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# Design, synthesis, and functional assessment of Cmpd-15 derivatives as negative allosteric modulators for the $\beta_2$-adrenergic receptor

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ABSTRACT

The $\beta_2$-adrenergic receptor ($\beta_2$AR), a G protein-coupled receptor, is an important therapeutic target. We recently described Cmpd-15, the first small molecule negative allosteric modulator (NAM) for the $\beta_2$AR. Herein we report in details the design, synthesis and structure-activity relationships (SAR) of seven Cmpd-15 derivatives. Furthermore, we provide in a dose-response paradigm, the details of the effects of these derivatives in modulating agonist-induced $\beta_2$AR activities (G-protein-mediated cAMP production and β-arrestin recruitment to the receptor) as well as the binding affinity of an orthosteric agonist in radio-ligand competition binding assay. Our results show that some modifications, including removal of the formamide group in the *para*-formamido phenylalanine region and bromine in the *meta*-bromobenzyl methylbenzamide region caused dramatic reduction in the functional activity of Cmpd-15. These SAR results provide valuable insights into the mechanism of action of the NAM Cmpd-15 as well as the basis for future development of more potent and selective modulators for the $\beta_2$AR based on the chemical scaffold of Cmpd-15.



## 1. Introduction

The $\beta_2$-adrenergic receptor ($\beta_2$AR) is a prototypical G protein-coupled receptor (GPCR) and is also an important therapeutic target for diseases such as cardiac arrhythmias, hypertension, and other cardiovascular diseases.[1,2] Drugs that target the $\beta_2$AR, such as β-blockers, are orthosteric β-adrenergic receptor antagonists that bind to the endogenous adrenaline binding site.[3–6] To date, all known β-adrenergic agonists and antagonists are orthosteric ligands. However, the possibility that allosteric modulators that potentiate or attenuate the activity of orthosteric ligands might possess enhanced therapeutic efficacy, selectivity, or other novel therapeutic properties has raised interest in identifying and characterizing such modulators for the $\beta_2$AR.

We recently carried out high throughput screening of DNA-encoded small-molecule libraries (DEL), comprising 190 million different unique compounds, against purified human $\beta_2$AR. This *in vitro* affinity based screening approach yielded the first allosteric β-blocker, named as Cmpd-15[7] (Scheme 1). The compound inhibits agonist-$\beta_2$AR induced G-protein activities (as measured via cAMP accumulation) and binds to inactive state form of the receptor with low micro-molar binding affinity. More recently, we co-crystallized and solved the structure of inactive $\beta_2$AR in complex with Cmpd-15, in the form of a polyethylene glycol-carboxylic acid derivative (Cmpd-15PA). The structure reveals that the $\beta_2$AR NAM Cmpd-15 ('allosteric β-blocker') binds to a pocket which is composed of the cytoplasmic ends of transmembrane segments 1, 2, 6 and 7 (TM1, TM2, TM6 and TM7) as well as intracellular loop 1 (ICL1) and helix 8 (H8). A proposed mechanism of allosteric antagonism of Cmpd-15 is that this modulator prevents $\beta_2$AR from coupling to Gs, and blocks the interactions with arrestins.[8] Herein we report in details the design and synthesis of seven Cmpd-15 derivatives, and present a more detailed dose dependent assessment for their ability to modulate agonist-mediated $\beta_2$AR activities in downstream functional and radioligand binding competition assays.

## 2. Design and synthesis of Cmpd-15 derivatives

The structure of Cmpd-15 is shown in Scheme 1. Guided by knowledge of the initial hits from our *in vitro* affinity-based selection with DEL described in our previous paper[7], we designed 7 derivatives, focused on strategic points on the chemical structure

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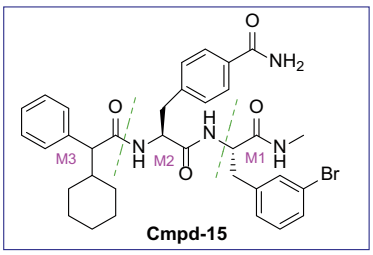


**Scheme 1.** Chemical structure of Cmpd-15.

of Cmpd-15; with the aim of investigating the influence of steric, hydrophilic, and hydrophobic features on NAM activity of the derivatives. For the convenience of our SAR analyses, the structure of Cmpd-15 is divided into three subunits (Scheme 1) similar to as previously described[7]: the (*meta*-bromobenzyl)methylbenzamide (M1), (*para*-formamido)phenyl-alanine (M2), and cyclohexyl-2-phenylacetamido (M3) regions. We made certain modifications in each region. For example, in region M1, an additional *meta*-bromine was introduced to the phenyl ring (derivative **15A5**), the *meta*-bromine was replaced with *meta*-fluorine (derivative **15A4**), and the *meta*-bromine was removed (**15A3**); in region M2, the *para*-formamide group was moved to the *meta*-position (**15A2**), and completely removed (**15A1**); in region M3, a methoxy group (**15A7**) and a hydroxyl group (**15A6**) were introduced to the *para*-position of the phenyl ring, respectively.

The synthetic route for the designed Cmpd-15 derivatives is outlined in Scheme 2. The chiral building block, substituted (*S*)-phenyl alanine **5a–5c**, was prepared through asymmetric phase transfer catalytic alkylation[9–11] of substituted benzyl bromide with diphenylamine glycine *tert*-butyl ester **3**. The latter was obtained by condensation of benzophenone with glycine *tert*-butyl ester in refluxing toluene and in the presence of boron trifluoride diethyl etherate. Under the catalysis of *O*-allyl-*N*-9-anthracene methyl bromide cinchonine (**3A**), **3** was alkylated by substituted benzyl bromide in toluene/chloroform (2:1) and at −40 °C, affording (*S*)-3-halobenzyl-2-diphenylimine glycine *tert*-butyl ester (**4a–4c**) in highly stereochemistry-controlled manner. For example, (*S*)-3-(3,5-dibromobenzyl)-2-diphenylimine glycine *tert*-butyl ester (**4a**) was obtained with 94.9% ee. After acidic hydrolysis in hydrochloric acid, **4a–4c** were converted into the corresponding *L*-phenyl alanines **5a–5c**, and then the amino group was protected with Fmoc, affording Fmoc-protected *L*-phenyl alanines **6a–6c.** The acidic hydrolysis didn't racemize the amino acids.[12] For example, the ee value of (9*H*-fluoren-9-yl)methyl (*S*)-(3,5-dibromo)-phenylalanine (**6a**) is 94.7%. In the next step, Fmoc-protected *L*-phenylalanine methylamides **7a-7d** were obtained by condensation of methylamine with the corresponding *L*-phenylalanines in the presence of *O*-benzotriazol-1-yl-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) and hydroxybenzotriazole (HOBt).[13] Upon treatment with piperidine in DMF,[13] the Fmoc group in **7a–7d** was removed smoothly, giving *L*-phenylalanine methylamides **8a–8d** with satisfactory yields.

With amines **8a–8d** in hand, we turn our attention to the remaining steps in the synthetic sequence. By employing HBTU/HOBt as the amide coupling agents, **8a–8d** were coupled with Fmoc-*L*-4-carbamoylphenylalanine (**9a**, $R_3$ = 4-formamide), Fmoc-*L*-3-carbamoylphenylalanine (**9b**, $R_3$ = 3-formamide) or Fmoc-*L*-phenylalanine (**9c**, $R_3$ = H), respectively, and dipeptides **10a–10f** were generated. Under the same reaction conditions used for preparation of **5a–5c**, the Fmoc group in **10a–10f** was taken off by piperidine and resulted in amines **11a–11f**. In the final step, **11a–11f** were reacted with 2-cyclohexyl-2-phenyl acetic acid (**14a**, $R_4$ = H), 2-cyclohexyl-2-(4-methoxyphenyl)acetic acid (**14b**, $R_4$ = 4-OMe), and 2-cyclohexyl-2-(4-hydroxyphenyl)acetic acid (**14c**, $R_4$ = 4-OH), respectively, affording the desired final products (**15A1-15A7**). While **14a** is commercial available, **14b** was prepared by alkylation of 2-(4-methoxyphenyl)acetic acid with cyclohexyl 4-methylbenzenesulfonate in the presence of *n*-butyllithium (Scheme 3). Upon treatment with boron tribromide, **14b** was demethylated to give **14c** in 55% yield. The identity of all the products and the intermediates was confirmed by their $^1$H NMR, $^{13}$C NMR and high-resolution mass spectrometry (HRMS) properties.

## 3. Pharmacological activity assays of Cmpd-15 derivatives

We conducted both cell-based assays and radioligand binding assays to characterize the pharmacological activity of seven derivatives of Cmpd-15 at the $\beta_2$AR in a more systematic way than the previous report.[7] The cell-based assays include the Promega GloSensor assay and the DiscoverX PathHunter assay. The Promega GloSensor assay was performed to measure agonist-induced cAMP production as a means of quantifying G-protein activation, and the DiscoverX PathHunter assay was performed to measure agonist-induced β-arrestin recruitment to the receptor. For the highly amplified GloSensor assay, HEK-293 cells endogenously expressing the $\beta_2$AR were pretreated with the derivatives and then read after the stimulation with the orthosteric agonist, isoproterenol (ISO). For the stoichiometric DiscoverX PathHunter assay, U2OS cells stably expressing the chimeric $\beta_2V_2$R, which has increased phosphorylation on C-terminus tail, leading to more stable interaction with β-arrestin than the native receptor, were pretreated with the derivatives, and then stimulated with ISO. Competition binding with $^{125}$I-cyanopindolol (CYP) was performed to determine the derivatives' ability to affect the binding of an orthosteric agonist to the $\beta_2$AR. Both the allosteric derivatives and the orthosteric agonist isoproterenol (ISO) were introduced to reconstituted in High-Density-Lipoparticles (HDLs, or nanodiscs). The orthosteric antagonist $^{125}$I-CYP was then introduced and allowed to compete with ISO in a dose-wise manner.

## 4. Results and discussion

All the Cmpd-15 derivatives were systematically evaluated for their ability to modulate agonist-induced $\beta_2$AR activities in a concentration-dependent manner by using two cellular functional assays (Gαs signaling via cAMP accumulation measurement and β-arrestin recruitment) as well as a competition radio-ligand binding assay. The results are summarized in Tables 1 and 2.

Table 1 is the summary of the data showing the maximum degree of inhibition of $E_{max}$ induced by a derivative relative to that of Cmpd-15. The data are consistent with the results in our previous report, in which we evaluated the inhibitory activity of these derivatives at a single concentration (50 μM).[7] Table 2 shows the maximum fold shift of ISO $IC_{50}$ or $EC_{50}$ values induced by the derivatives at 50 μM in $^{125}$I-CYP competition binding ($IC_{50}$) as well as both the GloSensor and PathHunter cell-based assays ($EC_{50}$).

The effect of Cmpd-15 and its derivatives **15A1–15A7** on $\beta_2$AR-mediated functional activities is presented in Figs. 1–3. After pretreatment with Cmpd-15 or its derivatives at various concentrations, $\beta_2$AR-mediated activity were measured in cells upon stimulation with isoproterenol (ISO) in a dose-dependent manner: cAMP production by the endogenously expressed $\beta_2$AR (Fig. 1) and β-arrestin recruitment to the exogenously expressed $\beta_2V_2$R (Fig. 2). A dose–response curve of ISO competition binding to the $\beta_2$AR reconstituted in HDL particles (nanodiscs) with radiolabeled $^{125}$I-CYP and ISO was obtained in the presence of 50 μM of Cmpd-15 or its derivatives (Fig. 3). For Cmpd-15, there were both a decrease in $E_{max}$ and a curve shift of the $EC_{50}$ to the right in both the cAMP