# Identification of a Novel Subtype-Selective $\alpha_{1B}$ -Adrenoceptor Antagonist

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report the discovery of 4-[(2-hydroxyethyl)amino]-6-methyl-2H-chromen-2-one (Cpd1), as an  $\alpha_{1B}$ -AR antagonist that has 10–15fold selectivity over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR. Through computational and site-directed mutagenesis studies, we have identified the binding site of Cpd1 in  $\alpha_{1B}$ -AR and propose the molecular basis of  $\alpha_{1B}$ -AR selectivity, where the nonconserved V197<sup>45.52</sup> residue plays a major role, with contributions from L314<sup>6.55</sup> within the  $\alpha_{1B}$ -AR pocket. By exploring the structure–activity relationships of Cpd1 at  $\alpha_{1B}$ -AR, we have also identified 3-[(cyclohexylamino)methyl]-6-methylquinolin-2(1H)-one (Cpd24), which has a stronger binding affinity than Cpd1, albeit with reduced selectivity for  $\alpha_{1B}$ -AR. Cpd1 and Cpd24 represent potential leads for  $\alpha_{1B}$ -AR-selective drug discovery and novel tool molecules to further study the physiology of  $\alpha_1$ -ARs.

# INTRODUCTION

G protein-coupled receptors (GPCRs) are a large family of transmembrane proteins that are activated by a variety of endogenous stimuli, such as neurotransmitters, ions, and peptides. They regulate numerous physiological processes and constitute highly druggable target proteins.<sup>1</sup> The  $\alpha_1$ -adrenoceptors ( $\alpha_1$ -AR),  $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR, and  $\alpha_{1D}$ -AR are members of the GPCR family that are activated endogenously by the catecholamines adrenaline (epinephrine) and noradrenaline.<sup>2</sup> Historically, the roles of  $\alpha_1$ -ARs in modulating cardiac physiology have been well studied and these receptors are clinically targeted.<sup>3</sup> However, the role of cell-type specific expression of specific  $\alpha_1$ -AR subtypes is unknown, and functional investigation of each subtype is hampered by the lack of validated, fully subtype-selective  $\alpha_1$ -AR antagonists.<sup>2,4–6</sup> Knowledge of the roles of these receptors has largely come from transgenic animal studies or using nonselective ligands.<sup>5,7–11</sup>  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR are believed to mediate opposing roles in many aspects of cardiac physiology, with  $\alpha_{1A}$ -AR stimulation being cardioprotective and chronic  $\alpha_{1B}$ -AR activation detrimental.<sup>10,12</sup>  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR are the major  $\alpha_1$ -ARs expressed in the central nervous system (CNS), with similarly opposing roles:  $\alpha_{1A}$ -AR activation enhances learning, memory, and neurogenesis, whereas prolonged  $\alpha_{1B}$ -AR stimulation or overexpression is neurodegenerative and proapoptotic.  $^{10,13}$  Recent studies using the selective  $\alpha_{1A}$ -AR agonists A-616003 and dabuzalgron have confirmed the cardioprotective effects of  $\alpha_{1A}$ -AR activation, and these compounds are considered clinical candidates to treat heart failure.  $^{14-16}$ 

No selective ligands, however, exist for  $\alpha_{1B}$ -AR.  $\alpha_{1B}$ -AR-selective antagonists have been actively pursued by several groups, resulting in the identification of (+)-cyclazosin,<sup>17,18</sup> conopeptide  $\rho$ -TIA,<sup>19</sup> L-765314,<sup>20</sup> and AH 11110A,<sup>21</sup> each of which reportedly exhibits no more than 10–24-fold selectivity

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**Figure 1.** Validating the  $\alpha_{1B}$ -AR selectivity of Cpd1 in binding and functional assays. (A) Chemical structure of Cpd1. (B) Equilibrium binding of the antagonist QAPB was inhibited by Cpd1 at WT  $\alpha_{1B}$ -AR (white circles) to a 30-fold greater extent than at WT  $\alpha_{1A}$ -AR (black circles) and  $\Delta 1$ –79  $\alpha_{1D}$ -AR (white squares) in COS-7 cells transiently expressing human receptors at 21 °C. (C–F) Phenylephrine (PhE) concentration response curves measuring intracellular Ca<sup>2+</sup> mobilization in the absence (red circles) or presence (blue circles) of 500  $\mu$ M Cpd1 in COS-7 cells stably expressing human WT  $\alpha_{1A}$ -AR (C), WT  $\alpha_{1B}$ -AR (D), WT  $\alpha_{1D}$ -AR (E) or transiently expressing  $\Delta 1$ –79  $\alpha_{1D}$ -AR (F) at 37 °C. Cpd1 was preincubated with the cells for 30 min before addition of PhE. Testing the antagonistic effects of Cpd1 on PhE-induced contraction of rat isolated (G) mesenteric arteries or (H) abdominal aorta. Points represent the mean ± SE of at least three independent experiments performed in duplicate. Refer to Table S1 for values.

for  $\alpha_{1B}$ -AR over other  $\alpha_1$ -adrenoceptor subtypes. A recent study on these reportedly subtype-selective  $\alpha_1$ -adrenoceptor ligands investigated 101 clinical drugs and laboratory compounds but did not identify a single ligand with preference for  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR.<sup>6</sup> There is, therefore, a need to develop new selective  $\alpha_{1B}$ -AR ligands to further characterize this important receptor and its roles in normal physiology and disease.

In the past decade, advances in GPCR structural biology have provided many structures of the  $\beta$ -ARs in both active and inactive states<sup>1,22</sup> and, more recently, structures of  $\alpha_{1A}$ -AR,<sup>23</sup>  $\alpha_{2A}$ -AR,<sup>24,25</sup>  $\alpha_{2B}$ -AR,<sup>26</sup> and  $\alpha_{2C}$ -AR.<sup>27</sup> These studies provide valuable information for understanding subtle structural differences between  $\beta$ - and  $\alpha_2$ -AR subtypes and have enabled structure-based drug discovery (SBDD) of novel selective ligands.<sup>28,29</sup> Recently, we determined the crystal structure of the  $\alpha_{1B}$ -AR bound to the modestly  $\alpha_{1B}$ -AR-selective inverse agonist (+)-cyclazosin,<sup>30</sup> representing an important resource to enable the development of  $\alpha_{1B}$ -AR-selective compounds.

Since researchers have so far failed to identify highly  $\alpha_{1B}$ -ARselective compounds, we have undertaken a study using fragment screening as an alternate avenue for selective hit identification. Fragment screening is a well-validated starting point for SBDD and involves screening small, chemically diverse libraries of low molecular weight (~300 Da) "fragments" for binding to a protein target.<sup>31</sup> Because of their small size, fragments usually bind with weak affinity, which often prevents testing in cell-based GPCR functional assays, instead requiring the use of biophysical methods to measure binding, such as ligand-observed nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR).<sup>32</sup> For these methods, the proteins of interest must be relatively pure and stable in solution over time. Due to the dynamic nature of GPCRs and their instability upon purification,<sup>3</sup> there are a few published examples of GPCR fragment

screening using biophysical studies,<sup>34–38</sup> with most of these requiring engineered receptor mutants.

In the current work, we aimed to use fragment screening methods to identify subtype-selective  $\alpha_1$ -AR ligands with novel chemical scaffolds and clear subtype selectivity. Screening was conducted by NMR against the stabilized  $\alpha_1$ -AR variants,  $\alpha_{1A}$ -AR-A4<sup>39</sup> and  $\alpha_{1B}$ -AR-B1,<sup>40</sup> using a subset of the Vernalis fragment library.<sup>41</sup> One fragment (Cpd1) was identified to preferentially bind to  $\alpha_{1B}$ -AR-B1 over  $\alpha_{1A}$ -AR-A4. A combination of ligand binding, receptor signaling assays, docking, molecular dynamics (MD) simulations, and site-directed mutagenesis studies was used to validate Cpd1 as an  $\alpha_{1B}$ -ARselective antagonist, to identify the Cpd1 binding pocket, and to determine the molecular basis of Cpd1 selectivity for  $\alpha_{1B}$ -AR. Finally, we probed the structure-activity relationship (SAR) of Cpd1 by screening an additional 23 commercially available analogues. The SAR screen identified Cpd24 to be a more potent analogue of Cpd1 with a weaker selectivity for  $\alpha_{1B}$ -AR. Rational design of further analogues, guided by the chemical structures of Cpd1, Cpd24, and the  $\alpha_{1B}$ -AR crystal structure, will help identify optimized ligands that retain the desired subtype selectivity. Such compounds will be important tools to probe the exact physiological roles of specific  $\alpha_1$ -AR subtypes and to examine their potential as targets for treating disease.

## RESULTS

Identification of 4-[(2-Hydroxyethyl)amino]-6-methyl-2H-chromen-2-one (Cpd1) as an  $\alpha_{1B}$ -AR-Selective **Antagonist.** We previously engineered stabilized  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR variants using Cellular High-throughput Encapsulation, Solubilization, and Screening (CHESS) and demonstrated that purified preparations of these receptors can be used to explore small-molecule ligand binding with STD NMR, a mainstay technique used for fragment screening.<sup>39</sup> CHESSstabilized variants of human  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR, named  $\alpha_{1A}$ -AR-A4<sup>39</sup> and  $\alpha_{1B}$ -AR-B1,<sup>40</sup> respectively, were used in this study. When expressed in COS-7 cells,  $\alpha_{1A}$ -ARA-4 and  $\alpha_{1B}$ -AR-B1 exhibit no signaling efficacy in response to agonist stimulation. However, the high stability and ligand-binding competency of  $\alpha_{1A}$ -AR-A4 and  $\alpha_{1B}$ -AR-B1 in solution makes them suitable candidates for NMR- or SPR-based fragment screening. Therefore, as a trial, 56 compounds from the Vernalis fragment  $library^{41}$  were screened against both receptors using NMR (data not shown). 4-[(2-hydroxyethyl)amino]-6-methyl-2H-chromen-2-one (Cpd1) (Figure 1A) was identified as a preliminary hit against both  $\alpha_{1A}$ -AR-A4 and  $\alpha_{1B}$ -AR-B1 (Figure S1A,B). However, using competition STD NMR, Cpd1 was shown to specifically bind and compete with epinephrine at  $\alpha_{1B}$ -AR-B1, but compete to a lesser degree with epinephrine at  $\alpha_{1A}$ -AR-A4 (Figure S1C,D), suggesting Cpd1 is a potential scaffold for the design of novel  $\alpha_{1B}$ -AR-selective ligands.

Since the screening and STD-NMR validation were performed on detergent-solubilized, stabilized receptor preparations, the activity of Cpd1 was validated in cell-based assays with human wild-type (WT)  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR and  $\alpha_{1D}$ -AR. COS-7 cells are fibroblast-like kidney cells from green African monkey and are well-established mammalian cell lines for assaying  $\alpha_1$ -AR activity.<sup>42,43</sup>The binding of Cpd1 to COS-7 cells transiently expressing WT  $\alpha_1$ -ARs was determined in a flow cytometry-based competition binding assay against the fluorescent antagonist QAPB (quinazoline piperazine BODI- PY, also termed BODIPY FL prazosin). Cpd1 partially displaced QAPB at  $\alpha_{1B}$ -AR expressing cells in a concentration-dependent manner with an estimated equilibrium inhibition constant (pK<sub>I</sub>) of 4.76 ± 0.11. In contrast, Cpd1 showed substantially weaker competition against QAPB at  $\alpha_{1A}$ -AR expressing cells (Figure 1B, Table S1). No detectable QAPB binding was observed for WT  $\alpha_{1D}$ -AR, which is known to be poorly expressed at the cell surface. An N-terminally truncated  $\alpha_{1D}$ -AR variant ( $\Delta$ 1–79  $\alpha_{1D}$ -AR), which has been reported to have improved cell-surface expression with minimal effects on ligand pharmacology,<sup>44</sup> was thus used for  $\alpha_{1D}$ -AR ligand-binding studies. Cpd1 could not compete with QAPB at this receptor variant (Figure 1B, Table S1).

All three  $\alpha_1$ -AR subtypes signal primarily through the  $G\alpha_{q/11}$ protein leading to the activation of phospholipase C (PLC), which catalyzes the formation of inositol triphosphate and diacylglycerol (DAG), resulting in the release of Ca<sup>2+</sup> from the endoplasmic reticulum.<sup>45</sup> We used a Ca<sup>2+</sup> mobilization assay as a measure of  $\alpha_1$ -AR activation downstream of  $G\alpha_{q/11}$ activation. COS-7 cells stably expressing either human WT  $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR, or  $\alpha_{1D}$ -AR, or transiently expressing  $\Delta 1$ -79  $\alpha_{1D}$ -AR were tested in this assay. At 500  $\mu$ M, Cpd1 caused a right-ward shift in the phenylephrine dose–response curve at  $\alpha_{1B}$ -AR expressing cells but did not change phenylephrine potency or efficacy at  $\alpha_{1A}$ -AR,  $\alpha_{1D}$ -AR, or  $\Delta 1$ -79  $\alpha_{1D}$ -AR expressing cells (Figure 1C–F).

Phenylephrine-induced  $\alpha_1$ -AR activation results in the accumulation of DAG, which activates protein kinase C (PKC), leading to the activation of the cAMP response element (CRE) binding protein to upregulate CRE-controlled genes.<sup>46–49</sup> The  $\alpha_{1B}$ -AR selectivity of Cpd1 was further confirmed in a CRE reporter assay, where Cpd1 was more potent at inhibiting phenylephrine-induced CRE response at  $\alpha_{1B}$ -AR compared to  $\alpha_{1A}$ -AR (Figure S2A, Table S1).

Since most preclinical drug evaluation efforts are carried out on rodents and considering interspecies variability in ligand pharmacology,<sup>50</sup> it is important to test whether Cpd1 can inhibit rodent  $\alpha_1$ -ARs. Cpd1 was tested on rat WT  $\alpha_1$ -ARs transiently expressed in COS-7 cells. As observed for the human WT receptors, Cpd1 showed selectivity for WT rat  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR (Figure S2B,C, Table S1). The ability of Cpd1 to inhibit phenylephrine-induced contraction of small resistance mesenteric arteries isolated from rats was tested.<sup>51</sup> At least one study has suggested that  $\alpha_{1B}$ -AR is the predominant  $\alpha_1$ -AR subtype expressed in mesenteric arteries and serves to promote vessel constriction upon catecholamine-induced activation.<sup>52</sup> As shown in Figure 1G, Cpd1 exhibited potent dose-dependent inhibition of phenylephrine-induced contraction of the mesenteric arteries. Cpd1 activity was then tested in abdominal aorta isolated from rats, a tissue whose contractions in response to phenylephrine are believed to result from the activation of  $\alpha_{1D}$ -AR.<sup>53</sup> Like the mesenteric artery data, Cpd1 was able to cause large, dose-dependent inhibition of phenylephrine-induced contractions of this tissue (Figure 1H). Given the lack of Cpd1 binding and antagonist activity at WT  $\alpha_{1D}$ -AR and  $\Delta 1-79 \alpha_{1D}$ -AR in COS-7 cells (Figure 1B,E,F), it is likely that rat abdominal aorta contractions are mediated by a combination of  $\alpha_1$ -AR subtypes, with these data suggesting that  $\alpha_{1B}$ -AR contributes significantly.

We examined the activity of Cpd1 to other members of the adrenoceptor family ( $\alpha_2$ -ARs and  $\beta$ -ARs) by performing bioluminescence resonance energy transfer (BRET)-based G protein activation assays.<sup>54,55</sup> The prototypical  $\alpha_2$ -AR agonist,



**Figure 2.** Docking and MD simulation studies of Cpd1 bound to  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR. Alternative views of docked Cpd1 (purple) in homology models of WT  $\alpha_{1A}$ -AR (A & B) and  $\alpha_{1B}$ -AR (C & D). Stability of docked Cpd1 in the MD simulations homology model of WT  $\alpha_{1A}$ -AR (E) and  $\alpha_{1B}$ -AR (F). Cpd1 remained stably bound to  $\alpha_{1B}$ -AR over 400 ns simulations but is not stable in the  $\alpha_{1A}$ -AR. Each simulation was replicated three times.

clonidine, resulted in a dose-dependent activation of the  $G\alpha_{i3}$  protein at WT human  $\alpha_{2A}$ -AR,  $\alpha_{2B}$ -AR, and  $\alpha_{2C}$ -AR (Figure S3A). At 500  $\mu$ M, Cpd1 did not have antagonist activity at  $\alpha_{2A}$ -AR or  $\alpha_{2C}$ -AR but caused a small right-ward shift in the clonidine dose-response curve at  $\alpha_{2B}$ -AR (Figure S3A). At WT human  $\beta$ -ARs, the prototypical  $\beta$ -AR agonist, isoprenaline, resulted in a dose-dependent activation of the  $G\alpha_{s}$  protein (Figure S3B). At 500  $\mu$ M, Cpd1 did not have antagonist activity at  $\alpha_{1}$ -AR,  $\beta_{2}$ -AR, or  $\beta_{3}$ -AR (Figure S3B).

In the mesenteric artery and aorta assays, the right-ward shift in the phenylephrine curve was significantly larger than that observed in the COS-7 cells (Figure 1G,H). While part of the right-ward shift in these assays is likely due to Cpd1 binding to  $\alpha_{1B}$ -AR, Cpd1 could also be inhibiting vasoconstriction by blocking  $\alpha_{2B}$ -AR, contributing to the larger right-ward shift compared to COS-7 cells expressing  $\alpha_{1B}$ -AR only. Indeed, it has been reported that  $\alpha_{2B}$ -AR is responsible for mediating vasoconstriction in some vascular beds.<sup>56</sup> Overall, our results confirm that Cpd1 is an antagonist that preferentially binds to  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR. Among all the other six adrenoceptors, Cpd1 also weakly binds to  $\alpha_{2B}$ -AR.

Ligand Docking and Molecular Dynamics Simulations Define Cpd1 Binding Site and Determinants of  $\alpha_{1B}$ -AR Selectivity. To understand the molecular basis of Cpd1 binding and selectivity for  $\alpha_{1B}$ -AR, ligand docking and MD simulations were performed. Homology models of WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR were made using the (+)-cyclazosin-bound  $\alpha_{1B}$ -AR crystal structure (PDB: 7B6W) as a template.<sup>30</sup> MD simulations were run on the (+)-cyclazosin-bound homology models of WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR, revealing that (+)-cyclazosin remained stably bound to the models over a 400 ns simulation (Figure S4A-D) and therefore the models were suitable for Cpd1 docking. Cpd1 was able to favorably bind in a nearly identical position into the cyclazosin-binding pocket of the homology models of both WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR (Figure 2A-D). The plane of the coumarin moiety in Cpd1 aligns parallel to transmembrane helix 3 of both  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR,

as seen with the quinazoline ring of (+)-cyclazosin bound to  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR<sup>30</sup> (Figure S4C,D). MD simulations were used to establish the stability of this predicted binding pose in both  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR. Cpd1 was stable in the docking site of  $\alpha_{1B}$ -AR during 3 replicate 400 ns simulations, evidenced by the low and consistent ligand root-mean-square deviation (RMSD) of Cpd1 heavy atoms (Figure 2F), similar to (+)-cyclazosin bound to the same receptor (Figure S4B). Conversely, docked Cpd1 was not stable in the  $\alpha_{1A}$ -AR (Figure 2E), whereas the RMSD of (+)-cyclazosin docked in the  $\alpha_{1A}$ -AR (Figure 2E), whereas the RMSD of (+)-cyclazosin docked in the  $\alpha_{1A}$ -AR model remained stable (Figure S4B). The increasing RMSD of Cpd1 over time during the  $\alpha_{1A}$ -AR simulation may indicate less favorable interactions between Cpd1 and  $\alpha_{1A}$ -AR at this proposed binding site compared to  $\alpha_{1B}$ -AR, consistent with our ligand-binding analysis.

We hypothesized that the selectivity of Cpd1 for  $\alpha_{1B}$ -AR is due to amino acid differences between  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR within the proposed binding site of Cpd1. The residues lining the binding pocket of Cpd1 in both receptors differ in extracellular loop 2 (ECL2) and the top of transmembrane domain 6 (TM6) (Figure 2A–D). In our models, the binding surface of Cpd1 in  $\alpha_{1B}$ -AR includes the nonconserved residues valine 197 (V197<sup>45.52</sup>) in ECL2 and leucine 314 (L314<sup>6.55</sup>) in TM6, which correspond to isoleucine (I178<sup>45.52</sup>) and methionine (M292<sup>6.55</sup>) in  $\alpha_{1A}$ -AR (superscripts refer to Ballesteros-Weinstein numbering<sup>57</sup>). We hypothesized that the longer side chains of I178<sup>45.52</sup> and M292<sup>6.55</sup> in  $\alpha_{1A}$ -AR create a narrower pocket that sterically hinders Cpd1 binding (Figure 2B,D). Together, our docking and MD studies revealed a likely Cpd1 binding pocket in  $\alpha_{1B}$ -AR and provided insight into the potential molecular determinants of the observed  $\alpha_{1B}$ -AR selectivity of this compound.

Validation of the Cpd1 Binding Pocket Using Site-Directed Mutagenesis. To confirm the importance of V197<sup>45.52</sup> and L314<sup>6.55</sup> for Cpd1 binding in  $\alpha_{1B}$ -AR and validate our docking and MD observations (Figure 2), we undertook site-directed mutagenesis of the predicted binding



Figure 3. Validation of the Cpd1 binding pocket in  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR by site-directed mutagenesis. (A) Equilibrium binding of QAPB in the presence of increasing concentrations of Cpd1 at WT  $\alpha_{1A}$ -AR or mutated  $\alpha_{1A}$ -AR (I178V, M292L, and I178V, M292L). (B) Equilibrium binding of QAPB in the presence of increasing concentrations of Cpd1 at WT  $\alpha_{1B}$ -AR or mutated  $\alpha_{1B}$ -AR (V197I, L314M, and V197I, L314M). QAPB binding was inhibited by Cpd1 at WT  $\alpha_{1B}$ -AR, with Cpd1 potency weakened by the presence of the mutations. (C & D) Intracellular Ca<sup>2+</sup> mobilization assays were used to test the effects of receptor mutations on Cpd1 inhibition of PhE (phenylephrine) binding. Cells were preincubated with Cpd1 for 30 min before addition of an EC<sub>50</sub> concentration of PhE to cells expressing  $\alpha_{1A}$ -AR variants (C) or  $\alpha_{1B}$ -AR variants (D). Points represent the mean  $\pm$  SE of at least three independent experiments performed in duplicate. Refer to Table S1 for values.

site. V19745.52 and L3146.55 were converted to the corresponding amino acids found in  $\alpha_{1A}$ -AR, generating  $\alpha_{1B}$ -AR (V197I),  $\alpha_{1B}$ -AR (L314M), and the double mutant  $\alpha_{1B}$ -AR (V197I, L314M). The corresponding mutations were also made on  $\alpha_{1A}$ -AR, generating  $\alpha_{1A}$ -AR (I178V),  $\alpha_{1A}$ -AR (M292L), and  $\alpha_{1A}$ -AR (I178V, M292L). Whole-cell QAPB saturation binding experiments showed that all mutated receptors expressed well at the cell surface and their expression levels were approximately double that of the respective WT receptor counterparts (Table S1). The equilibrium dissociation constant of the fluorescent antagonist QAPB  $(pK_D)$  was not significantly altered at any of the  $\alpha_{1A}$ -AR mutants (Table S1). In  $\alpha_{1B}$ -AR, the mutations L314M and V197I had little or no impact on QAPB affinity (Table S1). The equilibrium inhibition constant  $(pK_I)$  of prazosin, determined in competition binding assays against QAPB, was slightly, although significantly, enhanced at  $\alpha_{1A}$ -AR (M292L) and  $\alpha_{1A}$ -AR (I178V, M292L) compared to WT  $\alpha_{1A}$ -AR. Prazosin affinity for the  $\alpha_{1B}$ -AR mutants, however, was unchanged compared to WT  $\alpha_{1B}$ -AR (Table S1). Cpd1 caused the minimal displacement of QAPB at cells expressing each of the  $\alpha_{1A}$ -AR mutants (Figure 3A). Notably, all the  $\alpha_{1B}$ -AR mutants caused a substantial weakening of the affinities of Cpd1 (Figure 3B and Table S1), confirming the importance of the V197<sup>45.52</sup> and L314<sup>6.55</sup> side chains for Cpd1 binding to  $\alpha_{1B}$ -AR.

The antagonistic effects of Cpd1 were then tested on the WT and mutated receptors transiently expressed in COS-7 cells using a Ca<sup>2+</sup> mobilization assay. Before testing Cpd1, a phenylephrine dose–response curve was generated at each of the transiently expressed WT and mutated receptors to determine its  $EC_{50}$  (Figure S5 and Table S1). Similar to the stable cell lines, the potency of phenylephrine remained close

to 10 nM in COS-7 cells transiently expressing either WT  $\alpha_{1A}$ -AR or WT  $\alpha_{1B}$ -AR. The potency and efficacy of phenylephrine remained unchanged at COS-7 cells expressing  $\alpha_{1A}$ -AR (M292L),  $\alpha_{1A}$ -AR (I178V, M292L),  $\alpha_{1B}$ -AR (V197I), or  $\alpha_{1B}$ -AR (V197I, L314M), relative to their WT counterparts. Phenylephrine potency was significantly reduced at  $\alpha_{1A}$ -AR (I178V) and  $\alpha_{1B}$ -AR (L314M) (Figure S5 and Table S1). Cpd1 was then tested for its ability to inhibit phenylephrineinduced Ca<sup>2+</sup> mobilization at each of the WT and mutant receptors (for this assay, a phenylephrine concentration equal to its EC<sub>50</sub> was used). Consistent with the binding data (Figure 3B), the potency of Cpd1 in inhibiting phenylephrine-induced Ca<sup>2+</sup> mobilization was largely weakened at cells expressing either  $\alpha_{1B}$ -AR (V197I),  $\alpha_{1B}$ -AR (L314M), or  $\alpha_{1B}$ -AR (V197I, L314M), compared to WT  $\alpha_{1B}$ -AR (Figure 3D, Table S1), further validating the importance of V197<sup>45.52</sup> and L314<sup>6.55</sup> in Cpd1 binding to  $\alpha_{1B}$ -AR. Conversely, there was no significant change in the potency of Cpd1 in inhibiting phenylephrineinduced Ca<sup>2+</sup> signaling in cells expressing any of the mutant  $\alpha_{1A}$ -ARs compared to WT  $\alpha_{1A}$ -AR (Figure 3C, Table S1). Together, these data support the location Cpd1 binding site in  $\alpha_{1B}$ -AR predicted by the docking and MD study and validate the involvement of the V197<sup>45.52</sup> and L314<sup>6.55</sup> side chains in improving the binding affinity of Cpd1 for  $\alpha_{1B}$ -AR and thus increasing its selectivity over  $\alpha_{1A}$ -AR.

SAR Studies Identify a Higher Affinity Analogue of Cpd1. To further probe the SARs of Cpd1, and with the aim of increasing compound potency and fold- $\alpha_{1B}$ -AR selectivity, we selected a set of 23 commercially available analogues based around the coumarin (2*H*-chromen-2-one, 2*H*-benzopyran-2-one) core of Cpd1 (Cpds 2–24, Tables S2 and S3). Most of these compounds retain the core coumarin scaffold of Cpd1,



**Figure 4.** SAR screen of Cpd1 analogues and Cpd24 binding profile. QAPB equilibrium binding inhibition profile of Cpd1 and structural analogues tested at (A) WT  $\alpha_{1A}$ -AR or (B)  $\alpha_{1B}$ -AR. QAPB equilibrium binding inhibition profile of Cpd24 was tested at (C) WT  $\alpha_{1A}$ -AR and the  $\alpha_{1A}$ -AR mutants (I178V, M292L, and I178V, M292L) or (D) WT  $\alpha_{1B}$ -AR and the  $\alpha_{1B}$ -AR mutants (V197I, L314M, and V197I, L314M). Points represent the mean  $\pm$  SE of at least three independent experiments performed in duplicate. Refer to Tables S1 and S2 for values.

with a range of substitutions at position 4. Cpd23 and Cpd24 have a 6-methylquinolin-2(1H)-one core with substitution at position 3 in Cpd24 (Table S1).

Compounds were screened for their ability to (1) displace QAPB or  $[^{3}H]$ -prazosin at 500  $\mu$ M; (2) inhibit phenylephrineinduced receptor activation at 100  $\mu$ M; and (3) produce a response in a  $Ca^{2+}$  mobilization assay at 500  $\mu M$  in untransfected COS-7 cells or cells expressing either WT  $\alpha_{1A}$ -AR or WT  $\alpha_{1B}$ -AR (Tables S2 and S3). As expected, the prototypical nonselective  $\alpha_1$ -AR agonist phenylephrine and the antagonist phentolamine, fully displaced QAPB at both subtypes (Table S2). Cpd1, 14, 19, 20, 23, and 24 significantly displaced QAPB at both receptor subtypes, with Cpd24 causing full displacement (Table S2). Notably, Cpd19, 20, and 24 were able to displace QAPB more effectively than Cpd1 at  $\alpha_{1B}$ -AR expressing cells, suggesting higher affinity than Cpd1 (Table S2). Next, the ability of the compounds to act as antagonists in a functional assay was tested. Preincubating cells expressing either  $\alpha_{1A}$ -AR or  $\alpha_{1B}$ -AR with 100  $\mu$ M phentolamine or Cpd24 fully inhibited the Ca<sup>2+</sup> mobilization response elicited by 10 nM phenylephrine at both receptors (Table S2). At 100 µM, Cpd1, 7, 12, 17, 19, and 21 significantly inhibited phenylephrine-induced Ca<sup>2+</sup> signaling only at cells expressing  $\alpha_{1B}$ -AR, while Cpd10, 11, and 20 caused significant inhibition of phenylephrine-induced Ca<sup>2+</sup> signaling both in cells expressing either  $\alpha_{1A}$ -AR or  $\alpha_{1B}$ -ARs (Tables S2 and S3). None of the compounds screened caused significant  $\alpha_{1A}$ -ARselective inhibition of phenylephrine-induced Ca<sup>2+</sup> signaling at cells expressing  $\alpha_{1A}$ -AR over those expressing  $\alpha_{1B}$ -AR.

The SAR series of compounds were subsequently tested for their ability to induce an agonist response in cells expressing either WT  $\alpha_{1A}$ -AR or WT  $\alpha_{1B}$ -AR. Nontransfected COS-7 cells were used as a negative control. At 500  $\mu$ M, Cpd9, 12, and 17 produced nonreceptor-specific Ca<sup>2+</sup> mobilization responses in untransfected COS-7 cells (Table S4). Cpd24 also produced Ca<sup>2+</sup> mobilization responses in untransfected COS-7 cells, however, this was due to the higher concentration of dimethyl sulfoxide (DMSO, 5%) present in this sample, rather than nonreceptor-specific agonist activity. A similar calcium signal was observed when 5% DMSO alone was added to untransfected COS-7 cells (18.47 ± 1.05). Furthermore, Cpd13, 14, and 21 produced significant, although weak, agonist activity in cells expressing either  $\alpha_{1A}$ -AR or  $\alpha_{1B}$ -AR, while the remaining compounds did not have any agonist activity (Tables S2 and S3).

Based on the results of the initial, single-concentration screen, full competition dose-response curves were obtained against QAPB for Cpd14, 19, 20, 23, and 24 at cells expressing either WT  $\alpha_{1A}$ -AR or WT  $\alpha_{1B}$ -AR, to derive p $K_1$  values (Figure 4A,B). Relative to Cpd1, only Cpd24 showed significant improvement in affinity (~100-fold higher than Cpd1) toward both  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR, while the affinities of Cpd14 and Cpd23 were significantly reduced for  $\alpha_{1B}$ -AR. Despite the affinity improvement of Cpd24, its preference for  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR was smaller than Cpd1 (Figure 4 and Table S2).

The binding and functional data obtained for this SAR series of compounds indicate that the methyl group at position 6 of Cpd1 is important for its affinity and potency at  $\alpha_{1B}$ -AR. When this methyl group is removed (Cpd2), the compound's binding to inhibit the phenylephrine response is significantly attenuated at  $\alpha_{1B}$ -AR (Table S3). The importance of this 6methyl substituent of Cpd1 is also suggested in Cpds 3–5, where substitution at position 4 is similar to Cpd1, but the methyl at position 6 is replaced with a hydrogen: Cpds 3–5 did not significantly attenuate phenylephrine-induced Ca<sup>2+</sup> mobilization at either  $\alpha_{1A}$ -AR or  $\alpha_{1B}$ -AR. Replacement of the methyl group at position 6 with a hydroxyl group seems also not well tolerated, as in Cpd6 (Table S3). In Cpd7, where the moiety at position 4 is changed to a piperidine ring while the methyl group at position 6 remains compared to Cpd1, the functional  $\alpha_{1B}$ -AR antagonism is present, although it appears weaker than Cpd1 (Table S3). When an imidazole group is introduced to the coumarin side chain at position 4, while leaving the methyl group at position 6 (Cpd14), the  $\alpha_{1B}$ -AR antagonist activity of Cpd1 is lost and, instead, Cpd14 has weak agonist activity at both  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR (Table S2). The agonist activity at both receptors is lost when the methyl group of Cpd14 is replaced with a hydroxyl group at position 6, as seen in Cpd15–16 (Table S3). Off-target agonist activity is observed (in cells not transfected with  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR) when the methyl group in position 6 is replaced with an ethyl group as in Cpd17 (Tables S3 and S2). The presence of a methyl group at position 7 (Cpds 16 18, 19, and 20) may lead to the loss of the agonist activity seen in Cpd14 while having an additional methyl group at position 5 (Cpd19) seems to improve  $\alpha_{1B}$ -AR selectivity as an antagonist (Figure 4B and Tables S2 and S3). Interestingly, adding a 5-membered ring to form an Indane (Cpd21) results in weak agonist activity in cells expressing either  $\alpha_{1A}$ -AR or  $\alpha_{1B}$ -AR. Cpd21 also seems to have antagonist activity at  $\alpha_{1B}$ -AR (Table S3). The observed antagonist activity of Cpd21 may be due to the depletion of intracellular calcium stores or receptor desensitization, as the cells are preincubated with ligands before the addition of phenylephrine during the experiment. In this case, the cells were preincubated with an agonist (Cpd21) before the addition of another (phenylephrine), making interpretation of this result difficult. An amino furan substitution at position 4 (Cpd22) resulted in no detectable binding or Ca<sup>2+</sup> response at  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR. Cpds8–10 contain aminobenzene substitutions at position 4, which is extended by 1 and 2 carbons in Cpds11 and Cpds12, respectively. The aminobenzene group substitution alone (Cpd8) leads to weak but not significant antagonist activity at  $\alpha_{1B}$ -AR and this seems to be rescued by the addition of a hydroxyl group at C3 of the benzene ring (Cpd10) or extending the chain between the amine group and the benzene ring by one or two carbons as seen for Cpds11–12 (Table S3). Interestingly, the removal of the coumarin oxygen in Cpd1 and replacing it with a nitrogen (Cpd23) results in a very weak affinity ligand that loses most of its antagonist function and  $\alpha_{1B}$ -AR selectivity (Figure 4 and Table S2). However, introducing a cyclic moiety at position 3 of Cpd23 leads to Cpd24, a higher affinity ligand than Cpd1 with a preference for  $\alpha_{1B}$ -AR that is smaller than Cpd1. This highlights the importance of the coumarin scaffold for the  $\alpha_{1B}$ -AR selectivity observed with Cpd1.

The SAR study has identified Cpd19 as having similar affinity, antagonist potency, and  $\alpha_{1B}$ -AR selectivity profile to Cpd1. Cpd20 displayed a similar profile to Cpd19 albeit with weaker antagonist potency at  $\alpha_{1B}$ -AR (Figure 4A,B, Table S2). The study also identified that when the only change to Cpd1 is at position 4 to an imidazole aromatic ring, an  $\alpha_{1A}$ -AR agonist is the result (Cpd14), while Cpd19–20, which also contain the imidazole ring at position 4 but have additional methyl groups at position 5 (Cpd19) or 7 (Cpd20) result in antagonists. Therefore, subtle changes at positions 5 and 7 of the coumarin cause the shift from antagonist to agonist. The coumarin core scaffold appears to be important for  $\alpha_{1B}$ -AR selectivity, while having a cyclohexyl amino moiety at position 3 may confer higher affinity to the ligands as in Cpd24.

Characterization of Cpd24. To further understand the molecular determinants of the improved affinity of Cpd24, we investigated its binding profile at  $\alpha_{1A}$ -AR (I178V),  $\alpha_{1A}$ -AR (M292L), and  $\alpha_{1A}$ -AR (I178V, M292L), as well as at  $\alpha_{1B}$ -AR (V197I),  $\alpha_{1B}$ -AR (L314M), and  $\alpha_{1B}$ -AR (V197I, L314M) that belong to the Cpd1 binding site (Figure 3, Table S1). In competition binding assays against QAPB, Cpd24 had a similar binding profile to Cpd1 in each of the mutants (Figure 4C,D). The  $\alpha_{1A}$ -AR mutants had only subtle effects on Cpd24 binding affinity, with M292L being the only mutant with a significantly reduced affinity (Figure 4C, Table S1). Similar to results seen for Cpd1, the  $\alpha_{1B}$ -AR mutants had more pronounced effects on Cpd24 binding with V197I and the double mutant (V197I, L314M) having statistically significant reduced affinity (Figure 4D, Table S1). In docking and MD simulation studies with Cpd24, the quinolinone plane of Cpd24 binds parallel to TM3 in the  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR homology models. In both receptors, the cyclohexyl ring of Cpd24 projects upward and forms hydrophobic interactions with the aromatic side chain of  $W^{3.28}$  (W102<sup>3.28</sup> in  $\alpha_{1A}$ -AR and W121<sup>3.28</sup> in  $\alpha_{1B}$ -AR) at the top of TM3 (Figure 5). The cyclohexyl moiety is similarly positioned as the cis-decahydroquinoxaline ring of (+)-cyclazosin and is predicted to share similar receptor interactions (Figure S4C,D). These additional hydrophobic interactions may be driving the higher affinity of Cpd24 for  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR compared to Cpd1 (Figure 2). MD simulations validated the stability of the predicted binding pose of Cpd24 in  $\alpha_{1B}$ -AR (Figure 5C). Two out of three 400 ns simulations of Cpd24 showed that it remained stable in the binding site of  $\alpha_{1B}$ -AR, evidenced by the low and consistent ligand RMSD plotted over the duration of the simulations (Figure 5C), which closely resembled the RMSD of (+)-cyclazosin bound to the  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR (Figure S4B).

#### DISCUSSION

Emerging evidence implicates the importance of  $\alpha_1$ -ARs in the CNS, the cardiovascular, and the immune systems<sup>58,59</sup> and places them as potential therapeutic targets to treat various disorders. Subtype-selective  $\alpha_1$ -ARs ligands will be essential to study and fully exploit these receptors as therapeutic targets. Despite decades of research, ligands highly selective for  $\alpha_{1B}$ -AR are still lacking, and the physiological and pathophysiological roles of this receptor subtype remain incompletely understood.<sup>6,59</sup> While  $\alpha_{1B}$ -AR is nonselectively targeted (along with  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR) in cardiovascular and genitourinary diseases, its role in other important organs where it is much more abundantly expressed (such as the brain, liver, ovaries, spleen, and kidney<sup>60</sup>) remains to be explored. The discovery of a tool antagonist with greater than 10-fold selectivity for  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR would allow researchers to more confidently assign particular physiological actions of catecholamines to  $\alpha_{1B}$ -AR stimulation specifically. This, in turn, may reveal a role for selective  $\alpha_{\rm 1B}\text{-}{\rm AR}$  modulation as a means to treat disease. It is important to note that while  $\alpha_{1B}$ -ARpreferring antagonists already exist (see Introduction section) they are not sufficiently selective and new chemical scaffolds are needed for selective tool molecule development, which is what the present study aimed to address.

In this study, we have identified Cpd1, a novel smallmolecule antagonist with modest (10–15-fold) selectivity for  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR. The pharmacology of Cpd1 has been characterized using multiple approaches including fluorescent and radioligand-binding assays, intracellular signal-



**Figure 5.** Cpd24 docking and MD simulation studies on  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR. Docked structure of Cpd24 (green) in the homology models of (A) WT  $\alpha_{1A}$ -AR and (B) WT  $\alpha_{1B}$ -AR. (C) MD simulations were run on the Cpd24-bound  $\alpha_{1B}$ -AR homology model, revealing that Cpd24 remained stably bound in the  $\alpha_{1B}$ -AR in two out of three replicate 400 ns simulations.

ing assays (in vitro and ex vivo), ligand docking/MD simulations, and site-directed mutagenesis studies. We have also explored the SAR surrounding Cpd1. Collectively, the assays confirm the preferential binding of Cpd1 to  $\alpha_{1B}$ -AR over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR. Our computational studies suggest that in  $\alpha_{1B}$ -AR, Cpd1 makes contact with the partially nonconserved residues V197<sup>45.52</sup> in ECL2 and L314<sup>6.55</sup> in TM6 (Figures 2 and 3). In the crystal structure of  $\alpha_{1B}$ -AR bound to (+)-cyclazosin, both V197<sup>45.52</sup> and L314<sup>6.55</sup> constitute part of the binding pocket, with L314<sup>6.55</sup> forming hydrophobic interactions with the dimethoxyquinazoline moiety while V197<sup>45.52</sup> interacts with the furan ring (Figure S4).<sup>30</sup> In  $\alpha_{1B}$ -AR, Cpd1 occupies the orthosteric ligand-binding pocket in a similar way as (+)-cyclazosin, albeit making fewer interactions due to its smaller size.

We propose that the  $\alpha_{1B}$ -AR selectivity of Cpd1 is likely due to amino acid differences in the binding pocket of Cpd1 in  $\alpha_{1B}$ -AR versus  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR. Our modeling studies suggest that the longer side chains of corresponding residues in  $\alpha_{1A}$ -AR (I178<sup>45.52</sup> and M292<sup>6.55</sup>) sterically hinder Cpd1 binding as opposed to the relatively shorter side chain of V19745.52 and  $L_{314}^{6.55}$  in  $\alpha_{1B}$ -AR (Figure 2B,D). Indeed, ligand-binding pocket shapes and/or volumes have been shown to influence ligand affinity in both  $\beta_1$ -AR<sup>61</sup> and  $\alpha_{2A}$ -AR.<sup>24</sup> Mutagenesis of V197<sup>45.52</sup> and L314<sup>6.55</sup> in  $\alpha_{1B}$ -AR to the corresponding residues in  $\alpha_{1A}$ -AR (V197I and L314M) reduced the potency of Cpd1 at these mutants (Figure 3 and Table S1) which supports our binding model. However, our hypothesis was not supported by the mutagenesis of  $\alpha_{1A}$ -AR residues I178<sup>45.52</sup> and M292<sup>6.55</sup> to the corresponding  $\alpha_{1B}$ -AR residues (I178V and M292L) such that we could not improve Cpd1 binding (Figure 3 and Table S1). Given the low binding affinity of Cpd1, especially at  $\alpha_{1A}$ -AR, it is likely that I178V and M292L are not sufficient to allow stable binding of Cpd1. Other factors that may contribute to the selectivity of Cpd1 include the path Cpd1 may take into the binding pocket<sup>62,63</sup> or the involvement of highly flexible residues adjacent to V19745.52 in ECL2, which have been previously identified to contribute toward  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR selectivity.<sup>64</sup> Given the enormous difficulty in achieving drug selectivity between closely related GPCR subtypes,<sup>65</sup> and the lack of highly subtype-selective ligands for  $\alpha_{1B}$ -AR,<sup>4,6</sup> we explored the SAR space of Cpd1. The fragment-like size of this molecule allows for significant diversification,<sup>32</sup> potentially allowing the development of novel higher affinity  $\alpha_{1B}$ -AR-selective ligands.

Our identification of Cpd1 raises the prospect that the coumarin core structure may enable the development of new compounds in this class. Coumarins are "privileged structures" that possess a variety of pharmacological activities depending on their substitution pattern.<sup>66</sup> Coumarin derivatives with antitumor,<sup>67-69</sup> anticoagulant,<sup>70</sup> and antiviral<sup>71</sup> effects have been described. Other coumarin derivatives have pharmacological activity at a range of GPCRs including niacin, cannabinoid, histamine, 5-HT receptors and GPR55.72-Zhou et al. describe the replacement of the piperazinylquinazoline moiety of prazosin with various coumarinpiprazine rings to generate novel  $\alpha_1$ -AR antagonists.<sup>76</sup> However, these antagonists were only tested on the  $\alpha_{1A}$ -AR subtype and some had comparable or weaker  $pK_I$  values to prazosin. Other groups have also described coumarinpiperazine derivatives as serotonin and dopamine receptor binding ligands,<sup>77</sup> and to a lesser extent  $\alpha_1$ -AR ligands,<sup>78,79</sup> again focusing only on the  $\alpha_{1A}$ -AR subtype. Furthermore, a recent study showed that 7-hydroxycoumarin induces vasorelaxation in hypertensive rats and diminishes rat mesenteric arteries' responsiveness to  $\alpha_1$ -AR agonists challenge.<sup>80</sup> Thus, the potential of the coumarin scaffold for developing novel, selective  $\alpha_1$ -adrenoceptor ligands has precedent.

Testing of additional compounds containing the coumarin scaffold (Cpd2–22) did not identify any with significantly improved affinity and/or  $\alpha_{1B}$ -AR selectivity over Cpd1 (Figure 4A,B, Tables S2 and S3). However, Cpd24, with the structurally similar 6-quinolin-2(1*H*)-one core, had improved affinity toward both  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR and maintained a slight preference for  $\alpha_{1B}$ -AR. Our docking studies found that the cyclohexyl ring of Cpd24 forms hydrophobic interactions with the aromatic side chain of W121<sup>3.28</sup> in  $\alpha_{1B}$ -AR at the top of TM3, likely driving the higher affinity of Cpd24 in a similar way to the cyclohexyl ring of the decahydroquinoxaline moiety cyclazosin (Figure 5).<sup>30</sup> L314<sup>6.55</sup> is positioned deeply at the bottom of the orthosteric ligand-binding pocket in the  $\alpha_{1B}$ -AR crystal structure (Figure S4),<sup>30</sup> contributing to a hydrophobic patch that makes contact with the dimethoxyquinazoline moiety of inverse agonists such as cyclazosin and QAPB. While the involvement of L314<sup>6.55</sup> in ligand binding is confirmed for QAPB in the current study (Table S1), the presence of Leu at position 314 of  $\alpha_{1B}$ -AR appears to be less important for the binding of Cpd1 and Cpd24, as evident by a weaker reduction in binding affinities at the L314<sup>6.55</sup>M mutation compared with V197<sup>45.52</sup>I (Figures 3-4 and Table S1). On the other hand, M292<sup>6.55</sup>L in  $\alpha_{1A}$ -AR significantly affects Cpd24 binding (Figure 4C, Table S1). Although not evident in our models, this suggests that Cpd1 and Cpd24 may bind to a more superficial area within the orthosteric ligand-binding pocket in  $\alpha_{1B}$ -AR and make more intimate interactions with V197<sup>45.52</sup>. Indeed, the interaction of Cpd1 and Cpd24 with V197<sup>45.52</sup> may be the reason for the preferred binding of these compounds to the  $\alpha_{1B}$ -AR subtype, as the equivalent residues in both  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR are isoleucine rather than value. This residue (45.52) has been suggested to play a role in determining ligand subtype selectivity.<sup>27,30,64</sup> Zhao et al. also identified adjacent residues in ECL2 (G196<sup>45.51</sup> and T198) to play a role in antagonist selectivity at  $\alpha_{1B}$ -AR.<sup>64</sup> Furthermore, residue 6.55 (L314<sup>6.55</sup> in  $\alpha_{1B}$ -AR) has previously been identified to be determinant in  $\alpha_{1A}$ -AR subtype-selective agonist binding<sup>81</sup> and a contributor to  $\alpha_1$ -AR over  $\alpha_2$ -AR selectivity of piperazinylquinazoline compounds in  $\alpha_{1B}$ -AR.<sup>30</sup> In  $\alpha_{2A}$ -AR,  $\alpha_{2B}$ -AR, and  $\alpha_{1B}$ -AR, residue 6.55 has been identified as a key nonconserved residue that belongs to the ligand-binding pocket and controls adrenergic receptor diversity.<sup>2</sup>

When considering the data on rat isolated abdominal aorta, a tissue traditionally believed to highly express the  $\alpha_{1D}$ -AR subtype, Cpd1 potently inhibits phenylephrine-induced contractions (Figure 1H) despite showing no binding or antagonist activity in assays using COS-7 heterologously expressing  $\alpha_{1D}$ -AR (Figure 1B,E,F). This is in line with previous reports showing the contractions in rat aorta are attributable to more than one subtype of adrenoceptor.<sup>82,83</sup> This affirms the need to develop subtype-selective tools to clear misconceptions regarding the expression and functional significance of specific receptor subtypes in various tissues. Much of the older studies in the  $\alpha_1$ -AR field relied on nonspecific ligands, global gene knockout mice, or antibodies to assign  $\alpha_1$ -AR subtypes to specific tissues, resulting in a myriad of contradictory reports. Overall, this study has identified Cpd1 as an  $\alpha_{1B}$ -AR antagonist with 10–15-fold selectivity over  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR and revealed the molecular basis of  $\alpha_{1B}$ -AR selectivity, where the nonconserved V197<sup>45.52</sup> residue plays a major role and L314<sup>6.55</sup> is also possibly involved. Such a tool compound can be optimized and used to probe the exact physiological roles of specific  $\alpha_1$ -AR subtypes and to examine their potential as targets for treating disease.

#### METHODS

**Reagents.** Human  $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR,  $\alpha_{1D}$ -AR, and  $\Delta 1-79 \alpha_{1D}$ -AR mammalian expression vectors pCSC-receptor-strep-IRES-mCherry were purchased from GenScript. Prazosin, phenylephrine, phentolamine, isoprenaline, clonidine, and probenecid were purchased from Sigma-Aldrich. BODIPY FL prazosin (QAPB), Fluo-4 AM, and cell culture reagents were purchased from ThermoFisher Scientific. [<sup>3</sup>H]-Prazosin and Ultima Gold liquid scintillation vials were purchased

from PerkinElmer. Cpd1 and its analogues Cpds2–22 and Cpd24 were purchased from Vitas-M Laboratories and ChemSpace.Hit2Lead. Cpd23 was custom synthesized by Synthesis MedChem. Nano-Glo Luciferase Assay Substrate was purchased from Promega.

STD NMR. Stabilized human  $\alpha_{1A}$ -AR-A4 and  $\alpha_{1B}$ -AR-B1 were expressed and purified from *Escherichia coli* as previously described.<sup>30,39,40</sup> The fusion protein-cleaved  $\alpha_{1A}$ -AR-A4 samples were prepared containing 5  $\mu$ M receptor (for STD experiment) or 10  $\mu$ M (for STD competition experiments) in 500  $\mu$ L of phosphate buffer (50 mM potassium phosphate, 100 mM NaCl, pH 7.4) containing 0.01% Lauryl Maltose Neopentyl Glycol (LMNG), 20% deuterated glycerol, and 10% <sup>2</sup>H<sub>2</sub>O in 5 mm NMR tubes. The cleaved  $\alpha_{1B}$ -AR-B1 samples were prepared containing 5  $\mu$ M receptor (for STD experiment) or 20  $\mu$ M (for STD competition experiments) in 500  $\mu$ L of phosphate buffer (50 mM potassium phosphate, 100 mM NaCl, pH 7.4) containing 0.05% n-dodecyl- $\beta$ -d-maltopyranoside, 20% deuterated glycerol, and 10% <sup>2</sup>H<sub>2</sub>O in 5 mm NMR tubes. To avoid oxidation, all samples containing epinephrine were supplemented with 1 mM ascorbic acid. For STD-NMR experiments, samples contained 500  $\mu$ M Cpd1, and for the STD-NMR competition experiments 500  $\mu$ M epinephrine was used, with and without the addition of 600  $\mu$ M Cpd1 ( $\alpha_{1A}$ -AR-A4) or 100  $\mu$ M Cpd1 (for  $\alpha_{1B}$ -AR-B1). All NMR spectra were acquired at 25 °C on a 700 MHz Bruker Avance IIIHD spectrometer equipped with a cryogenically cooled triple resonance probe. STD-NMR data were acquired with a saturation time of 3 s, using a train of 50 ms Gaussian pulses with a B1 field of 130 Hz, separated by 4 ms delays.<sup>84</sup> The on- and off-resonance frequencies were -1 and 71.4 ppm, respectively. To suppress residual protein and water signals, a spin-lock pulse of 40 ms and excitation sculpting<sup>8</sup> with gradients were employed, respectively. The relaxation delay between transients was set to 3.5 s. A total of 512 transients were averaged over 32,000 data points and a spectral width of 16 ppm. Prior to Fourier transformation, data were multiplied by an exponential function with 2 Hz line-broadening and zero-filled once. Data were analyzed with TopSpin 3.4 and MestReNova 10.0.2.

Flow Cytometry-Based Binding Assays. COS-7 cells grown in 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and Dulbecco's modified Eagles medium (DMEM) were seeded at 300,000 cells/well in a 6-well plate. The next day, the cells were transiently transfected in the 6-well plate with 5  $\mu$ g of receptor-IRESmCherry DNA constructs per well, using Lipofectamine 2000 transfection reagent as per the manufacturer's instructions. After 48 h, the cells were resuspended in 2 mL phenol red-free DMEM at 20  $^{\circ}$ C, and 50  $\mu$ L of the cell suspension was added to each well of a clear v-bottom 96-well plate. For saturation ligand-binding assays, a further 50  $\mu$ L of phenol red-free DMEM containing varying concentrations (0, 3.125, 6.25, 12.5, 25, 50 nM) of QAPB was added to relevant wells. For competition ligand-binding assays, 12.5 nM of QAPB with or without varying concentrations of competing ligands was added to relevant wells. Total binding was defined by the wells containing QAPB only, whereas nonspecific binding was defined by wells containing QAPB and 100  $\mu$ M phentolamine. The cells were incubated with ligands for 1 h at 21 °C prior to detection of bound QAPB to mCherry-positive cells with flow cytometry using a Cytoflex LX cell analyzer (Beckman Coulter). Flow cytometry data were then analyzed in FlowJo to obtain QAPB mean fluorescence intensity (MFI) values from mCherry-positive cells. For each well, at least 5000 cells were used for data analysis. Data were then plotted in Graphpad Prism. Data represent the mean  $\pm$  SEM of three independent biological replicate experiments each performed in duplicate technical measurements.

Whole-Cell [<sup>3</sup>H]-Prazosin Binding Assays. Competition ligandbinding assays using [<sup>3</sup>H]-prazosin were performed on COS-7 cells stably expressing WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR. Cells were seeded at 17,000 cells per well into 96-well culture plates and allowed to grow overnight at 37 °C, 5% CO<sub>2</sub>. Media were aspirated and the COS-7 cells were washed with 200  $\mu$ L of PBS before radioligand binding. Cells were incubated with 100  $\mu$ L of HEM buffer (20 mM HEPES, 1.4 mM EGTA, 12.5 mM MgCl<sub>2</sub>, 150 mM NaCl, pH 7.4) containing 2 nM [<sup>3</sup>H]-prazosin with or without test ligand in triplicate for 1 h at RT. Nonspecific binding was determined in the presence of 1 mM phentolamine. Cells were then quickly washed twice with 200  $\mu$ L of cold PBS and lysed with 50  $\mu$ L of 0.2 M NaOH for 30 min. The lysed cells in each well were then transferred to individual 6 mL plastic scintillation vials, and a total of 5 mL Ultima Gold liquid scintillation cocktail (PerkinElmer) was added to each vial and incubated for 30 min before measurement on a TriCarb  $\beta$ -Counter (PerkinElmer) for 3 min per vial. Data represent the mean ± SEM of three independent experiments performed in technical triplicates. Statistical analysis was performed using one-way ANOVA with statistical significance valued at P < 0.05.

Ca<sup>2+</sup> Mobilization Assays. Intracellular Ca<sup>2+</sup> mobilization assays were performed on COS-7 cells either stably or transiently expressing  $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR,  $\alpha_{1D}$ -AR, or  $\Delta 1-79 \alpha_{1D}$ -AR. For stable cell lines, cells were seeded at 35,000 cells per well into 96-well culture plates and allowed to grow overnight at 37 °C, 5% CO<sub>2</sub>. For transient cell lines, cells were seeded in 6-well plates at 300,000 cells per well and allowed to grow overnight at 37 °C, 5% CO<sub>2</sub>. The next day, the cells were transfected with receptor DNA (5  $\mu$ g per well) using Lipofectamine 2000 transfection reagent as per the manufacturer's instructions. After 24 h, the cells were seeded at 35,000 cells per well into 96-well culture plates and allowed to grow overnight at 37 °C, 5% CO<sub>2</sub>. On the day of the experiment, cells were washed twice with Ca<sup>2+</sup> assay buffer (150 mM NaCl, 2.6 mM KCl, 1.2 mM MgCl<sub>2</sub>, 10 mM D-glucose, 10 mM HEPES, 2.2 mM CaCl<sub>2</sub>, 0.5% (w/v) BSA, and 4 mM probenecid, pH 7.4) and then incubated in  $Ca^{2+}$  assay buffer containing 1 mM Fluo-4-AM for 1 h in the dark at 37 °C and 5% CO2. Extracellular fluo-4-AM was then removed by washing the cells twice with 100  $\mu$ L of Ca<sup>2-</sup> assay buffer. Fluorescence was measured for 1.5 min after the addition of 10× concentrated phenylephrine or test compounds in a Flexstation plate reader (PerkinElmer) using an excitation wavelength of 485 nm, emission wavelength of 520 nm, and a final volume of 200  $\mu$ L per well. To test the antagonist activity of the compounds, they were incubated with the cells for 30 min before stimulation with the agonist phenylephrine. All data are initially normalized to the peak response elicited by 3  $\mu$ M ionomycin. When measuring the inhibitory effects of compounds on the phenylephrine response, data are further normalized to the response elicited by an EC50 concentration of phenylephrine. The phenylephrine  $E_{\rm max}$  is  ${\sim}80\%$  in cells stably expressing WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR and ~25% in cells stably expressing WT  $\alpha_{1D}$ -AR relative to the response elicited by 3  $\mu$ M ionomycin. Transient expression of WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR and  $\Delta 1$ -79  $\alpha_{1D}$ -AR receptors results in a phenylephrine  $E_{\text{max}}$  of ~25 and ~10% for WT  $\alpha_{1D}$ -AR (Figures 1C-E and S5). Data represent the mean  $\pm$ SEM of three independent experiments performed in technical duplicates. Statistical analysis was performed using one-way ANOVA with statistical significance valued at P < 0.05.

cAMP Response Element (CRE) Reporter Assay. COS-7 cells stably expressing either  $\alpha_{\rm 1A}\text{-}{\rm AR}$  or  $\alpha_{\rm 1B}\text{-}{\rm AR}$  were plated at 25,000 cells/ well in a 96-well CellBind plate (Corning) and transfected the next day with plasmid encoding pCRE  $\beta$ -gal as a CRE-controlled reporter of cAMP activity.<sup>86</sup> 24 h post-transfection, the medium was removed, and increasing concentrations of phenylephrine or 5  $\mu$ M forskolin, made up in DMEM, were added to the respective wells. The stimulation was carried out for 6 h at 37 °C, 5% CO<sub>2</sub>, after which the medium was aspirated and the plates were kept frozen at -80 °C until further analysis. Development of the plates required the plates and assay buffers to be first thawed to room temperature. 25  $\mu$ L of assay buffer 1 (100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 2 mM MgSO<sub>4</sub>, and 0.1 mM  $MnCl_2$ ) was added into each well and shaken at room temperature for 10 min before adding 100  $\mu$ L of assay buffer 2 (identical to buffer 1 with the addition of 0.5% Triton X-100 and 40 mM  $\beta$ mercaptoethanol) into each well and further shaken for 10 min at room temperature. 25  $\mu$ L of substrate for  $\beta$ -gal, chlorophenol red  $\beta$ -Dgalactopyranoside (Roche Applied Science), was added into each well with shaking until a color change was observed. The plate was then read and the absorbance was measured at 570 nm on a Bio-Rad plate reader. Phenylephrine  $EC_{50}$  values were initially determined to be 200 nM for  $\alpha_{1A}$ -AR and 800 nM for  $\alpha_{1B}$ -AR. To assess the antagonist activity of Cpd1, varying concentrations (ranging from 100 nM to 500

 $\mu$ M) were tested for inhibiting the response elicited by EC<sub>50</sub> concentrations of phenylephrine (Figure S2) and assayed as described above. Data were normalized to the response induced by 5  $\mu$ M forskolin (100%) and DMEM medium (0%). Data were then further normalized to the response elicited by the EC<sub>50</sub> concentration of phenylephrine. Data represent the mean ± SEM of four independent experiments conducted in triplicates.

BRET-Based G Protein Activation Assays. COS-7 cells were plated in a six-well plate at 300,000 cells/well in complete DMEM. Cells were transfected 24 h later. For  $\alpha_2$ -ARs, cells were transfected with 750 ng/well of receptor DNA and 250 ng/well of Gai3 BRET biosensor DNA. For  $\beta$ -ARs, cells were transfected with 2  $\mu$ g/well of receptor DNA and 2  $\mu$ g/well of G $\alpha$ s BRET biosensor DNA.<sup>55</sup> 24 h later, cells were washed using phosphate-buffered saline (PBS) and resuspended in phenol red-free DMEM supplemented with 10% (v/ v) FBS, 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, and 25 mM HEPES (complete PRF-DMEM). Cells were plated in white opaque 96-well microplates (PerkinElmer) at 40,000 cells/well in 100  $\mu$ L. 24 h later, PRF-DMEM was aspirated and replaced with 80  $\mu$ L of assay solution (ratio of 1 µL Nano-Glo Luciferase Assay Substrate (Promega): 450  $\mu$ L PRF-DMEM for a 1.11× assay solution). Plates were equilibrated at 37 °C for 10-15 min in a PHERAstar FSX microplate reader (BMG LABTECH) (LUM 535 450 optics module). 10 µL of either 5% DMSO or 5 mM Cpd1 (made up in 1× assay solution with a ratio of 1  $\mu$ L Nano-Glo Luciferase Assay Substrate (Promega): 500  $\mu$ L PRF-DMEM) was then added to the cells and incubated for 20 min at 37 °C. After restarting the reader for ~3 min, the reader was paused, the plate ejected, and 10  $\mu$ L of 10× clonidine or isoprenaline dilutions or vehicle manually dispensed before reads allowed to continue for 1 h. All assay solutions used for  $\beta$ -ARs also contained 0.1% ascorbic acid to prevent degradation of isoprenaline. The BRET ratio was defined as the ratio of the acceptor emission (530-535 nm) over the donor emission (450-480 nm). Net BRET ratios were calculated as the difference in the ratio of ligand-treated and vehicle-treated conditions. The area under the BRET curve was calculated to generate agonist dose-response curves, and data were normalized to the maximum signal observed for each agonist.

Functional Assays in Rat Isolated Arteries. Male or female Sprague-Dawley rats (250-350 g) were obtained from the Biomedical Animal Facility, University of Melbourne, Victoria, Australia. All animals were group-housed in a climate-controlled facility  $(21 \pm 1 \ ^{\circ}C)$  with a 12 h dark/light cycle and free access to food and water. Rats were deeply anesthetized by inhalation of 5% isoflurane (Baxter Healthcare, Australia) in O<sub>2</sub> and then euthanized by rapid decapitation. The abdomen was opened and approximately 10 cm of jejunum and its attached vascular fat and the descending abdominal aorta was removed and pinned out on a Silastic-bottomed Petri dish filled with ice-cold physiologic salt solution (PSS) with the following composition (119 mM NaCl; 4.69 mM KCl; 1.17 mM MgSO<sub>4</sub>·7H<sub>2</sub>O; 1.18 mM KH<sub>2</sub>PO<sub>4</sub>; 5.5 mM glucose (11 mM glucose for aorta); 25 mM NaHCO<sub>3</sub>; 2.5 mM CaCl<sub>2</sub>·6H<sub>2</sub>O; 0.026 mM EDTA saturated with carbogen (95% O2; 5% CO2) at pH 7.4. Second- or third-order mesenteric arteries (250-350  $\mu$ m internal diameter) or abdominal aorta were isolated from the surrounding fat, connective tissue, and nerves, and  $\sim 2$  mm long segments of arteries were mounted in separate myograph chambers (Model 610 and 620 M; Danish Myo Technology, Denmark) containing PSS at 37 °C for isometric force measurement as described previously.<sup>87,88</sup> Contractile responses were recorded with LabChart 7 and a PowerLab 4/30 A/D converter (AD Instruments Pty Ltd., Australia). To normalize to the basal conditions, the vessels were passively stretched according to a normalization protocol and adjusted to a diameter setting of 90% of that determined for an equivalent transmural pressure of 100 mmHg. After allowing the tissues to equilibrate for 30 min, the arteries were exposed to a potassium depolarizing solution (124 mM K<sup>+</sup> replacing Na<sup>+</sup> in PSS; termed KPSS) and noradrenaline (10  $\mu$ M) for 2 min. A second exposure to KPSS solution (only) was used to provide a reference contraction. Contraction responses were assessed by performing cumulative response curves to phenylephrine (0.001300  $\mu$ M) in the absence (control) or presence of different concentrations of Cpd1 (100, 300, and 500  $\mu$ M).

**Homology Modeling.** Homology models of WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR were made using the stabilized  $\alpha_{1B}$ -AR crystal structure bound to (+)-cyclazosin (PDB: 7B6W)<sup>30</sup> using the ICM Homology package (Molsoft LLC). The stabilized  $\alpha_{1B}$ -AR template structure was primed for modeling by deleting the DARPin fusion protein.<sup>30,89</sup> WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR models were made using the "full refinement" tool, which uses the biased probability Monte Carlo algorithm and pairwise sequence alignment method<sup>90</sup> with the stabilized  $\alpha_{1B}$ -AR crystal structure. Modeling was conducted with the cocrystallized ligand, (+)-cyclazosin, in the binding site and unresolved regions of the template structure (the N-terminus, ICL3, and C-terminus) deleted from our models ( $\alpha_{1A}$ -AR residues: 1–18, 219–261, 329–466;  $\alpha_{1B}$ -AR residues: 1–37, 238–283, 351–520).

**Computational Docking and MD Simulations.** Ligand binding poses in the homology models of WT  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR were generated using computational docking with ICM-Pro (Molsoft LLC). Docking was performed by creating a 25 × 25 × 25 Å box centered around (+)-cyclazosin. The most energetically favorable ligand docking outputs were collected, but some higher energy poses were also retained after being visually inspected.

MD simulations were performed using DESMOND MD simulation systems<sup>91</sup> with the imported receptor–ligand structures from docking. The receptor–ligand complex was preprocessed and minimized using the protein preparation wizard tool before being placed in a  $10 \times 10 \times$ 10 Å membrane environment of a 1-palmitoyl-2-oleoyl-*sn*-glycero-3phosphocholine bilayer with water and salt. Simple point-charge water models were used with 0.15 M Na<sup>+</sup> and Cl<sup>-</sup> ions in addition to 11 Cl<sup>-</sup> ions to neutralize the receptor. The protein and membrane environment was then relaxed through a series of MD simulations as previously described in Deluigi et al.<sup>30</sup> Productions NP<sub>7</sub>T simulations were then conducted at 300 K and 1 atm for 300 or 400 ns with a recording interval of 300 or 400 ps, respectively.

The membrane surface tension was set to 4000 bar/Å. MD data were gathered using VMD 1.9.386. Protein and ligand RMSD values were obtained using the RMSD visualizer tool. RMSD of ligands and protein backbone residues are relative to the receptor in its initial frame after the MD relaxation protocol described above.<sup>30</sup>

# ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.3c00767.

Competition STD-NMR data for Cpd1; Cpd1 activity in the CRE assay at human  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR and at QAPB binding and Ca<sup>2+</sup> mobilization assays rat  $\alpha_{1A}$ -AR and rat  $\alpha_{1B}$ -AR; Cpd1 activity in cell-based assays for  $\alpha_2$ -ARs and  $\beta$ -ARs; (+) cyclazosin docking and MD simulations when bound to  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR; phenylephrine dose-response curves in Ca<sup>2+</sup> mobilization assays using cells transfected with  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR, and site-directed mutated versions of each; pharmacological characterization of ligands at  $\alpha_1$ -ARs; structures, QAPB binding inhibition, and Ca<sup>2+</sup> mobilization characteristics of Cpd1 derivatives; structures, [3H]-prazosin binding inhibition and Ca<sup>2+</sup> mobilization characteristics of Cpd1 derivatives; and compound effects on non-receptor-expressing cells in Ca<sup>2+</sup> mobilization assays (PDF)

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A.A., M.D., B.D., R.E.H., P.R.G., A.P., R.A.D.B, D.K.C., and D.J.S. conceptualized and designed the research. M.D. prepared receptor protein samples for fragment screening, which was performed by B.D., R.H., H.S. and R.E.H. T.M.V., P.R.G., and D.J.S. prepared independent receptor samples and performed competition STD-NMR experiments. A.A., A.W., Y.Z., and D.J.S. performed and analyzed all cell-based receptor assays and receptor mutagenesis. A.H.B. performed and

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analyzed the data from, the ex vivo blood vessel contractile assays. L.A.Z. performed, and analyzed the data from, the docking and MD simulations, with input from D.K.C. and D.J.S. A.A., D.K.C., and D.J.S. chose compounds to order and synthesize based on the data. D.J.S. supervised the project. A.A., L.A.Z., and D.J.S. wrote the paper. All authors except A.W., Y.Z., B.D., R.H., and H.S. edited the paper.

## Notes

The authors declare no competing financial interest.

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# ABBREVIATIONS

AC, adenylate cyclase; ANOVA, analysis of variance; AR, adrenoceptor; BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; CHESS, cellular high-throughput encapsulation solubilization and screening; CNS, central nervous system; CRE, cAMP response element; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagles medium; DMSO, dimethyl sulfoxide; ECL, extracellular loop; GPCR, G protein-coupled receptor; MD, molecular dynamics; MFI, mean fluorescence intensity; NMR, nuclear magnetic resonance; PhE, phenylephrine; PLC, phospholipase C; QAPB, Quinazoline Piperazine BODIPY; RMSD, root mean-square deviation; SAR, structure–activity relationship; SBDD, structure-based drug design; SPR, surface plasmon resonance; STD, saturation transfer difference; TM, transmembrane domain; WT, wild type

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