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Allosteric modulator potentiates $\beta_2$AR agonist–promoted bronchoprotection in asthma models

Seungkirl Ahn,¹ Harm Maarsingh,² Julia K.L. Walker,¹,3 Samuel Liu,¹ Akhil Hegde,³ Hyeje C. Sumajit,² Alem W. Kahsai,¹ and Robert J. Lefkowitz¹,4,5

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Asthma is a chronic inflammatory disease associated with episodic airway narrowing. Inhaled $\beta_2$-adrenergic receptor ($\beta_2$AR) agonists ($\beta_2$-agonists) promote — with limited efficacy — bronchodilation in asthma. All $\beta_2$-agonists are canonical orthosteric ligands that bind the same site as endogenous epinephrine. We recently isolated a $\beta_2$AR-selective positive allosteric modulator (PAM), compound-6 (Cmpd-6), which binds outside of the orthosteric site and modulates orthosteric ligand functions. With the emerging therapeutic potential of G-protein coupled receptor allosteric ligands, we investigated the impact of Cmpd-6 on $\beta_2$AR-mediated bronchoprotection. Consistent with our findings using human $\beta_2$ARs, Cmpd-6 allosterically potentiated $\beta_2$-agonist binding to guinea pig $\beta_2$ARs and downstream signaling of $\beta_2$ARs. In contrast, Cmpd-6 had no such effect on murine $\beta_2$ARs, which lack a crucial amino acid in the Cmpd-6 allosteric binding site. Importantly, Cmpd-6 enhanced $\beta_2$-agonist-mediated bronchoprotection against methacholine-induced bronchoconstriction in guinea pig lung slices, but — in line with the binding studies — not in mice. Moreover, Cmpd-6 robustly potentiated $\beta_2$-agonist–mediated bronchoprotection against allergen-induced airway constriction in lung slices obtained from a guinea pig model of allergic asthma. Cmpd-6 similarly enhanced $\beta_2$-agonist–mediated bronchoprotection against methacholine-induced bronchoconstriction in human lung slices. Our results highlight the potential of $\beta_2$AR-selective PAMs in the treatment of airway narrowing in asthma and other obstructive respiratory diseases.

Introduction

Asthma is a chronic airway disease that affects millions of people worldwide and is the most common chronic disease in children (1). Although asthma is an airway inflammatory disease, it is also characterized by airway hyperresponsiveness and remodeling. The most notable feature of the disease is episodic narrowing of the airways resulting in reversible airflow obstruction known as bronchospasm, asthma attack, or asthma exacerbation. Asthma exacerbation can be provoked by various triggers, including allergens, cold air, exercise, air pollution, and certain chemicals (2, 3). The most common symptoms experienced by asthmatics are those associated with airway narrowing, including wheezing, shortness of breath, chest tightness, and cough. The first line treatment for these symptoms is inhalation of a bronchodilator (4).

The standard of asthma therapy is a combination of inhaled bronchodilators and antiinflammatory drugs. The main bronchodilators are $\beta_2$-adrenergic receptor ($\beta_2$AR) agonists ($\beta_2$-agonists). Inhaled $\beta_2$-agonists are used in the prevention and reversal of airway narrowing by promoting airway smooth muscle relaxation and subsequent improvement of airflow (5). The $\beta_2$AR is a prototypical G-protein coupled receptor (GPCR) that is expressed in airway smooth muscle cells where its activation increases intracellular levels of cyclic AMP via Gas-mediated activation of adenyl cyclase (5). The increased levels of cAMP subsequently promote airway smooth muscle relaxation mainly by activation of protein kinase A (PKA) (6, 7), but also via activation of exchange proteins directly activated by cAMP (Epac) (8–10).

$\beta_2$-agonists are fundamental to asthma treatment, providing both prophylaxis (bronchoprotection) and rescue medication (bronchodilation) for many asthmatics. However, several decades of basic, translational, clinical, and epidemiological research has revealed that $\beta_2$-agonists provide suboptimal control for up to 50% of asthmatics (11, 12). Moreover, chronic use of $\beta_2$-agonists has been associated with loss of bronchoprotection, persistent safety concerns, and worsening of asthma control (5). Long-term use of inhaled $\beta_2$-agonists can lead to reduced responsiveness to these medications in individuals with asthma (5, 13) due, in part, to desensitization and downregulation of the $\beta_2$AR (14, 15).

Most drugs targeted to GPCRs bind to the receptor’s canonical orthosteric site, where endogenous ligands bind. All asthma drugs aimed at the $\beta_2$AR are also classic orthosteric ligands (e.g., fenoterol and albuterol [a.k.a. salbutalmol]). Unlike orthosteric ligands,
with deleterious effects. Moreover, a strong β_2AR PAM may reduce or obviate the need for β_2-agonists. Indeed, β_2AR PAM has the potential to relieve asthmatic airway symptoms during asthma exacerbations through increasing responsiveness of β_2AR signaling to endogenous levels of adrenaline, which are impaired in asthmatics during asthma exacerbations (19, 20). Thus, PAMs of the β_2AR might greatly improve the treatment of asthma. However, there have been no such agents available.

We recently isolated a β_2AR-specific PAM, compound-6 (Cmpd-6), by DNA-encoded library (DEL) screening utilizing the purified receptor occupied by a potent orthosteric agonist and reconstituted into high-density lipoprotein (HDL) particles (a.k.a. nanodiscs) (21). Cmpd-6 displays positive cooperativity with orthosteric agonists, enhancing their binding to the receptor and potentiating agonist-induced downstream cAMP production (21). Physicochemical characteristics of Cmpd-6 can be found in Supplemental Table 1.
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and intracellular loop (ICL) 2 in a conformation only observed in the core of the receptor and locks transmembrane (TM) helices 3 and 4. The recently determined crystal structure of a Cmpd-6 derivative bound to the agonist-activated human β₂AR (Figure 1). Since we obtained minimal effects of Cmpd-6 lysine (K)-142. Since we obtained minimal effects of Cmpd-6 at the human β₂AR (22). Thus, the action of Cmpd-6 is quite distinct from that of orthosteric β₂-agonists.

In the current study, we set out to assess whether the functional PAM activity of Cmpd-6 that we previously observed in cellular signaling systems would translate to potentiation of β₂AR structures (22). Thus, the action of Cmpd-6 is quite distinct from that of orthosteric β₂-agonists.

Results

Positive allosteric effects of Cmpd-6 on albuterol-mediated responses at the human β₂AR in functional assays. Allosteric modulators often show probe-dependency, displaying preferentially higher cooperativity with a specific orthosteric agonist over others (16–18). Before evaluating the effect of Cmpd-6 on β₂-agonist–induced airway relaxation, we confirmed cooperativity of Cmpd-6 with albuterol (a.k.a. salbutamol), one of the most commonly used short-acting β₂-agonists employed for asthma treatment (5). In the presence of Cmpd-6, stimulation of the β₂AR with albuterol leads to dramatic increases in cAMP production (Figure 1A) and β-arrestin recruitment to the receptor (Figure 1B) in HEK-293 cells, compared with the vehicle (DMSO) control condition. The increases in the maximal response to albuterol are even more robust than those observed with isoproterenol stimulation, as expected, given the partial agonist activity of albuterol. Cmpd-6 also shifts the albuterol dose–response curve to the left in an in vitro competition binding experiment by potentiating the binding affinity of albuterol for the β₂AR in a concentration–dependent fashion (Figure 1C). However, the shift induced by Cmpd-6 is weaker than with isoproterenol (Figure 1D), which is also consistent with the partial agonist activity of albuterol. Taken together, these data confirm the robust cooperativity between Cmpd-6 and albuterol at the human β₂AR. This led us to an examination of its allosteric activity in albuterol-induced airway relaxation in an animal model.

Minimal positive allosteric effects of Cmpd-6 on albuterol-induced bronchodilation in mice. To examine the effectiveness of Cmpd-6 for enhancing β₂AR-mediated function in airway smooth muscle, we employed 2 different, but complementary, in vivo assays developed and routinely used in our laboratory (13, 23). To test whether Cmpd-6 offered bronchoprotection in the absence of an exogenous β₂ agonist, Cmpd-6 was administered before delivery of increasing doses of methacholine, an agonist for muscarinic acetylcholine receptors that induces airway constriction. Secondly, to test the effect of Cmpd-6 on bronchodilation, we preconstricted murine airways with methacholine and administered increasing doses of albuterol in the absence and presence of Cmpd-6. As shown in Figure 2A, airway resistance increased similarly in Cmpd-6–treated and vehicle-treated mice, illustrating no bronchoprotective effect of Cmpd-6. Similarly, there was no enhancing effect of Cmpd-6 on bronchodilation, as evidenced by the nearly identical reduction in airway resistance with increasing doses of albuterol in the absence and presence of Cmpd-6 (Figure 2B). These findings demonstrate that β₂AR-mediated bronchodilation is not enhanced by Cmpd-6 in mice, which was unexpected based on our in vitro results with the human β₂AR (Figure 1).

One of the critical amino acids for Cmpd-6 binding to the β₂AR, F-133, is not conserved in the murine β₂AR. The recently determined crystal structure of a Cmpd-6 derivative bound to the agonist-activated human β₂AR indicates that 2 amino acids in the binding site are essential for binding of Cmpd-6: phenylalanine (F)-133 and lysine (K)-142 (22). Since we obtained minimal effects of Cmpd-6 on bronchoprotection or albuterol-induced bronchodilation in our...
In fact, we observed minimal cooperativity of Cmpd-6 with a β2-agonist in a competition binding assay with isolated membranes from HEK-293 cells transiently expressing the murine β2AR (Figure 3C). On the other hand, we found that mutation of V-133 of the murine β2AR to its human counterpart F-133 completely rescued the PAM activity of Cmpd-6 back to the activity observed at the human receptor, as assessed by cooperativity of ligand binding (Figure 3C). We have previously shown that Cmpd-6 behaves like a robust allosteric agonist by further enhancing the high constitutive (basal) cAMP production by the exogenously overexpressed murine model study, we inspected whether these 2 amino acids were conserved in the murine receptor. F-133 and K-142 provide multiple hydrophobic interactions and a hydrogen bond with Cmpd-6, respectively, crucial for Cmpd-6 binding to the human β2AR (22). One of these, F-133, is replaced with valine (V) in the murine β2AR (Figure 3A). Figure 3B illustrates how substitution of F-133 with V-133 in the Cmpd-6 binding site on the murine β2AR hinders Cmpd-6 binding to the murine receptor. The smaller aliphatic side chain of V-133 no longer provides hydrophobic contacts with Cmpd-6 as the larger aromatic ring of F-133 does for Cmpd-6’s binding to its pocket.

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Our murine model is likely attributable to its weak PAM activity at the murine β2AR due to the crucial single amino acid difference from the human receptor in the Cmpd-6 allosteric binding site. F-133 is conserved in the guinea pig β2AR and Cmpd-6 serves as a positive allosteric modulator at the guinea pig β2AR. Since we could not employ the murine system to evaluate the effect of Cmpd-6 on β2AR-mediated airway relaxation, we looked for alternative systems to continue our study with Cmpd-6. Fortunately, we discovered that both of the key amino acids, F-133 and L-142 of the human β2AR, are conserved in the receptor from guinea pig (Figure 4A), another animal model that is relevant for studying asthma (24, 25). The PAM activity of Cmpd-6 at the guinea pig β2AR was tested. Cmpd-6 displayed strong and dose-dependent cooperativity with the β2 agonist, isoproterenol, and increased its binding to the receptor, exhibited by the leftward shift of the β2AR in the absence of orthosteric agonist stimulation (21). This interferes with our ability to assess the positive allosteric effect of Cmpd-6 when the overexpressed β2AR is further stimulated with an agonist. Accordingly, we examined the effect of Cmpd-6 on the constitutive activity of overexpressed receptors. The dramatic difference due to a single amino acid change observed in the ligand binding assay (Figure 3C) was precisely recapitulated when we examined the effect of Cmpd-6 on cAMP production induced by the constitutive activity of transiently overexpressed human or murine WT or V133F mutant β2ARs (Figure 3D). Mutation of a single residue, V-133 to F-133 of the murine β2AR, completely recovered the positive allosteric effect of Cmpd-6 on β2AR receptor signaling, thus entirely salvaging the almost absent Cmpd-6 PAM activity observed at the murine WT receptor. These results demonstrate that the minimal effect of Cmpd-6 on airway relaxation in our murine model is likely attributable to its weak PAM activity at the murine β2AR due to the crucial single amino acid difference from the human receptor in the Cmpd-6 allosteric binding site.

**F-133 is conserved in the guinea pig β2AR and Cmpd-6 serves as a positive allosteric modulator at the guinea pig β2AR.** Since we could not employ the murine system to evaluate the effect of Cmpd-6 on β2AR-mediated airway relaxation, we looked for alternative systems to continue our study with Cmpd-6. Fortunately, we discovered that both of the key amino acids, F-133 and L-142 of the human β2AR, are conserved in the receptor from guinea pig (Figure 4A), another animal model that is relevant for studying asthma (24, 25). The PAM activity of Cmpd-6 at the guinea pig β2AR was tested. Cmpd-6 displayed strong and dose-dependent cooperativity with the β2 agonist, isoproterenol, and increased its binding to the receptor, exhibited by the leftward shift of the
isoproterenol dose-response curve in a competition binding assay (Figure 4B). We also confirmed Cmpd-6’s PAM activity in cAMP production induced by the constitutive (enhanced basal) activity of the overexpressed guinea pig β2AR, which is equivalent to what we observed with the overexpressed human receptor (Figure 4C).

We next examined the cooperativity of Cmpd-6 with other β2-agonists utilized to relieve asthmatic symptoms, including albuterol and fenoterol (5) (Figure 4, D and E). At the guinea pig β2AR, Cmpd-6 exhibited expected levels of cooperativity with tested β2-agonists except salmeterol (Figure 4F). Cmpd-6 showed cooperativity with salmeterol that was stronger than expected given its partial-agonist activity; this may be due to probe dependency. Probe dependency is difficult to predict and results in allosteric ligands that display greater or lesser cooperativity with different orthosteric agonists. Taken together, these findings demonstrate that Cmpd-6 enhances β2-agonist binding to, and activation of, the guinea pig β2AR.

Positive allosteric effects of Cmpd-6 on fenoterol-induced bronchoprotection against methacholine-induced airway constriction in guinea pig lung slices. Since one of the most important therapeutic applications of β2-agonists is their effect on β2AR-mediated bronchoprotection (5), the positive in vitro findings regarding the action of Cmpd-6 on the guinea pig β2AR prompted us to examine its effect on β2-agonist-mediated bronchoprotection of intrapulmonary airways in intact guinea pig lung slices using video-assisted microscopy. We first studied whether Cmpd-6 would enhance the bronchoprotection induced by fenoterol — a short-acting full agonist for the β2AR — against airway constriction induced by methacholine, an agonist for muscarinic acetylcholine receptors.

Methacholine induced a concentration-dependent constriction of airways in lung slices obtained from naive guinea pigs with an EC50 value of 0.25 ± 0.06 μM (Figure 5A). Whereas treatment with fenoterol (Figure 5, A and B) Lung slices were incubated with 1, 10, or 100 μM fenoterol (A) or 25 μM Cmpd-6 (B), and airway constriction to increasing concentrations of methacholine (MCh) was determined by measuring the luminal area as a percentage of baseline (C and D). The effect of 25 μM Cmpd-6 in combination with 1 μM (C) or 10 μM (D) fenoterol was compared with that at a 10-fold higher fenoterol concentration by itself (10 and 100 μM, respectively). All curve fits were generated using the software program GraphPad Prism. Data are represented as mean ± SEM obtained from 6 guinea pigs. Statistical analyses were performed using a paired 2-tailed Student’s t test: *P < 0.05 and **P < 0.01 regarding the shift of the MCh EC50 values compared with control; ‡P < 0.01 and §P < 0.01 regarding the additional shift of the MCh EC50 values in the presence of Cmpd-6 compared to 1 μM and 10 μM fenoterol, respectively (see also Table 1).

Table 1. Effects of fenoterol in the absence and presence of Cmpd-6 on methacholine-induced airway constriction in guinea pig lung slices

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCh Emax (%)</th>
<th>MCh EC50 (μM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
<td>0.25 ± 0.06</td>
<td>6</td>
</tr>
<tr>
<td>25 μM Cmpd-6</td>
<td>100.0 ± 2.0</td>
<td>0.28 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>1 μM Fenoterol</td>
<td>107.7 ± 1.9</td>
<td>0.37 ± 0.08</td>
<td>6</td>
</tr>
<tr>
<td>1 μM Fenoterol + 25 μM Cmpd-6</td>
<td>112.3 ± 9.5</td>
<td>0.59 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>10 μM Fenoterol</td>
<td>107.0 ± 1.0</td>
<td>0.56 ± 0.03</td>
<td>6</td>
</tr>
<tr>
<td>10 μM Fenoterol + 25 μM Cmpd-6</td>
<td>100.9 ± 3.2</td>
<td>0.98 ± 0.12</td>
<td>6</td>
</tr>
<tr>
<td>100 μM Fenoterol</td>
<td>102.5 ± 1.6</td>
<td>1.00 ± 0.15</td>
<td>6</td>
</tr>
</tbody>
</table>

*A P < 0.05; †P < 0.01 compared with control; ‡P < 0.01 compared with 1 μM fenoterol; §P < 0.05 compared with 1 μM fenoterol; ‡P < 0.01 compared with 10 μM fenoterol; ‡P < 0.05 compared with 10 μM fenoterol using a paired 2-tailed Student’s t test. Data are represented as mean ± SEM of n experiments. MCh, methacholine; Emax, maximal constriction induced by methacholine; MCh EC50, methacholine concentration (in μM) inducing 50% of the maximal response.
ol (1, 10, and 100 μM) did not affect the maximal airway constriction induced by methacholine, it reduced the sensitivity toward methacholine in a dose-dependent way as indicated by the rightward shift of the methacholine-induced airway constriction response curve and increased EC\textsubscript{50} values (Figure 5A and Table 1). Treatment with the β\textsubscript{2}AR selective PAM Cmpd-6 (25 μM) by itself did not affect the maximal airway constriction (E\textsubscript{max}) or sensitivity (EC\textsubscript{50}) toward methacholine compared with control (Figure 5B and Table 1).

Importantly, cotreatment with Cmpd-6 enhanced the bronchoprotective effect of 1 μM fenoterol, so that the combined drugs provided the same level of protection as achieved at a 10-fold higher concentration (10 μM) of fenoterol by itself (Figure 5C and Table 1). Similarly, the bronchoprotective effect of 10 μM fenoterol was also enhanced in the presence of Cmpd-6 — again, to the same level of protection as observed at a 10-fold higher concentration (100 μM) of fenoterol alone (Figure 5D and Table 1). These results are consistent with our previous finding that Cmpd-6 potentiates the binding affinity of fenoterol for the guinea pig β\textsubscript{2}AR approximately 10-fold in in vitro binding assays (Figure 4D). Thus, compared with just fenoterol, the same rightward shift in methacholine-induced constriction could be obtained with a 10-fold lower concentration of fenoterol when it was combined with Cmpd-6. The fact that Cmpd-6 is able to augment the responsiveness of the airway smooth muscle to fenoterol by 10-fold suggests that Cmpd-6 may have utility as an adjunctive agent to β\textsubscript{2}-agonists for the treatment of bronchoobstruction in respiratory diseases like asthma.

Positive allosteric effects of Cmpd-6 on fenoterol-induced bronchoprotection against allergen-induced airway constriction in lung slices from a guinea pig model of asthma. The therapeutic potential of Cmpd-6 as an adjunctive therapy to treat airway narrowing in asthma was subsequently tested in lung slices obtained from guinea pigs that were actively IgE-sensitized to ovalbumin using Al(OH)\textsubscript{3} as the adjuvant. Guinea pig models of acute and chronic allergic asthma display characteristics similar to those found in human subjects with asthma, such as airway hyperresponsiveness, eosinophilic inflammation, early and late asthmatic reactions, mucus hypersecretion, and airway remodeling (24). Indeed, guinea pigs are a very relevant species to study pharmacological drug targets in asthma, because the anatomy of the airway, pathophysiology — including mast cell mediators released upon allergen challenge — and receptor pharmacology of guinea pigs are very similar to those of humans (24, 25).

In sensitized guinea pigs, exposure to the allergen (ovalbumin) leads to crosslinking of ovalbumin-specific IgE to the high-affinity IgE receptors, FcεRI, on mast cells, resulting in the release of various mast cell mediators, including histamine (26–28). In line with this, we found that ovalbumin induced a concentration-dependent constriction of airways in lung slices obtained from ovalbumin-sensitized guinea pigs. Ovalbumin induced a full airway constriction with an EC\textsubscript{50} value of 8.0 ± 2.4 ng/mL (Figure 6 and Table 2). Compared with control, pretreatment of lung slices with 0.1 μM fenoterol induced a 190-fold rightward-shift of ovalbumin-induced airway constrictions without affecting the maximal constriction (Figure 6 and Table 2). Importantly, the bronchoprotective effect of 0.1 μM fenoterol was greatly enhanced in the presence of the β\textsubscript{2}AR PAM Cmpd-6 (25 μM). Compared with treatment with 0.1 μM of fenoterol alone, the cotreatment with Cmpd-6 greatly reduced the maximal constriction from 103.7% ± 2.4% to 19.9% ± 7.7% airway closure – offering almost full protection against allergen-induced airway narrowing. Interestingly, the bronchoprotective effect of 0.1 μM fenoterol plus 25 μM Cmpd-6 was identical to that of a 10-fold higher concentration of fenoterol (1 μM) by itself (Figure 6 and Table 2), consistent with the lung-slice findings observed using methacholine as the contractile agent. These findings show that cotreatment with Cmpd-6 greatly enhanced the bronchoprotective effect of fenoterol against allergen-induced airway constriction.

### Table 2. Effects of fenoterol in the absence and presence of Cmpd-6 on ovalbumin-induced airway constriction in lung slices obtained from a guinea pig model of allergic asthma

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OVA E\textsubscript{max} (%)</th>
<th>OVA EC\textsubscript{50} (log ng/mL)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.8 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>5</td>
</tr>
<tr>
<td>0.1 μM Fenoterol</td>
<td>103.7 ± 2.4</td>
<td>2.8 ± 0.3\textsuperscript{a}</td>
<td>4</td>
</tr>
<tr>
<td>0.1 μM Fenoterol + 25 μM Cmpd-6</td>
<td>15.9 ± 7.4\textsuperscript{b}</td>
<td>4.4 ± 0.2\textsuperscript{c}</td>
<td>5</td>
</tr>
<tr>
<td>1 μM Fenoterol</td>
<td>11.7 ± 1.3\textsuperscript{b}</td>
<td>4.1 ± 0.4\textsuperscript{b}</td>
<td>5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P < 0.001 compared with control, \textsuperscript{b}P < 0.001, \textsuperscript{c}P < 0.01, \textsuperscript{d}P < 0.05 compared with 0.1 μM fenoterol using a 1-way ANOVA followed by Bonferroni’s posthoc test. Data are represented as mean ± SEM of n experiments. OVA, ovalbumin; E\textsubscript{max}, maximal constriction induced by ovalbumin; OVA EC\textsubscript{50}, log of the ovalbumin concentration (ng/mL) inducing 50% of the maximal response.
Positive allosteric effects of Cmpd-6 on fenoterol-induced bronchoprotection against methacholine-induced airway constriction in human lung slices. Since asthma is a human disease, it was essential to assess the ability of Cmpd-6 to enhance β2 agonist-mediated bronchoprotection in human tissue. We therefore tested the ability of Cmpd-6 to enhance β2 agonist-mediated bronchoprotection against methacholine-induced airway narrowing in human lung slices. As one would expect based on classic pharmacologic principles, Figure 7 shows that 10 μM fenoterol offered better protection against methacholine-induced bronchoconstriction than 1 μM fenoterol. (Figure 7 and Table 3). In the presence of 1 μM fenoterol, the maximal methacholine-induced airway constriction was 47.2% ± 5.2% with a pD2 value of 6.99 ± 0.10. The bronchoprotection by 10 μM fenoterol was enhanced as shown by lower maximal methacholine-induced constrictions, 21.7% ± 2.2%, and a rightward shift of the pD2 value to 5.77 ± 0.33 (Figure 7 and Table 3). In other words, the airway luminal area at the highest methacholine concentration was larger with 10 μM fenoterol (79.5% ± 2.8% open) than with 1 μM fenoterol (55.7% ± 6.2% open). Importantly, Figure 7 shows that when 25 μM Cmpd-6 was added to 1 μM fenoterol, the effectiveness of the β2-agonist in preventing methacholine-induced bronchoconstriction was greatly enhanced, reducing the maximal methacholine-induced constriction to 26.5% ± 4.4% and inducing a rightward shift of the pD2 value to 5.52 ± 0.24 (Figure 7 and Table 3). Additionally, the bronchoprotective effect of 1 μM fenoterol plus 25 μM Cmpd-6 was indistinguishable from that of 10 μM fenoterol (Figure 7 and Table 3), demonstrating a 10-fold enhancement of the β2-agonist responsiveness. The result that Cmpd-6 enhances β2 agonist-mediated bronchoprotection in human lung slices is consistent with that observed in guinea pig lung slices. Moreover, the functional relevance of Cmpd-6 in humans that was implied by the results of the human cell in vitro positive cooperativity binding and signaling studies was confirmed by these findings in ex vivo human airways.

Discussion

Previously, we showed that Cmpd-6 enhances binding of the orthosteric ligand, isoproterenol, at the β2AR and potentiates downstream cAMP production (21). Here, we demonstrate the therapeutic potential of Cmpd-6 to enhance airway smooth muscle relaxation in response to β2-agonists commonly used in the treatment of asthma. Our in vitro studies using guinea pig β2AR show that Cmpd-6 improves agonist binding of fenoterol, albuterol, and salmeterol to the guinea pig β2AR and enhances cAMP production. Additionally, the positive allosteric effect of Cmpd-6 on the physiological function of β2AR was demonstrated; we showed that Cmpd-6 augmented the fenoterol-induced bronchoprotection against methacholine-induced bronchoconstriction in guinea pig lung slices. In fact, when Cmpd-6 was added, the same bronchoprotective effect could be achieved with a 10-fold lower dose of the β2 agonist. To test if the positive allosteric bronchoprotective effect of Cmpd-6 was pathophysiologically relevant, we used lung slices from a guinea pig model of allergic asthma and showed that Cmpd-6 robustly enhanced fenoterol-induced bronchoprotection against allergen-induced airway constriction. Importantly, we demonstrated that the positive allosteric effect of Cmpd-6 on fenoterol-induced bronchoprotection was also present in human tissue, where the combination of Cmpd-6 and fenoterol again offered the same level of protection as that observed with a 10-fold higher dose of the β2 agonist. Taken together, these findings demonstrate that the β2AR-selective PAM, Cmpd-6, enhances agonist binding to, and signaling of, guinea pig and human β2ARs and potentiates β2 agonist-mediated bronchoprotection in naive and allergic guinea pig lung slices and human lung slices. These results suggest that β2AR PAMs, like Cmpd-6, could have important clinical utility for the treatment of airway narrowing in asthma.

Table 3. Effects of fenoterol in the absence and presence of Cmpd-6 on methacholine-induced airway constriction in human lung slices

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCh Emax (%)</th>
<th>MCh pD2 (–log M)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM Fenoterol</td>
<td>47.2 ± 5.2</td>
<td>6.99 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td>1 μM Fenoterol + 25 μM Cmpd-6</td>
<td>26.5 ± 4.4A</td>
<td>5.52 ± 0.24</td>
<td>4</td>
</tr>
<tr>
<td>10 μM Fenoterol</td>
<td>21.7 ± 2.8B</td>
<td>5.77 ± 0.33A</td>
<td>4</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 compared with 1 μM fenoterol using a 1-way ANOVA followed by Bonferroni’s posthoc test. Data are presented as mean ± SEM of n experiments. MCh, methacholine; Emax, maximal constriction induced by methacholine; pD2, –log of the methacholine concentration (in M) inducing 50% of the maximal response.
Recently, an increasing number of positive and negative allosteric modulators for GPCRs have been described (16-18); although, to date, only 2 have reached the clinic (29, 30). Rather than directly stimulating or inhibiting biological effects, allosteric modulators exert their effects by altering receptor responsiveness to orthosteric agonists. Allosteric compounds offer a number of potential therapeutic advantages compared to classical orthosteric ligands, including improved safety, reduced off-target effects, and increased efficacy or potency of orthosteric ligands. Drug safety may be improved since the effects of PAMs are saturable and therefore exhibit a ceiling effect. Thus, the risk of overdosing is reduced and is only manifest when an orthosteric agonist is present (17, 18). Off-target effects are reduced by PAMs compared with orthosteric agonists because the former bind with greater specificity among closely related receptor subtypes. Orthosteric binding site regions are highly conserved among receptor subtypes within a receptor family since these subtypes all bind the same endogenous agonist(s). On the other hand, allosteric regions of receptor subtypes are evolutionarily less conserved, and thus allosteric compounds provide greater binding specificity among closely related receptor subtypes.

In asthma, the addition of PAMs like Cmpd-6 could improve the efficacy and reduce unwanted side effects of currently prescribed β2-agonists. In terms of efficacy, PAMs for the β2AR should reduce the dose of β2-agonists needed to obtain clinically relevant outcomes (e.g., bronchoprotection) and enhance the physiological response to currently prescribed doses of β2-agonists. PAMs might also potentiate the airway effects of endogenous epinephrine in asthmatics. These scenarios might well result in better clinical outcomes, such as better asthma control and reduced tachyphylaxis for patients that progressively become less responsive to β2-agonists. Adverse drug responses to inhaled β2-agonists are dose dependent and in some cases drug specific (31, 32). Despite the development and use of β2-agonists in asthma and COPD that display clear selectivity toward β2ARs over β1ARs, there remains some degree of cross-reactivity, particularly at higher treatment doses. Typical β2AR-mediated cardiac effects, such as arrhythmias, increased myocardial oxygen demand, and sudden death are still a concern in asthmatic patients, despite using selective β2-agonists. While some of these cardiac events may be β2AR-mediated, off-target β2AR activation certainly plays a significant role (31, 32). The strong selectivity of Cmpd-6 for the β2AR over the closely related β1AR would preclude any increase in β1AR-mediated side effects associated with its use. The risk for these cardiac side effects may even decrease if β2AR-selective PAMs permit a lower effective dose of β2-agonist to be prescribed.

Although Cmpd-6 alone, in the absence of fenoterol, did not reduce methacholine-induced airway constriction in guinea pig lung slices, this result does not rule out the possibility that monotreatment with Cmpd-6 could have beneficial effects in asthma. Humans, unlike the isolated lung slices used in our experiments, have circulating levels of epinephrine, the βAR binding and signaling effects of which would be improved by PAMs. The fact that nonselective βAR-blockers are contraindicated in individuals with asthma (33) supports the notion that endogenous epinephrine offers a modicum of bronchodilation in this disease, whereas endogenous epinephrine alone is clearly not able to adequately counteract airway narrowing in asthmatics. This may be explained by the observation that levels of catecholamines, including epinephrine, fail to rise normally during an asthmatic exacerbation (19, 20) and in response to exercise in individuals with exercise-induced asthma (34). It is tempting to speculate that PAM-induced enhancement of βAR responsiveness to endogenous epinephrine may protect against the development and/or severity of asthma exacerbations. Thus, a strong βAR-specific PAM may improve asthma control by endogenous epinephrine and thus reduce or obviate the need for long-term use of inhaled β2-agonists without increasing the risk for epinephrine-induced adverse effects via other adrenergic receptor subtypes.

The mechanisms of βAR tachyphylaxis in asthma are not well understood and we did not use a model of βAR tachyphylaxis; thus, it is difficult to speculate as to the ability of Cmpd-6 to impact its development and/or progression. Possible molecular mechanisms for functional βAR tachyphylaxis include reduced density of cell surface receptors — such as an imbalance in ratios of receptor production to degradation and/or internalization to recycling (35, 36) — phosphorylation-mediated uncoupling of receptors from downstream Gs/CAMP signaling (37-39), increased activity of endogenous receptor desensitization by β-arrestins (13, 15, 40), some combination of all 3, or an, as yet, undiscovered mechanism (5, 14, 41). Given that Cmpd-6 enhances β2-agonist-mediated bronchoprotection while also enhancing agonist-induced β-arrestin recruitment to the β2AR, we would not anticipate that Cmpd-6 would reduce receptor desensitization. However, additional study of receptor tachyphylaxis mechanisms is needed and doing so in the context of PAMs is warranted.

PAMs like Cmpd-6 would likely be more effective in treating asthma if they were Gs-biased, since β-arrestin signaling is proinflammatory in murine models and β-arrestin binding leads to receptor desensitization. β-arrestin2 is required for the development and perpetuation of the asthma phenotype in mice (23, 42). Murine lung expression of β-arrestin2 is upregulated by allergen sensitization and challenge (13). β-arrestin2 mediates agonist-specific βAR desensitization in airway smooth muscle (15, 40), and knockout of β arrestins prevents β2-agonist-induced functional tachyphylaxis (15, 23, 40, 42). Thus, future efforts to develop Cmpd-6 analogs that are Gs-biased would be worthwhile.

In human asthma, the hallmark signs and symptoms, including airway constriction, are caused by release of histamine and leukotrienes from mast cells and other inflammatory cells (43) as well as by release of acetylcholine from airway neurons and from nonneuronal sources, such as airway epithelium and inflammatory cells (44). Allergen-induced airway constriction in our guinea pig model is mainly caused by histamine released from activated mast cells (27, 28). Allergens cause mast cell activation and degranulation via allergen-specific IgE crosslinking with the high-affinity IgE receptor, FcεRI (26). Our observation that fenoterol provided better bronchoprotection against ovalbumin-induced, compared with methacholine-induced, airway constriction could be explained by the findings that fenoterol reduces immediate antigen-induced histamine release from human (45-47) and guinea pig (48) mast cells and is better at countering tracheal contractions induced by histamine than by methacholine.
βCmpd-6, enhances the bronchoprotective effect of the β2AR. Our findings regarding Cmpd-6 specificity toward the β2AR are consistent with previous studies that showed species-dependent PAM activities toward other GPCRs (51-53). Since allosteric sites generally exhibit a greater variation between species than the orthosteric sites (54, 55), species-dependent effects may be even more prevalent for allosteric modulators. During the allosteric drug development process, species-specific differences would need to be considered not only in screening efforts (25, 56), but also in selection of appropriate animal models that relate to the pathophysiology and pharmacology of the human disease. Our studies further indicate the importance of determining the location of allosteric binding sites by structural approaches.

In conclusion, we demonstrate that the β2AR-selective PAM, Cmpd-6, enhances the bronchoprotective effect of the β2-agonist fenoterol against methacholine-induced airway narrowing in guinea pig and human lung slices as well as against allergen-induced airway narrowing in guinea pig lung slices. Our study suggests that PAMs like Cmpd-6 hold promise as adjuncts to β2-agonists to improve control of airway narrowing in asthma. Given the exceptional β2AR selectivity and ceiling effect of PAMs, Cmpd-6 may improve the pharmacological treatment of asthma and other respiratory diseases by increasing the bronchoprotective response to β2-agonists, lowering the effective dose of β2-agonists, and producing fewer side effects.

**Methods**

**Materials.** Compound-6 was synthesized as previously described (21). With the exception of BI-167107, which was synthesized as previously described (57), all of the orthosteric β2AR ligands used, methacholine, and ovalbumin were purchased from Sigma-Aldrich and sourced at a 95% or greater purity. All other chemicals were obtained from Sigma-Aldrich unless otherwise indicated. Mammalian expression plasmids for human and murine β2ARs were previously described (36) and obtained from GeneScript. The plasmid for the guinea pig β2AR was generated by insertion of the de novo synthesized coding region of the receptor into pcDNA3 through 5'-EcoRI and 3'-NotI sites by GENewIZ. The V133F mutant of the murine β2AR was created using the Quikchange Site-Directed Mutagenesis Kit (Agilent). The V133F mutation was verified by sequencing. The coding regions of all plasmids were also sequenced for their authentication.

**Cell culture and transfection.** Human embryonic kidney-293 (HEK293) cells (ATCC) stably expressing the GloSensor CAMP reporter (58) and HEK293T cells for the Tango assay with the β2V2R chimeric receptor (59) were maintained at 37°C and 5% CO2 in a humidified condition. Cells were cultured in standard MEM (Gibco) supplemented with 10% FBS and penicillin/streptomycin (GeminiBio) together with proper selection antibiotics, 100 μg/mL Hygromycin B (Invitrogen) for the GloSensor cells and 300 μg/mL Zeocin (Invitrogen), 100 μg/mL Hygromycin B, and 5 μg/mL of puromycin (Gibco) for the Tango cells. Expi293F suspension cells (Invitrogen) were maintained in Expi293 Expression Medium (Thermo Fisher Scientific) at 37°C and 8% CO2 in a humidified condition while shaking the culture flask. Each construct for expression of the β2AR from different species was transiently transfected into HEK293 GloSensor cells using FuGENE 6 (Promega) for cAMP production measurement and Expi293F cells using ExpiFectamine (Invitrogen) for membrane isolation according to the manufacturer’s instructions. All the assays and preparations were performed at approximately 48 hours after transfection.

**Measurements of CAMP production.** HEK293 cells stably expressing the GloSensor luciferase enzyme (Promega), in the absence or presence of transient transfection with each of the constructs for expressing the β2AR from different species, were plated at a density of approximately 80,000 cells on each well of a 96-well, white clear-bottom plate. At 20–24 hours after plating the cells, the GloSensor reagent (Promega) was prepared and added onto the cells in each well according to the manufacturer’s instructions. The plate was moved to a humidified incubator at 27°C, and after 1 hour incubation, cells were then treated with Cmpd-6 at an indicated concentration or a vehicle (DMSO) diluted in HBSS (Sigma-Aldrich), supplemented with 20 mM Hepes, pH 7.4, 0.05% BSA, and 100 μM 3-isobutil-1-methytxanilne (IBMX) (Sigma-Aldrich). After cells were further incubated for 20 minutes, the extent of luminescence signal was read using a ClarioStar microplate reader (BMG Labtech). When endogenously expressed β2AR in HEK293 cells was stimulated with a serial dilution of a β2 agonist, a luminescence reading was performed 10 minutes after stimulation.

**Measurement of β-arrestin recruitment.** β-Arrestin2 recruitment to the receptor was measured using the previously described Tango assay (59). HEK293T cells stably expressing the β2V2R tethered to the tetracycline transactivator (tTA) transcription factor with a Tobacco Etch Virus (TEV) protease cleavage site as a linker. The β2V2R is a chimeric receptor created by fusion of the C-terminal region of the V2 vasopressin receptor to the C-terminal-truncated β2AR to improve the extent of β-arrestin recruitment to the receptor while retaining the pharmacological profile of the WT β2AR (35). These Tango cells are also stably express human β-arrestin2 fused to the TEV protease and the tTA-driven luciferase reporter. At 20–24 hours after cells were plated on a 96-well, white clear-bottom plate at a density of approximately 50,000 cells per well, they were treated with either Cmpd-6 at 25 μM or a vehicle (DMSO) diluted in HBSS (Sigma-Aldrich), supplemented with 20 mM Hepes, pH 7.4, and 0.05% BSA. After 20 minutes incubation at 37°C, cells were stimulated with a serial dilution of a β2 agonist, followed by 6 hours further incubation at 37°C in a humidified condition. Then, after the plate was briefly cooled down to the ambient tem-
perturbation, the Bright-Glo reagent (Promega) was added following the manufacturer’s instructions to read chemiluminescence signals using a ClarioStar microplate reader (BMG Labtech).

**Radioligand Competition Binding.** Plasma membranes from the Expi293F cells (Thermo Fisher Scientific) transiently expressing each of the WT or mutant receptors were prepared as described previously (60). With each of the prepared membranes, radioligand competition binding assays were performed using a radiolabeled antagonist, [125I]-cyanopindolol (CYP) (2,200 Ci/mmol; PerkinElmer) at a concentration of 60 pM. Reactions started upon mixing the isolated membranes together with 125I-CYP, Cmpd-6 at varying concentrations, and a serial dilution of a competitor β2-agonist in an assay buffer (75 mM Tris-HCl, pH 7.4, 2 mM EDTA, pH 8.0, 12.5 mM MgCl2, 0.1% BSA, and 1 mM ascorbic acid), as indicated on each figure. Reaction mixtures were incubated for 90 minutes at ambient temperature to reach the equilibrium state. Assays were then terminated by rapid filtration of the reaction mixtures onto GF/B glass-fiber filters (Brandel) treated with 0.3% polyethyleneimine and washed with 8 mL of a cold binding buffer (75 mM Tris-HCl, pH 7.4, 2 mM EDTA, pH 8.0, 12.5 mM MgCl2) using a harvester (Brandel). The extent of 125I-CYP bound to the β2AR in isolated membranes was measured using a WIZARD 2 2-Detector Gamma Counter (PerkinElmer). Data were expressed as specific binding obtained by subtraction of nonspecific binding determined in the presence of high-affinity propranolol at 20 nM.

*Mice.* Seven-to-14-week-old male C57BL/6J naive mice (n = 5–7) were purchased from the Jackson Laboratory and were housed in pathogen-free temperature- and humidity-controlled facilities at Duke University. Mice were euthanized at the end of airway responsiveness measurement.

**Airway responsiveness measurements in vivo.** Airway hyperresponsiveness (AHR) was measured using the forced oscillation technique as described previously (42). In brief, anesthetized (sodium pentobarbital 85 mg/kg, i.p.), tracheotomized, and skeletal muscle-relaxed (pancuronium bromide 0.25 mg/kg, i.v.) mice were i.v. administered with Cmpd-6 (50 nM in 100% DMSO; 10 mg/kg), or equivalent volume of 100% DMSO as vehicle control for 10 minutes before the start of the bronchoprotection or bronchodilation protocol as described below. For the bronchoprotection protocol, bronchospasm was induced by jugular vein i.v. administration of increasing doses of methacholine (25, 50, 100, 200, 400 μg/kg) or ovalbumin by injecting a suspension containing 100 mg/mL ovalbumin and 100 mg/mL Al(OH)3 in saline. Each animal was injected with 1.0 mL of suspension: 0.5 mL was injected i.p. and 0.5 mL was divided over 7 s.c. sites close to lymph nodes in the neck, paws, and lumbar regions (24). Animals were euthanized at least 4 weeks after sensitization.

**Guinea pig lung slices.** Precision-cut lung slices were prepared as described previously (61, 62). A 3% solution of low melting-point agarose (Euthal, Patterson Veterinary) was added to prevent postmortem airway constriction. After filling, the lungs were covered with ice for 30 minutes to solidify the agarose for slicing. Lungs were removed and cylindrical tissue cores (diameter 15 mm) were prepared followed by slicing the tissue in ice cold lung slice buffer (1.8 mM CaCl2, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, 1.2 mM NaHPO4, 16.7 mM glucose, 26.1 mM NaHCO3, 25.2 mM Hepes, pH = 7.2) using a tissue slicer (CompresstomeTM VF-300 micrometre, Precision Instruments). Lung slices were cut at a thickness of 500 μm and washed several times with slicing buffer to remove debris and washout the isoproterenol. Slices were incubated overnight in a 60 mm dish in sterile incubation buffer: MEM composed of lung slice buffer supplemented with 0.5 mM sodium pyruvate, 1 mM glutamine (Gibco), MEM-amino acids mixture (1:50), MEM-vitamins mixture (1:100; Gibco), and penicillin-streptomycin (1:100; Gibco), pH 7.2, at 37°C in a CO2- and humidity-controlled atmosphere.

After washing the slices in medium, individual guinea pig lung slices were mechanically maintained with a Teflon ring with an inner diameter of 7 mm and covered with 1 mL of incubation buffer. Airway responsiveness to increasing concentrations of methacholine (10 nM to 3 mM, using cumulative concentrations in half-log increments) or ovalbumin (1 pg/mL to 1 mg/mL, using cumulative concentrations in log increments) was measured in lung slices from naive or IgE-sensitized guinea pigs, respectively, using video-assisted microscopy (Nikon Eclipse TS 100) as previously described (61). Lungs slices were incubated with various concentrations of the β2-agonist fenoterol (0.1, 1, 10, and 100 μM) and/or Cmpd-6 (25 μM) before the addition of methacholine or ovalbumin. Image acquisition software (NIS-Elements, Nikon) was used to quantify airway luminal area. Images of the airways were acquired every 2 seconds during the whole course of the experiment, starting 2 minutes before the addition of any agent to allow for baseline measurements of the airway caliber. For each methacholine or ovalbumin concentration, the maximal airway constriction was expressed as percentage of the initial (baseline) airway luminal area and plotted against that concentration. The maximal constriction (Emax, percentage airway closure) and concentration of methacholine or ovalbumin inducing 50% of the maximal response (EC50) were determined for each concentration-response curve.
**Human lung slices.** Precision-cut human lung slices were purchased from AnaBios and stored in a liquid nitrogen tank. Lung tissue was obtained postmortem and consent for research was obtained by AnaBios for all donors. Characteristics of the donors of the lung tissue are shown in Supplemental Table 2. Slices were rapidly thawed following instructions from AnaBios. In short, slices were washed 3 times in DMEM/F12 medium supplemented with penicillin-streptomycin (1:100) followed by a wash in incubation buffer (1.8 mM CaCl2, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, 1.2 mM NaHPO4, 16.7 mM glucose, 26.1 mM NaHCO3, 25.2 mM Hepes, 0.5 mM sodium pyruvate, 1 mM glutamine, MEM-amino acids mixture (1:50), MEM-vitamins mixture (1:100) and penicillin-streptomycin (1:100), pH = 7.2) and subsequently placed overnight in incubation buffer at 37°C in a CO2- and humidity-controlled atmosphere.

After washing, individual human lung slices were mechanically maintained with a Teflon ring with an inner diameter of 7 mm and covered with 1 mL of incubation buffer. Airway responsiveness to increasing concentrations of methacholine (1 nM to 300 μM, using cumulative concentrations in half-log increments) was measured using video-assisted microscopy (Nikon Eclipse TS 100). Lungs slices were incubated with the β2-agonist fenoterol (1 and 10 μM) for 30 minutes before the addition of methacholine. For the condition where fenoterol and Cmpd-6 were used, lung slices were incubated with 25 μM Cmpd-6 5 minutes before the addition of 1 μM fenoterol. Image acquisition software (NIS-Elements, Nikon) was used to quantify airway luminal area. The initial airway intraluminal area was assessed after fenoterol incubation and before methacholine incubation. Airway constriction in response to each methacholine concentration was expressed as percentage of the initial airway luminal area. The maximal constriction (Fmax, percentage airway closure) to methacholine and −log of the concentration of methacholine inducing 50% of the maximal response (pD2) were determined for each concentration-response curve.

**Statistics.** Statistical differences were determined using a 1-way ANOVA followed by either Bonferroni’s (Tables 2 and 3) or Tukey’s (Figure 3D) posthoc test. General linear model repeated measures ANOVA with Tukey’s posthoc test was used to determine differences in airway responsiveness in vivo (Figure 2). Differences were considered to be statistically significant when P < 0.05. All other analyses were performed using paired 2-tailed Student’s t tests. All curve fits were generated using the software program GraphPad Prism.

**Study approval.** All animal care and experimental procedures complied with the animal protection and welfare guidelines and were approved by the Institutional Animal Care and Use Committee of Palm Beach Atlantic University or Duke University and are reported in compliance with the ARRIVE guidelines (63).

**Data availability.** Data are available in the Supporting Data Values XLS file. All data needed to evaluate the conclusions of this study are available in the paper. Cells for the Tango β-arrestin recruitment assay were a gift from Gilad Barnea (Brown University, Providence, Rhode Island, USA). Cells for the GloSensor cAMP production assay can be obtained upon request to RJL and used for academic research with a standard academic material transfer agreement (MTA).

**Author contributions**
SA, HM, JKLW, SL, AH, and RJL conceived and designed experiments. SA, HM, JKLW, SL, AH, and HCS performed experiments and analyzed the data. AWK synthesized the allosteric compound (Cmpd-6). SA, HM, JKLW, and RJL wrote the manuscript. All authors contributed feedback to the manuscript and approved the submitted version. SA, HM, and JKLW share the first author position. The alphabetical order of their last names was used to assign the authorship order among these authors.

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