs1–4. The anti-FLAG mAb negative control gave no response as expected.

The concentration response curves for the mAbs in the Hit Hunter cAMP assay were shifted to the right by the β_1AR antagonist propranolol by varying degrees, confirming that the effect in functional assays was mediated by the β_1AR. A very low level of stimulation of cAMP was observed with both isoprenaline and the mAbs on the CHO.K1 parental cell line that was not transfected with turkey β_1AR (data not shown), although the effects were very small compared with the response in cells stably transfected with β_1AR. In both cases, this effect could be blocked by propranolol suggesting that the background response was likely mediated by endogenous β-adrenergic receptors in this cell line.

MAb1 and MAb3 were selected for pharmacological characterization based on their contrasting physical properties. The EC_{50} for MAb1 activation of the wild-type β_1AR receptor in this assay using stable transfected cells was in the range of 0.1–0.36 nM in the absence of propranolol compared with 3.9–33 nM in the presence of 10 μM propranolol (**Fig. 3B**), whereas MAb3 was not significantly propranolol sensitive (**Fig. 3C**). However, we observed a significant decrease in the maximum response to MAb3 in the presence of propranolol. This is consistent with non-competitive antagonism and would be expected if propranolol and MAb3 are binding to different sites on the receptor.

Homology between turkey and human in ECL2 is the same as between turkey and mouse. Receptor sequence alignment between turkey, mouse and human β_1AR is shown in **Figure 4** with corresponding ECL homologies indicated beneath. To determine whether the antibodies would cross-react with human β_1AR, the cAMP assay was repeated on CHO cells transiently transfected with human wild-type β_1AR. All the antibodies that were able to activate the turkey receptor were also found to stimulate signaling of the cAMP pathway via the human receptor. MAb1 and MAb3 in particular showed similar agonist responses at both human and turkey WT receptors, with EC_{50} values of 10.75 nM and 1.93 nM at the turkey receptor and 9.4 nM and 3.6 nM at the human receptor for MAb1 and MAb3, respectively (**Fig. 3D**).

The functional properties associated with antibody format were also evaluated using fragments produced by pepsin [$F(ab')_2$] or papain (Fab) enzymatic digestion. It was observed that the dimeric structure of the $F(ab')_2$ format maintained similar agonist activity to the intact mAb from which it was derived (**Fig. 3E and 3F**). However, agonism of the receptor was greatly reduced for the monomeric Fab format, which had a reduced Emax, whereas a similar Emax (EC_{50} = 62.9 nM and 27.3 nM) was observed for the corresponding $F(ab')_2$ of MAb1 and MAb3 despite a reduction (half-log to a log) in potency. On further investigation, it was confirmed that the Fab is capable of completely blocking the agonist activity of its corresponding IgG.

Effects of mAbs on the modulation of cAMP response to isoprenaline

In addition, we investigated the modulation of cAMP response by the mAbs to isoprenaline. Thus, concentration response curves

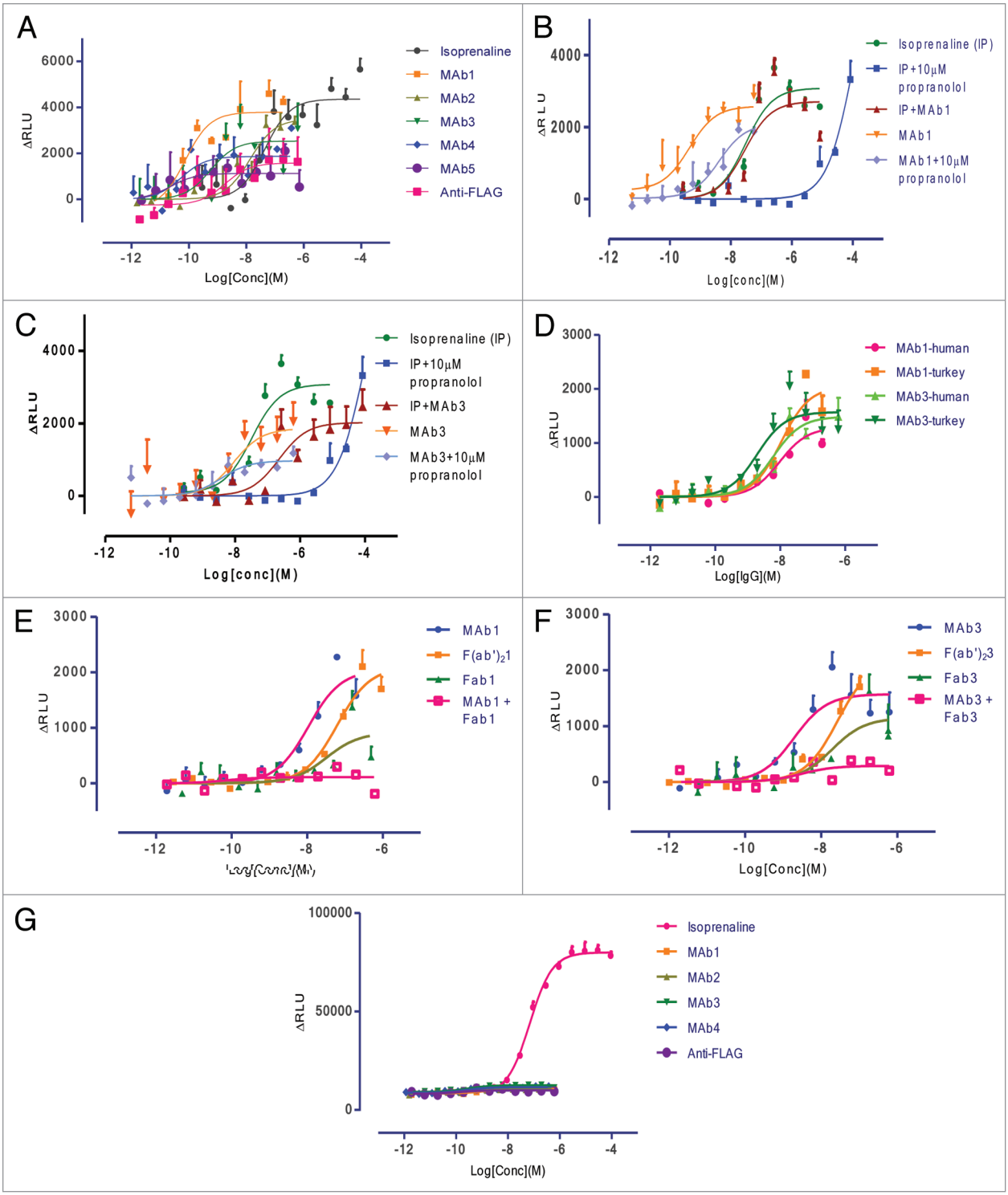

Figure 3. For figure legend, see page 252.

were evaluated in the cAMP assay for isoprenaline where the potency of isoprenaline could be reduced in the presence of 10 μM propranolol. For the example shown in **Figure 3B and C**, the EC_{50} values were 30.6 nM and 103.8 μM, respectively. Similarly, concentration response curves for isoprenaline were investigated in the absence and presence of MAb1 and MAb3. There was no significant effect of MAb1 on the potency of isoprenaline, EC_{50} = 32.1 nM vs. 28.6 nM (**Fig. 3B**). On the other hand, MAb3 reduced the potency of isoprenaline 10-fold, to EC_{50} = 224.9 nM (**Fig. 3C**). In contrast there was no significant effect on either isoprenaline potency or efficacy in the presence of either Fab (data not shown), suggesting that it is the dimeric format of

Figure 3 (See previous page). (**A**) Functional characterization of mAbs evaluating the stimulation of cAMP production through the turkey wild-type β_1AR receptor for MAbs 1–5 alongside isoprenaline and anti-FLAG (isotype control) using the cell-based HitHunter cAMP assay where data points represent the mean of triplicate experiments measuring relative luminescence units (RLU). (**B**) The HitHunter cAMP assay was performed in the absence and presence of 10 μM propranolol to evaluate the effect of this antagonist ligand on mAb potency. Data points have been normalized and represent the mean of triplicate data samples. Where a) the potency of isoprenaline was reduced (EC_{50} from 30.6nM to 103.8μM) in the presence of 10μM propranolol; b) the MAb1 EC_{50} value is reduced from 0.1 nM to 32.9 nM; and c) dose response curves for isoprenaline to evaluate potential for modulating agonist response were also investigated in the absence and presence 500nM MAb1. (**C**) The HitHunter cAMP assay was performed in the absence and presence of 10 μM propranolol to evaluate the effect of this antagonist ligand on mAb potency. Data points have been normalized and represent the mean of triplicate data samples. Where a) the potency of isoprenaline was reduced (EC_{50} from 30.6nM to 103.8μM) in the presence of 10μM propranolol; b) the MAb3 EC_{50} is unaffected; and c) dose response curves for isoprenaline to evaluate potential for modulating agonist response were also investigated in the absence and presence 500nM MAb3. (**D**) cAMP stimulation of both human (solid black circle) and turkey (solid black square) wild-type β_1AR receptors by two antibodies, thus demonstrating species cross-reactivity by showing similar functional potencies. Data point values have been normalized representing the mean of triplicate samples. (**E and F**). Antibody fragments for MAb1 and MAb3 were generated and evaluated for functional properties using the cAMP HitHunter cell based assay where (a) the antibody format is indicated by the denotation of IgG, $F(ab')_2$ and Fab. Data point values have been normalized representing the mean of triplicate samples; and (b) the Fab fragment antagonizes its corresponding IgG in the cell-based HitHunter cAMP assay; Data point values have been normalized representing the mean of triplicate samples. (**G**) β-arrestin independent cAMP signaling. Having demonstrated that the mAbs were also cross-reactive with the human wild- type β_1AR receptor, dose response curves for the anti-β_1AR mAbs were then assessed alongside an irrelevant antibody (anti-FLAG) in the DiscoverX PathHunter kit that utilizes frozen cell-division arrested cells stably transfected with both wild type human β_1AR and β-arrestin. Samples were evaluated in triplicate.

MAb1 and MAb3 that causes the conformational change leading to an effect on the functional activity of isoprenaline.

Effects of the β_1AR mAbs on β-arrestin recruitment

Agonist activation of β_1AR results in the recruitment of β-arrestin to the receptor as demonstrated in the enzyme fragment complementation assay. The EC_{50} for isoprenaline in this assay was found to be in the range 63.9 nM ± 15.8 (**Fig. 3G**). In contrast, MAb1 and MAb3, which potently stimulated G protein signaling to increase cAMP levels, had no effect in the β-arrestin recruitment assay at concentrations up to 1000-fold greater than their respective EC_{50} values in the cAMP assay.

Effects of the β_1AR mAbs on radioligand competition binding

Inhibition binding studies using the orthosteric antagonist [^{3}H] dihydroalprenolol (DHA) were used to characterize the interactions of both mAbs separately and in combination with the β_1AR agonist isoprenaline and the antagonist propranolol at the turkey WT β_1AR receptor. Both isoprenaline and propraanolol fully inhibited the specific binding of [^{3}H]-DHA in a concentration-dependent manner (**Fig. 5A**) with estimated K_I values of 100 nM ± 0.14 and 0.8nM ± 0.1, respectively, in agreement with previously published data (http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?objectId=28). To determine the interaction of the mAbs with the β_1AR, the effect of both MAb1 and MAb3 on the equilibrium binding of [^{3}H]-DHA was determined (**Fig. 5B**). MAb1 did not alter the binding of [^{3}H]-DHA, indicative of neutral cooperativity between the binding of the mAb and radioligand (α = 1). MAb3 caused a modest, concentration-dependent inhibition of [^{3}H]-DHA binding, yielding estimates of K_B = 16 nM and α = 0.3, indicative of weak negative allosteric modulation. It is important to note that this value concerns the nature of the interaction / analysis. The affinity (and cooperativity) estimate from the data provided reflect the fact that the inhibition is incomplete because the interaction is allosteric rather than competitive. Similar observations have been reported for other receptor-ligand interactions, such as M1 receptor allosteric modulation.[26]

Subsequent experiments were designed to assess the effect of these mAbs on the affinity of isoprenaline and propranolol binding to the receptor. Increasing concentrations of MAb1 yielded a concentration-independent increase in the affinity of isoprenaline ($P < 0.01$; F-test; **Fig. 5C and D**). As such, these data could not be modeled using the extended ternary complex model, but instead the simple single site model employed suggested that isoprenaline affinity was increased 5-fold in the presence of MAb1 ($\alpha \approx 5$). Similar concentrations of MAb1 had no significant effect on the affinity of propranolol ($P = 0.18$; F-test), indicative of neutral cooperativity ($\alpha = 1$) for this antibody. Due to the lack of concentration-dependent effects on the binding of any of the ligands, it was impossible to estimate the affinity of MAb1 for the β_1AR.

In contrast, increasing concentrations of MAb3 yielded a progressive leftward shift for both the isoprenaline and propranolol binding curves, suggesting that MAb3 induced concentration-dependent increases in their affinity. Analysis according to the extended ternary complex model yielded an estimate of the positive cooperativity between MAb3 and isoprenaline (α = 9) and propranolol (α = 3; **Fig. 5E and F**). As expected, the weak negative allosteric modulation of MAb3 with respect to [^{3}H]-DHA was reflected by a modest, concentration-dependent reduction in specific [^{3}H]-DHA binding (**Fig. 5E and F**).

Antibody-receptor interaction

MAb3 was prioritized for evaluation by fSEC analysis to test the potential effect on receptor dimerization given that this mAb binds to ECL2. These experimental conditions were repeated and extended using the corresponding Fab. Both antibody formats altered the fSEC profile of β_1AR, indicating that antibody-antigen complexes had formed and were intact in the detergent.

Size determination demonstrated that β_1AR alone elutes as a 110 kDa species (as a GFP fusion molecule), whereas β_1AR + MAb3 elutes as a 390 kDa species and β_1AR+Fab3 elutes as a 170 kDa species after subtraction of the molecular weight of the antibody format (**Fig. 6A**). These data suggests that MAb3 causes a size change consistent with an IgG binding two receptor molecules and are suggestive of receptor dimerization. As expected, Fab3 binds to a single β_1AR molecule based on the changes on protein size. It should be noted that any antibody aggregation would change the molecular weight of the complex,

which in turn would lead to changes in the elution time of the receptor. However, it is also unlikely that aggregated antibody would be able to still bind receptor. Dimerization can be caused by bivalent binding, i.e., to an epitope on each receptor molecule within a dimer (or oligomer). This is distinct from dimer-specific binding where a novel binding site (or neo-epitope) is created by the interacting receptor monomers. Subsequently, Fab3 was prioritized for Biacore affinity measurement, which indicated a KD of 43nM for Fab3 (**Fig. 6B**).

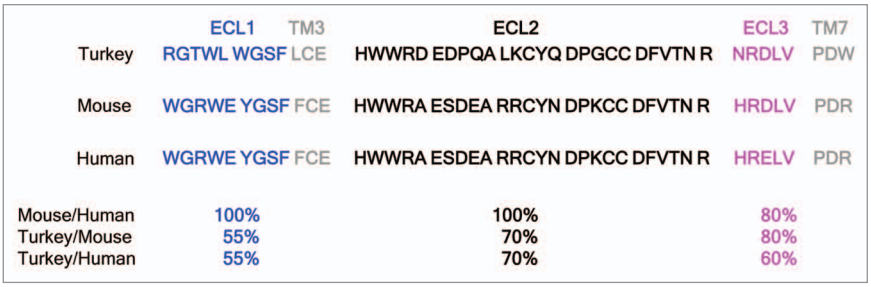

Figure 4. The amino acid sequences for ECL1, ECL2, and ECL3 for turkey, mouse and human β_1AR are highlighted in blue, black and pink respectively. The corresponding % homology is shown below for comparison between these different species.

In vivo effects of β_1AR MAb3

MAb3 was selected for evaluation of its effects in a rat cardiovascular model that assesses heart rate and blood pressure because this antibody expressed well, demonstrated a good level of receptor activation in the cell-based assays, and we were able to map its corresponding epitope to ECL2. Given the level of homology between inter-species β_1AR ECL sequences and the observed in vitro activity seen at turkey and human receptors, we would anticipate activity at the rat receptor. Cardiovascular responses to the MAb3 and its IgG1 isotype control are shown in **Figure** 7. Administration of MAb3 caused no significant change in blood pressure, but a significant ($P < 0.05$, Friedman's test) increase in heart rate starting 20 min after the onset of administration and persisting for the remainder of the experimental period, whereas administration of the corresponding IgG1 isotype control caused no significant change in blood pressure or heart rate. Hence, the integrated (0–240 min) increase in heart rate was significantly ($P < 0.05$, Mann-Whitney U-test) greater in the group given MAb3 than in the IgG1 isotype control group. While no significant change in systemic arterial blood pressure was observed, it is nonetheless possible that there were peripheral microvascular effects of the antibody. The observed tachycardic effect with MAb3 would be consistent with β_1AR agonism.

Discussion

A thermostabilized β_1AR has been used to generate functional anti-β_1AR mAbs following in vivo immunization that employed a strategy of DNA priming followed by protein boosting. A differentiating feature of the StaR platform is that the mutations can influence the stability and conformation of the protein providing correctly folded antigen for antibody discovery. The stabilizing mutations are conformation specific depending on the pharmacology of the ligand used StaR generation and can be identified in regions across the receptor, but mainly affect helix-to-helix interactions. It is important to note that for antibody generation, we deliberately avoid incorporating mutations in the extracellular domains of the N terminus and the ECLs.

Five stable hybridomas that demonstrated specific binding for β_1AR StaR protein were identified and four of these hybridoma clones produced mAbs that exhibited functional activity as observed in cAMP signaling assays. MAb5 did not exhibit functional properties in either the cAMP signaling assay or the β-arrestin assay and was not progressed further in this study. At least two of the anti-β_1AR mAbs (MAb3 and MAb4) map to an epitope on ECL2. These mAbs could either represent two difference sequences that bind to the same epitope or that the mAbs themselves are two clones of the same antibody. In fact, restriction fragmentation confirmed that these were different mAbs. It has been reported that autoantibodies mapping to the β_1AR ECL2 are highly pathogenic in a variety of cardiomyopathy conditions, inducing aortic dysfunction and ventricular arrhythmia,[27,28] thought to be mediated by pro-apoptotic effects.[29] Additionally, β_1AR autoantibodies have been described as potent agonists of the ERK1/2 pathway[30] and increase tumor necrosis factor secretion in RAW264.7 macrophages, which is thought to be PKA-dependent.[31] A review summarizing the role and mechanisms of cardiac autoantibodies in cardiomyopathy demonstrates that β_1AR autoantibodies play a significant role in the pathophysiology of heart failure.[32] Recently, the cyclic peptide COR1, designed to block anti-β_1AR autoantibodies by mimicking the tertiary structure of ECL2, has shown efficacy in a rat model of autoimmune cardiomyopathy.[33] Subsequently, a Phase 1 clinical study has demonstrated safety in humans and good pharmacokinetic properties (NCT 01043146).[34]

A number of groups have described a critical role for ECL2 in the binding of orthosteric and allosteric ligands to GPCRs,[35] including ligand activation of the C5a receptor[36] and residues that contribute to ligand specificity between the muscarinic M_2 and M_5 receptor subtypes.[37] It is also thought that receptor antibodies generated by immunization can act in a different way to autoantibodies isolated from patient sera.[38] The agonist activity attributed to β_1AR autoantibodies is less prone to induce receptor desensitization than classical agonist ligands[39] and, when bound at the same time as natural agonists, the autoantibodies are able to modulate the receptor response.[38]

β_1AR autoantibodies have been postulated to bind to conformational epitopes that may represent a domain separate to the ligand binding pocket,[43] and this domain is thought to be part of ECL2. Although ECLs are not involved directly in agonist binding, it has been proposed that ECL2 in the form of a helix reaches into the ligand binding pocket and can contact the ligand.[40,41] Disulfide bonds essential for maintaining this domain out of the ligand binding pocket, which enables natural ligand binding,[40] have been hypothesized to be sited within these

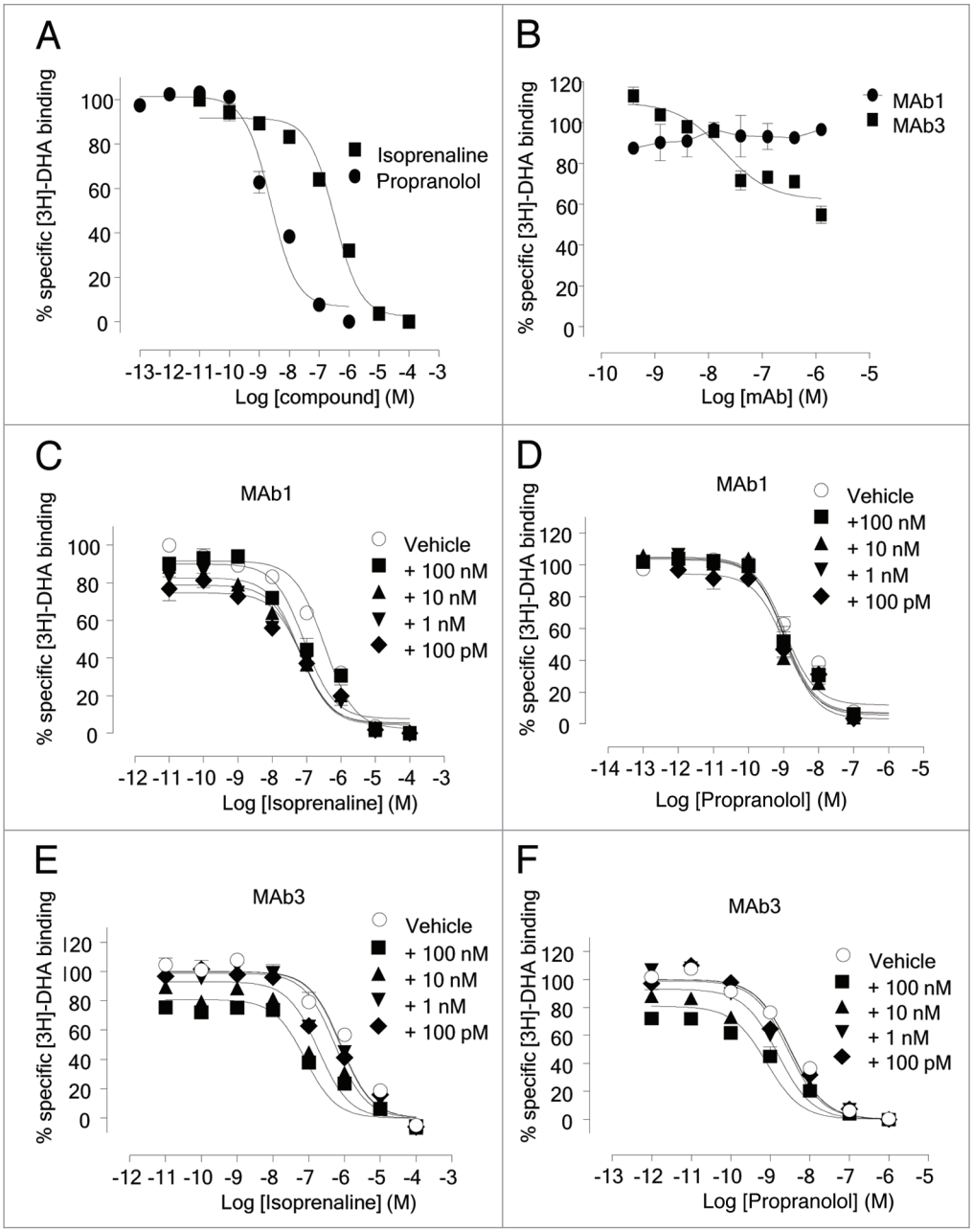

Figure 5. Radioligand competition binding evaluations for the effect of (**A**) isoprenaline and propranolol and (**B**) MAb1 and MAb3 on specific [^{3}H]-DHA binding to HEK293-β_1AR (wild-type receptor) cell membranes. Data points represent the mean of two independent experiments. Inhibition of specific [^{3}H]-DHA binding by (**C**) isoprenaline and (**D**) propranolol in the presence of increasing concentrations of MAb1 to HEK293-β_1AR (wild-type receptor) cell membranes. Data points represent the mean of two independent experiments. Inhibition of [^{3}H]-DHA specific binding by (**E**) isoprenaline and (**F**) propranolol in the presence of increasing concentrations of MAb3 to HEK293-β_1AR (wild-type receptor) cell membranes. Data points represent the mean of two independent experiments (where each data point was performed in triplicate).

antibody epitopes.[42] Hence, conformational changes to this loop would be anticipated to affect receptor activity.

The mode of action identified here for MAb3, which also interacts with ECL2, may be due to the ability of the antibody to stabilize an agonist conformation or through an ability to prevent ECL2 from interacting with the ligand binding site. Modulation of receptor agonism has previously been proposed for stimulatory autoantibodies against ECL2, and the hypothesis is based on the observation that autoantibodies decrease not only ligand affinity, but also the maximal capacity of equilibrium radioligand binding to the receptor in a dose-dependent fashion.[43] A recent review has documented progress made in determining the role of extracellular loops in ligand binding and the subsequent activation of GPCRs,[41] drawing upon the advances made in GPCR crystal structure determination and associated molecular modeling studies that suggest ECL2 can play a role in ligand selectivity within a diverse range of GPCRs, such as the muscarinic receptors, β_1AR and β_2AR, A_{2A} receptor and CXCR4. Finally, the cAMP signaling data obtained in our study correlates with other observations that β_1AR autoantibodies and isoprenaline-mediated effects are not identical with regard to the maximum increase in current amplitude for rat ventricular cardiomyocytes.[39]

It is evident from our results that the interaction of MAb1 with β_1AR is very different from that of MAb3. MAb1 does not change the affinity of [^{3}H]-DHA, but exerts a positive effect on isoprenaline binding by a factor of 5-fold, suggesting a positive allosteric modulation of isoprenaline binding, although surprisingly there was no significant effect of MAb1 on the potency of isoprenaline (EC_{50} = 32.1 nM vs. 28.6 nM). MAb1 was neutral with regard to propranolol in the binding affinity assay. Given that MAb1 does not directly affect the binding of propranolol, the blocking effects of propranolol on antibody activation in the cAMP assay may be mediated by changes in the receptor's ability to be activated in the presence of antagonist. In addition, western blot analysis suggests that MAb1 is a conformationally sensitive antibody.

In contrast, MAb3 appears to act as an agonist. The antibody could bind in the same position (orthosteric) as isoprenaline, or that the antibody may bind to a distinct location on the receptor (allosteric site), creating a conformational change that affects the orthosteric ligand binding pocket. The latter mechanism is more likely because MAb3 caused a 10-fold reduction (EC_{50} = 224.9nM) in the potency of isoprenaline, despite significantly enhancing the binding affinity of isoprenaline. A similar example has been reported for small molecule allosteric modulators of cannabinoid receptors where opposite effects were described for agonist binding vs. agonist function. Although this seems counter-intuitive, these allosteric modulators had differential effects on affinity and potency. These small molecule ligands increased binding of the radioligand [^{3}H]-CP55,940 while acting as insurmountable antagonists in function at the CB_1 receptor, i.e., a reduction in signaling efficiency,[44] and even other, more complex states of allosteric modulation have been described.[45,46]

None of the mAbs in this study had any effect in the β-arrestin recruitment assay at concentrations up to 1000-fold the EC_{50} in the cAMP assay. There are, however, many examples of differences between the degree of receptor activation through G proteins and β-arrestin recruitment. This dissociation between G protein activation (leading to cAMP signaling) and β-arrestin recruitment is known as biased agonism[47] and suggests that the mAbs stabilize a different active conformation to isoprenaline, which can signal down both pathways. Only one other example of a biased agonist mAb has been described to our knowledge, namely, an anti-mGluR7 mAb that triggers receptor internalisation[48] via a pertussis toxin-insensitive pathway that does not involve $G\alpha_i$ and is not dependent on the cAMP signaling pathway. The mAb divalent format was required for internalisation, prompting the authors to propose that the IgG caused a conformational change involving receptor dimers thereby inducing the internalisation process.

GPCR dimerization can be associated with changes in ligand binding affinity, through inter-receptor positive or negative cooperativity. The β_1AR mAbs described here are all divalent, providing the possibility for receptor oligomerisation and, in the case of MAb3, can alter the potency of the agonist ligand. Epitope binning via pair-wise competition and peptide mapping indicate that MAb3 binds to ECL2, but it binds to a completely different epitope from MAb1. MAb1 itself is conformationally sensitive as determined by its lack of binding to receptor protein in Western analysis, where receptor protein will be denatured and only linear epitopes exposed. It is possible that the β_1AR mAbs are able to stabilize conformational states associated with dimeric forms of the receptor in a similar manner as has been hypothesized for the angiotensin AT1 receptor.[18]

Although it is known that SDF-1 triggers CXCR4 dimerization, activating the JAK/STAT pathway,[49] and MCP-1 induces functional responses through dimerization of CCR2,[50] there is mixed evidence in the literature that receptor dimerization is linked to activation of GPCRs. Receptor dimerization has been described as a critical step in chemokine signaling.[51] The bivalent format of mAbs allows for the potential to induce receptor dimerization, but this does not necessarily lead to a functional response, e.g., anti-CCR5 mAb CCR5–02. This mAb does not compete for ligand binding to the receptor, whereas the

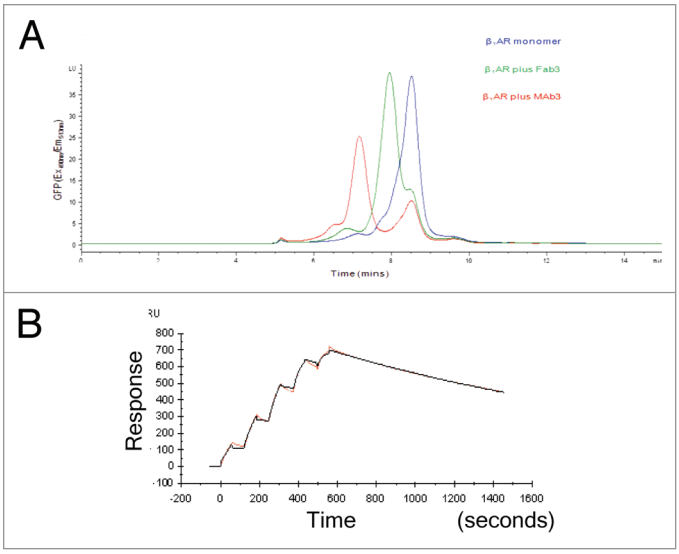

Figure 6. (**A**) Evaluation of antibody-receptor interaction by fSEC analysis using a β_1AR-GFP fusion construct. The data indicates that MAb3 (red trace) changes the motility of the receptor (blue trace) where the size change is consistent with an IgG binding two receptor molecules. Similarly, an additive size change is observed for Fab3 (green trace) representing a Fab fragment binding one receptor molecule. (**B**) Affinity evaluation of antibody-receptor interaction for the corresponding Fab fragment of MAb3 by SPR analysis.

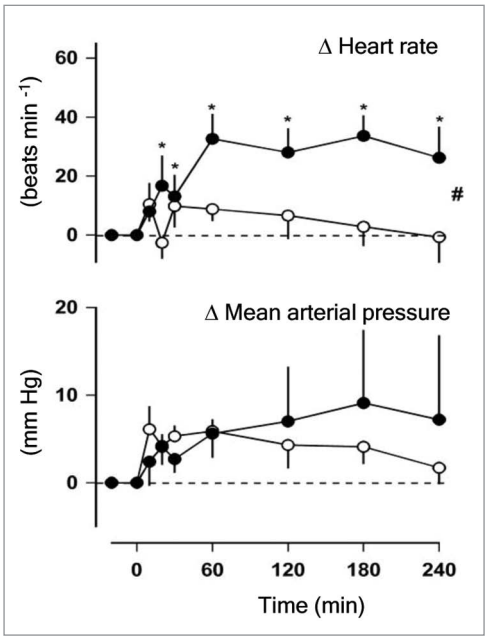

Figure 7. In vivo assessment of MAb3-β_1AR using a freely-moving conscious rat model for the evaluation of cardiovascular responses. Open circles represent the isotype control at 3 mg/kg and filled circles represent MAb3 at 3 mg/kg. The increases in heart rate and blood pressure have been plotted over a time course of 4 h. Values are mean and vertical bars indicate SEM, n = 6. * Friedman's test, $P < 0.05$. # Mann-Whitney Test, $P < 0.05$.

anti-CCR2 mAb CCR2–02 produces an agonist response and does compete with ligand binding to the receptor.[52]

In conclusion, four unique agonist mAbs have been isolated that bind to different epitopes on the limited extracellular surface of β_1AR and appear to possess different mechanisms of action relating to the nature of interaction with the receptor. Further study would be necessary to elucidate these differences in antibody-receptor interactions, such as co-crystallization studies using the corresponding Fabs of MAb1 and MAb3, which could shed further light on the role of ECL2 in ligand-induced activation of GPCRs. By interrogating the interaction of these activating antibodies with the receptor, valuable insights into the structure-function of GPCR activation would be gained.

Rat and mouse ECL homology are 100% identical, hence cross-reactivity of the murine β_1AR mAbs with the rat receptor is expected. In vivo studies of β_1AR suggest the amplitude in cellular responses can be used to predict the cardiovascular properties of small molecule β-blockers,[53] where isoprenaline is used as a small molecule positive control. As with the cAMP assay, we observe a response from a therapeutically relevant dose of antibody that is lower than that of the full isoprenaline response. It was notable that there was a tachycardic effect following administration of MAb3 to conscious rats, which could be explained by β_1AR agonism, but this was not associated with any significant change in systemic arterial blood pressure. β_1AR agonism might have been expected to have caused a rise in blood pressure due to concurrent positive inotropic and chronotropic effects. Hence, the lack of change in blood pressure might suggest either that the cardiac effects were insufficient to increase cardiac output or there were underlying, opposing, regional vascular effects to offset any change in blood pressure. It would, therefore, be desirable to further investigate regional hemodynamic effects of this anti-β_1AR mAb activity in vivo, in order to more fully understand the cardiovascular effects observed at this therapeutically-relevant dose.

Unlike other antigen production methods, DNA immunization has the unique advantage that the antigen is produced in the native environment of the host animal. This maximizes the likelihood of the protein forming its native structure via intracellular synthesis with correct post-translational modifications, three-dimensional folding and trafficking to the cell surface with correct presentation. However, workers in the field report that it may only produce low levels of antibodies when using the WT receptor, which can impede the success of identifying a therapeutic. The method we used here employs the Gene Gun where DNA delivery is via the bombardment of the skin with DNA-coated microparticles. We followed this with a short protein boost prior to the fusion process, thereby reserving the usage of recombinant StaR protein and enabling a less protein resource-intensive route to identifying functional antibodies.

In this study, the use of a thermostabilized receptor in both DNA and protein immunogen format has demonstrated the ability to generate functionally active mAbs to a GPCR, where β_1AR presents a far smaller extracellular surface area than other GPCRs, such as CXCR4, for which there are a number of mAbs in development (and where the mAbs are generally antagonizing the receptor by targeting the N terminus). Despite only identifying five stable hybridomas, we demonstrated that four of these mAbs exhibit agonist activity, suggesting that the quality of the GPCR antigen not only has to be biochemically pure, but also biophysically pure, i.e., correctly folded and enriched for biologically relevant epitopes.

Targeting peptide receptors and other GPCRs with a large ligand binding domain, such as chemokine and Family B receptors, with an antibody is a useful alternative approach to generating small molecule drugs. A recent example of this has been the reporting of antagonist mAbs directed to the glucose-dependent insulinotropic polypeptide receptor (GIP).[54] Similarly, orphan receptors (for which there are no known ligands) and GPCRs with emerging biology (such as the adhesion subfamilies) present a challenge in drug development. Attempts to identify small molecule agonists or antagonists for these generally fail; however, a functional mAb provides an alternative approach to identifying drugs to such targets. The generation of purified StaR proteins and the corresponding StaR cDNA for immunization is an emerging technology that may provide the means to develop therapeutic mAbs to clinically important GPCR targets.[55] The study presented here has attained initial proof-of-concept, and provided valuable initial insight into the mechanism of action of these mAbs that show different interactions with the receptor as reflected by the varied pharmacological profiles mapping to different epitopes.

Materials and Methods

β_1AR StaR protein

The thermostabilized turkey (*Meleagris gallopavo*) β_1AR StaR, also called β_1AR-m23,[15] was used as the antigen. This stabilized receptor contains eight amino acid changes (C116L increased expression; C358A at the C terminus of Helix 8 removed palmitoylation and were previously included to help protein crystallization; and R68S, M90V, Y227A, A282L, F327A and F338M were added to increase the thermostability). The receptor was expressed in insect cells using the baculovirus system and purified in 0.1% (v/v) decylmaltoside (Anatrace) with 0.1 mM alprenolol (Sigma-Aldrich) bound as previously described.[16]

Immunization and isolation of mAbs

A β_1AR StaR cDNA construct that included the full-length native N-terminus of β_1AR was prepared. This was used for genetic immunization of 3 female balb/c mice with Aldevron Freiburg's proprietary Gene Gun system (Aldevron Freiburg GmbH, formerly GENOVAC GmbH), implementing a primary challenge of cDNA, followed by 3–6 boosts of cDNA and a further boosting with purified β_1AR StaR protein. The strength of the β_1AR immune response was monitored by flow cytometry and ELISA analysis of the sera. All animals gave significant responses and were sacrificed after a minimum of 100 d. Lymph nodes were harvested and isolated lymphocytes used to generate hybridoma fusion clones with mouse myeloma Sp2 cells using standard hybridoma methods. 1000 clones were picked from one fusion. Hybridoma supernatants were screened for specific binding to β_1AR by ELISA and to the extracellular domains of the receptor by flow cytometry analysis. Once stable hybridoma

cell lines had been established and monoclonality confirmed, the resulting expressed IgGs were also validated by flow cytometry analysis, and progressed to small-scale IgG production and isotype identification by an ELISA-based method using the Biozol isotype kit for mice (Southern Biotech).

Antibody specificity ELISA

β_1AR StaR protein was immobilized via capture of the C-terminal His6 tag to nickel-coated 96-well Nunc Immobilizer plates (Thermo Scientific). Plates were coated with 5 μg/ml antigen solubilised in 0.1% (v/v) DM (Anatrace) and 0.1 mM alprenolol (Sigma-Aldrich). Serial dilutions of mouse sera or purified IgG were prepared and evaluated for antibody binding. Bound antibody was detected with goat anti-mouse horseradish peroxidise (HRP)-conjugate (Cell Signaling Technology) using the TMB (3,3′,5,5′-Tetramethylbenzidine) liquid substrate system (Sigma-Aldrich). The absorbance was read at 655 nm. To determine mAb specificity, binding to β_1AR was compared with that of A_1 StaR (adenosine A_1 receptor), A_{2A} StaR (Adenosine A_{2A} receptor) and an unrelated StaR (Orexin 1). The unrelated Orexin 1 StaR also provided a control for potential binding to the His-tag. The presence of antigen successfully coated to the plate well was confirmed using a mouse anti-Penta His tag mAb (Qiagen).

Western blotting

β_1AR StaR protein was diluted to 14 μg/ml and 22 μl of the sample mixed with 2μl 1 M DTT and 8 μl 4x LDS sample buffer (Expedeon Protein Solutions Ltd) to load 0.3 μg protein per well and subjected to electrophoresis on a 4–20% Tris-Glycine SDS PAGE Novex pre-cast gel (InVitrogen). Western blot transfers were made onto nitrocellulose membrane (Invitrogen) and probed with monoclonal IgG at 10 μg/ml. Bound antibody was detected with secondary anti-mouse HRP conjugate (Sigma-Aldrich) and the ECL detection system (Thermo Scientific) with exposure of Amersham Hyperfilm ECL autoradiograph film (GE Healthcare) to the nitrocellulose membrane.

Flow cytometry analysis to identify hybridoma clones of interest

The level of immune response was evaluated for binding to β_1AR-expressing cells using flow cytometry and determination of the sera titer. Binding for the resulting mAbs was also confirmed in this manner. Mouse anti-sera or mAbs binding cells expressing β_1AR StaR was detected using goat anti-mouse PE conjugate (BD Bioscience) and the background/ specificity evaluated on cells transiently transfected with an irrelevant cDNA. This was compared with the binding profile of the pre-immune bleed to monitor the strength of immune response. The hybridoma fusion performed for this study focused on identifying antibodies that bound to extracellular epitopes, hence a cell-based ELISA (developed in-house at Aldevron) was employed for sample testing and flow cytometry was used for further confirmation of positive hybridoma supernatants.

Measurement of cAMP stimulation using the HitHunter XS$^+$ cAMP assay

The DiscoveRX HitHunter assay kit (#90–0075L) is a sensitive detection assay for the stimulation of the secondary messenger cAMP.[56] A CHO cell line stably expressing the turkey wild type β_1AR was used in the HitHunter assay. In addition, to investigate the activity of the antibodies against the human receptor, CHO cells were transiently transfected with human wild type β_1AR cloned into pcDNA3.1 mammalian expression vector (gift from Dr Chris Tate, Laboratory of Molecular Biology, Cambridge, UK). Cells were plated in 96-well format at densities of 4000–25 000 cells/well and cAMP measurement was determined according to the recommendations of the DiscoverX 2 Step Reagent Addition Protocol, with each data point performed in triplicate. To investigate the effects of antagonist on antibody responses, propranolol (final concentration of 10^{-4} M) was added to the cells for 30 min prior to the addition of ligands/antibody. Plates were read on a Polarstar instrument (Omega, BMG Labtech) measuring relative luminescence signal in 96-well Costar plates. RLU data was plotted against final ligand/IgG concentrations and analyzed on GraphPad Prism using a nonlinear fit.

Measurement of β-arrestin recruitment

The DiscoveRX PathHunter eXpress assay kit (#93–0488E2) was employed for the evaluation of β-arrestin recruitment using cells stably transfected with both wild type human β_1AR and β-arrestin (#93–0446E1). This assay measures receptor activation via G-protein dependent and independent signaling because GPCR mediated β-arrestin signaling may occur regardless of G-protein coupling. It is possible to identify agonism, antagonism and allosteric modulation if the receptor recruits β-arrestin. Cells stably transfected with both wild type human β_1AR and β-arrestin were used for the assay. The assay was performed according to the manufacturer's instructions.[57] Isoprenaline was used as a positive control and anti-FLAG mouse mAb was used for the negative control, with each data point performed in triplicate. Luminescence was read on a Polarstar plate reader and data analyzed using GraphPad Prism.

Cell membrane preparation for radioligand binding assays

Membranes were prepared from β_1AR transiently-expressing HEK293 cells. Cells were transfected with β_1AR cDNA using Genejuice (Merck Biosciences). After 48 h, cells were harvested by trypsin, centrifuged (350 g, 5 min) and the resulting cell pellet stored at −80° C until required. To prepare membranes, the pellet was resuspended in 20 ml buffer (20 mM HEPES, 10 mM EDTA, pH 7.4) and homogenized using an Ultra-Turrax homogenizer for 10 s at the maximum setting. The homogenate was centrifuged (350 g for 15 min), supernatant collected, stored on ice and the pellet resuspended in 20 ml buffer. This process was repeated twice prior to centrifugation at 40 000 g for 45 min. The resultant pellet was resuspended in 20 ml of storage buffer (20 mM HEPES, 0.1 mM EDTA pH 7.4) and protein content determined by bicinchoninic acid (BCA) analysis. The homogenate was diluted to 2 mg/ml aliquots and stored at -80 °C until required.

Radioligand binding studies

Radioligand binding studies with [^{3}H]-DHA were used to determine the effects of antibodies on the binding of agonist and antagonist ligands to the receptor. HEK293-β_1AR cell membranes (5 μg/well) were incubated with ligand (concentration range) in Kreb's buffer (118 mM NaCl, 8.5 mM HEPES, 4.7 mM KCl, 4 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11 mM glucose; pH 7.4)

for 2 h at 25° C. Non-specific binding was defined by 0.1 mM alprenolol. Saturation binding data were modeled using a single site hyperbolic equation to determine the ligand K_D. The K_D value of [^{3}H]-DHA was determined to be 3.3 nM ± 0.34 (n = 2) and was used in subsequent competition studies.

Competition binding studies

For competition binding studies, 250 µl Kreb's buffer containing [^{3}H]-DHA (7 nM) and HEK293-β_1AR cell membranes (5 µg/well) were incubated for 4 h (25° C) with a range of concentrations of either propranolol, isoprenaline or mAb in the presence or absence of multiple, fixed concentrations of mAb or Fabs. The reaction was terminated by rapid filtration through 96-well GF/B filter plates pre-soaked in 0.1% (v/v) polyethyleneimine solution (PEI) using a Tomtec liquid handler and washed with ddH_2O. Plates were left to dry prior to addition of Safe Scint liquid scintillant (LabLogic). Plates were sealed and bound radioactivity measured using a Microbeta (PerkinElmer). Data was normalized to % specific binding for analysis. For competition by isoprenaline and propranolol, data were modeled using a single site binding model. IC_{50} values were converted to K_B values using the K_D value determined by saturation binding.[58]

To determine the effect of mAbs alone against [^{3}H]-DHA binding, data were modeled with an allosteric ternary complex model[59,60] allowing the determination of α, the cooperativity factor, governing the interaction between [^{3}H]-DHA and the mAb. For studies examining the effect of MAb3 binding on the affinity of propranolol or isoprenaline, data were modeled according to an extended ternary complex model that describes the interaction between two orthosteric ligands (i.e., [^{3}H]-DHA and propranolol or isoprenaline) and a putative allosteric ligand (MAb3). Due to the very limited effect of MAb1 on the binding of ligands, curves for this study were modeled according to the single site model and the resultant K_B values compared by F-test for significance.

Antibody epitope mapping

Epitope binning by surface plasmon resonance

Epitope mapping using pair-wise competition was performed using a Biacore T200 instrument and NTA sensor chip (GE Healthcare). The assay was performed in PBS, 0.1% (v/v) DM, 0.1 mM alprenolol, 0.05 mM EDTA pH 7.4 at 25° C. Each assay cycle began with the capture of β_1AR StaR protein (0.5 µM, 1 min injection) followed by the injection of the first antibody, followed by the second antibody (0.5 µM, 5 min each). Finally, the chip surface was regenerated with 1.0 M imidazole (Sigma-Aldrich), 0.1% (v/v) DM (15 min).

Peptide mapping

Peptides were synthesized that encode the extracellular domains of turkey β_1AR, including the N terminus (Nt) and the first (ECL1), second (ECL2) and third (ECL3) extracellular loops. To orientate the peptide, a C-terminal Cys residue was added where it was not already present in the native sequence. Two peptides were synthesized that represented the proximal and distal regions of extracellular loop 2 (ECL2a and ECL2b) using the position of the first Cys residue to segment the domain. The sequences were as follows: Nt, MGDGWLPPDC GPHNRSGGGG ATAAPTGSRQ VSAELLSQQC; ECL1, RGTWLWGSFL C; ECL2, MHWWRDEDPQ ALKCYQDPGC CDFVTNC; ECL2a, MHWWRDEDPQ ALKC; ECL2b, YQDPGCCDFV TNC; ECL3, VNVFNRDLVP DC (Cambridge Research Biochemicals). Each peptide stock solution, at 10 mg/ml dissolved in DMSO (Sigma-Aldrich), was diluted in PBS for immobilization to maleimide-activated plate surfaces (Pierce, Thermo Scientific) at 50 µg/ml. Plates were incubated overnight at 4° C sealed with adhesive microplate lids (Greiner, SLS). Wells were then washed with PBS and simultaneously blocked along with underivatized sites in PBS/3% (w/v) dried Marvel skimmed milk powder plus freshly prepared 100 µg/ml cysteine (Pierce) in PBS for 1–2 h at room temperature. The blocking solution was discarded and then mAb at 1 µg/ml concentration was added and the plate incubated for 1 h at room temperature. The wells were then washed with PBS/0.1% (w/v) BSA, 0.05% (v/v) Tween 20 (Sigma-Aldrich) and then with PBS. Bound antibody was detected with goat anti-mouse HRP-conjugate (Cell Signaling Technology) using the TMB (3,3′,5,5′-Tetramethylbenzidine) liquid substrate system (Sigma-Aldrich) and absorbance read at 655 nm.

Restriction digest analysis to evaluate clone diversity

This method can be used to evaluate antibody gene diversity by restriction enzyme fragmentation pattern. Total RNA was extracted from hybridoma cells and cDNA of the variable regions (V_H and V_L) of the IgG were synthesized and amplified by RT-PCR (Genscript) for cloning. To demonstrate gene diversity, ~1 µg of miniprep DNA from individual colonies for each mAb were digested with NlaIV restriction enzyme (NEB) at 37° C for 3 h, enzyme was heat-inactivated at 65° C for 20 min, then DNA fragments were electrophoresed on a 1.5% agarose gel along with EZ Load 100 bp Molecular Ruler (Bio-Rad).

SPR analysis of affinity by Biacore

Normally, affinity measurement would involve mAb immobilization on the chip followed by antigen injection to avoid avidity effects. This proved to be impossible however due to high unspecific binding of the receptor to the chip surface. The use of NSB reducer (dextran) added to the sample failed to prevent problems with unspecific binding. We tried to resolve this using a number of different strategies, including that used in the identification of a suitable Fab for co-crystallization of $A_{2A}R$,[61] namely, to coat the chip in anti-mouse Fc, capture the antibody fragment and then flow the purified receptor over as analyte.

The experiment was run on a Biacore T200 instrument at 10° C in PBS buffer, pH 7.4, (GE Healthcare) supplied with 0.1 mM alprenolol, 0.1% DM, 0.05 mM EDTA. The receptor was captured on an NTA chip (GE Healthcare) to a level of ca. 1500 resonance units (RU). The Fab fragments were injected at five concentrations in the range 0.25–4 µM in the single cycle format. The kinetic constants and equilibrium dissociation constant were obtained by fitting the data to 1:1 interaction model using Biacore evaluation software (GE Healthcare).

Antibody fragment generation

For Fab fragmentation, 4 mg of protein G purified IgG were digested using the papain-based Fab preparation kit (Pierce). The procedure was performed according to the instructions provided by the supplier. Digestion was performed for 16 h at 37 °C and the crude digest was subjected to purification using a Protein

A column, removing Fc fragments and undigested material (Fc). For $F(ab')_2$ digestion, 2.3 mg protein G purified IgG was digested using the Ficin-based $F(ab')_2$ preparation kit (Pierce) using the manufacturer's recommended protocol. Digestion was performed for 28 h at 37° C after which undigested material was removed using a Protein A column. QC was performed by gel electrophoresis prior to protein concentration determination and SEC to remove undigested material and excess cysteine with a buffer exchange into PBS.

Study of antibody-receptor interactions by fSEC analysis

β_1AR-m23 receptors C-terminally tagged with EGFP were transiently expressed in human HEK293T cells using GeneJuice transfection reagent (Merck) according to the manufacturer's guideline. Briefly, for each transfection, $4x10^6$ cells were seeded in a 10 cm plate and incubated overnight at 37° C incubator. Next day, cells were transfected with 6 μg of plasmid DNA encoding β_1AR-m23-EGFP receptor. After 40 h post-transfection, cells were harvested and washed in 10 ml of PBS. The pellets were re-suspended in 850 μL of buffer containing 50 mM HEPES pH7.5/150 mM NaCl/0.5 mM EDTA supplemented with a cocktail of protease inhibitors (Roche Applied Science). The total protein content was quantified using BCA assay and 1 mg aliquots of the suspension were prepared and pelleted. For each fSEC run, pellets were re-suspended in 190 μL of buffer with or without 1 μM of antibodies. The samples were incubated at room temperature for 1 h to allow antibody binding and following equilibration to 4° C, 10 μl of 20% (w/v) n-Dodecyl β-D-maltoside (DDM) was added for solubilisation. The samples were incubated at 4° C for 1 h. The crude lysates were centrifuged at 50,000 rpm for 30 min prior to application of 50 μl samples to a BioSep-SEC-s3000 column (Phenomenex). The flow rate was set at 1 ml/min with the total run time of 15 min where the mobile phase contained 50 mM HEPES pH7.5/150 mM NaCl/0.5 mM EDTA/0.025% DDM. The size quantification was based on the calibrations provided by the manufacturer.

In vivo assessment of anti-β_1AR mAb activity

These studies were conducted at the University of Nottingham (UK) under approval from the local ethics committee and approved Home Office Project License protocols. Under anesthesia (fentanyl and medetomidine, 300 μg/kg i.p. of each, supplemented as required), 12 male Sprague-Dawley rats were instrumented with intravascular catheters implanted in the distal aorta (via the caudal artery) for recording arterial blood pressure and heart rate, and in the jugular vein (3 catheters) for drug administration. Reversal of anesthesia and postoperative analgesia was provided by atipamezole (1 mg/kg s.c.) and buprenorphine (0.02 mg/kg s.c.). Experiments commenced 24 h after the surgical procedure, when the rats were fully conscious and freely-moving. Rats were divided into 2 experimental groups to evaluate MAb3 at 3 mg/kg vs. IgG1 control at 3 mg/kg. Bolus doses were administered in a volume of 0.6 mL at a pump speed of 1 mL/h, over a period of 36 min. Infusions were administered in a volume of 0.4 mL at a pump speed of 0.4 mL/h, over a period of 60 min. The day following catheterization, continuous recordings of cardiovascular variables were made using a customized, computer-based system (Instrument Development Engineering Evaluation (IDeeQ), Maastricht Instruments), connected to a transducer amplifier (Gould model 13-4615-50). Raw data were sampled every cardiac cycle and stored to disc for later analysis off-line.

Baseline (control) values were taken as the average of the 10 min period prior to antibody administration; thereafter the values represent sequential averages (3 × 10 min, 1 × 30 min, 3 × 60 min) for the remainder of the recording period. Data were analyzed using non-parametric analysis of variance (Friedman test) for within-group comparisons, and the Mann-Whitney U-test for between-group comparisons, applied to the integrated (0–240 min) areas. $P < 0.05$ was taken as significant.

Disclosure of Potential Conflicts of Interest

Authors Hutchings CJ, Cseke G, Osborne G, Zhukov A, Koglin M, Jazayeri A, Pandya-Pathak J, Weir M, Marshall FH are employees of Heptares Therapeutics Ltd, a drug discovery company focused on GPCR NCE and antibody drug discovery. Langmead C is a shareholder of Heptares Therapeutics Ltd.

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