Potent Prearranged Positive Allosteric Modulators of the Glucagon-like Peptide-1 Receptor

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Drugs that allosterically modulate G protein-coupled receptor (GPCR) activity display higher specificity and may improve disease treatment. However, the rational design of compounds that target the allosteric site is difficult, as conformations required for receptor activation are poorly understood. Guided by photopharmacology, a set of prearranged positive allosteric modulators (PAMs) with restricted degrees of freedom was designed and tested against the glucagon-like peptide-1 receptor (GLP-1R), a GPCR involved in glucose homeostasis. Compounds incorporating a trans-stilbene comprehensively outperformed those with a cis-stilbene, as well as the benchmark BETP, as GLP-1R PAMs. We also identified major effects of ligand conformation on GLP-1R binding kinetics and signal bias. Thus, we describe a photopharmacology-directed approach for rational drug design, and introduce a new class of stilbene-containing PAM for the specific regulation of GPCR activity.

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G protein-coupled receptors (GPCRs) are critical for organism homeostasis by converting signals encoded by extracellular molecules into an appropriate cell response.[1] Classically, ligands bind the extracellularly located orthosteric face of the GPCR, leading to conformational changes and activation of second messenger pathways.[1] GPCRs are also subject to allosteric regulation, whereby small molecular entities influence orthosteric activation.[2] Such allosteric modulators are therapeutically desirable, as they demonstrate excellent specificity and selectivity.[24]

Recently, we described allosteric optical control of the glucagon-like peptide-1 receptor (GLP-1R),[3] a prototypical class B GPCR involved in the maintenance of blood glucose levels and a blockbuster target for type 2 diabetes treatment.[4] As peptide ligands that target the GLP-1R must be injected, the development of orally available small-molecule GLP-1R activators is a priority. In our previous work, an azobenzene-containing molecular photoswitch termed PhotoETP was designed and synthesized,[5] based upon an “azologue”[6] of the known GLP-1R positive allosteric modulator (PAM) 4-(3-(benzoxyl)phenyl)-2-(ethylsulfanyl)-6-(trifluoro-methyl)pyrimidine (BETP).[7] Notably, signaling responses to glucagon-like peptide-1 (GLP-1) degradation products could be sculpted by the application of blue light to induce formation of the trans-isomer.[3] We hypothesized that maximal GLP-1R activation by PAMs requires a molecule with fewer degrees of freedom. As the molecular conformations/interactions required for allosteric activation are largely unknown, we thought that this may be achievable by “pre-arranging” the molecule.

To produce PAMs with improved activity at the GLP-1R, the PhotoETP azobenzene diazene unit, which exhibits non-binary photostationary states,[5] was replaced with a C=C fragment to introduce a stilbene that displays single isomers. A range of prearranged molecules were synthesized and tested for their ability to potentiate GLP-1R signaling responses. In all cases, compounds incorporating the trans-stilbene outperformed those with cis-stilbene, in addition to native BETP. As such, we introduce a photopharmacology-based strategy to direct the rational design of prearranged PAMs, with broad applicability to the allosteric regulation of GPCRs involved in health and disease.

BETP adopts a conformationally free benzyl ether when bound,[3] suggesting that, upon activation of the GLP-1R by orthosteric ligands, it is able to rearrange its shape to fully engage the allosteric site (Figure 1a). This motion is restricted by light in PhotoETP, which presents a photosomerizable azobenzene in place of the O-benzyl group (Figure 1b). We reasoned that replacement of the diazene bridge in PhotoETP...
by a C–C moiety to introduce a stilbene would allow production of prearranged PAMs where the cis- and trans-states are mimicked, but without complications arising from photostationary states. Stilbenes have the added advantage of being more drug-like than azobenzenes and may demonstrate better metabolic stability in terms of double-bond cleavage in the intestine and the possible liberation of reactive anilines. Furthermore, BETP uses an ethyl sulfoxide as a leaving group that is replaced by covalent cysteine attachment on the receptor to give ethyl sulfenic acid as an unstable and, therefore, quickly-cleared leaving group. It also has been reported to undergo covalent labelling towards the formation of the Glp-1R positive allosteric modulator

To obtain BMTP, that is, the methyl analogue of the lead compound BETP, as a first model compound, boronic ester 1 was coupled under Suzuki–Miyaura conditions with chloropyrimidine 2 to give BMTP in 94% yield (Scheme 1a).imoto et al. published the synthesis of BMTP and SETP in a comparable synthetic sequence. Commencing with benzyltriphenylphosphonium bromide (4) and 3-bromobenzaldehyde (5) that underwent a non-stereoselective Wittig reaction at room temperature in THF with LiHMDS as base, cis- and trans-bromo stilbene 6 could be separated through flash column chromatography (FCC) and isolated in 79% overall yield (Scheme 1b). Single crystals of trans-6 were obtained, which unambiguously provided the isomeric identity, and this was further confirmed by the coupling constant $J_{HH}$ of the olefinic protons through $^1$H NMR spectroscopy, being approximately 12 and 16 Hz for cis- and trans-isomers, respectively. Next, Miyaura coupling with $B_3$pin$_3$ using $PdCl_2(dppf)$ under standard conditions, employing DMSO as the solvent and KOAc as the base, gave access to cis- and trans-boronic ester 7 in good yields (78 and 77%, respectively), which were subsequently subjected to Suzuki–Miyaura Pd cross-coupling with chloropyrimidine 2 to yield the cis- and trans-thioether precursor 8 in good 76% and excellent 95% yields, respectively. Progressing towards SETP, thioether 6 was monooxidized with one equivalent of mCPBA to give cis- and trans-SETP in 91 and 89%, respectively.

To progress to the ethyl sulfoxide, thioether 6 was oxidized with two equivalents of mCPBA to give the corresponding sulfoxide 9 (yield for cis: 95%; trans: 85%), which was subjected to aromatic substitution with a mixture of ethylsulfide/sodium thioethanolate to ethyl thioether 10 (cis: 89%; trans: 80%) in a microwave reactor before final installation of the ethyl sulfoxide with one equivalent of mCPBA to obtain cis- and trans-SETP in 74 and 85% yields, respectively. Lastly, we obtained a crystal structure of BETP by leaving a DMSO solution open to the atmosphere for 2 weeks (Scheme 1c), providing atomic coordinates and showing that BETP is stable for this timeframe in solution at room temperature and under benchtop light.
As expected, \( \text{BETP} \) and \( \text{BMTP} \) induced strong cytosolic Ca\(^{2+}\) allosterically enhanced cAMP responses, it was the only PAM that enhanced the Ca\(^{2+}\) response to GLP-1(7–36)NH\(_2\) when pre-incubated, as previously described.\(^{24}\) This may reflect depletion of accessible intracellular Ca\(^{2+}\) stores at a rate dependent on the intrinsic activity of the PAM (Figure S4).

PAMs increase receptor affinity for extracellular ligand, coupling to intracellular effectors, or both.\(^{11}\) We employed a time-resolved Förster resonance energy transfer (TR-FRET) approach to measure ligand-receptor binding in real-time, whereby FITC-conjugated orthosteric ligand binding to SNAP-GLP-1R self-labeled with Lumi4-Tb leads to an increase in FRET.\(^{13}\) For further details of this technique, see Section 6.6 in the Supporting Information. We determined that GLP-1R PAMs substantially slow down dissociation of GLP-1(7–36)NH\(_2\)-FITC, increasing residence time\(^{14}\) (Figure 3a), and also decreasing the rate of association (Figure 3b). The overall effect was to increase binding affinity of GLP-1(7–36)NH\(_2\)-FITC approximately fivefold (Figure 3c). \( \text{trans-SETP} \) and \( \text{trans-SMTP} \) increased the residence time and binding affinity more than their cis-counterparts.

The GLP-1R undergoes extensive internalization after agonist stimulation, and ongoing cAMP generation by internalized receptors may play a role in GLP-1R signaling.\(^{18} \) We hypothesized that the PAMs described here, which unlike peptide ligands are membrane-permeating, might access and modulate the behavior of pre-internalized ligand–receptor complexes (Figure 3d). After complete GLP-1R internalization with 100 nM GLP-1(7–36)NH\(_2\)-FITC (Figure 3e) and washout of remaining extracellular ligand, a steady reduction in FRET was detected, indicative of ligand–receptor dissociation within endosomes. However, when \( \text{BETP}, \text{BMTP}, \) and prearranged PAMs were applied to the cell immediately post-washout, marked reductions in dissociation from endosomal receptors were observed (Figure 3f, g). This effect was most marked with \( \text{trans-SMTP} \).

In the present study, we describe a photopharmacology-inspired strategy for the rational design of potent GLP-1R PAMs based upon the allosteric photoswitch Phe-Val-Val-Val.\(^{20}\) By restricting degrees of freedom using stilbenes, it could be shown that compounds prearranged as their trans-isomers were more potent than other PAMs, including \( \text{BETP} \). Such findings likely reflect the requirement for bound PAMs to undergo fine conformational changes at the allosteric site for full activation in response to orthosteric binding.\(^{26}\) Prearrangement may circumvent this by stabilizing molecule dynamics, allowing PAMs to adopt an allosteric ‘ON’ state when unbound. Moreover, replacement of the ethyl sulfoxide moiety with a methyl sulfoxide maintained full activity, whilst promoting cheaper and faster access to PAMs and prearranged PAMs from commercially available chemicals in high yields. Although the differences in signaling described here for the prearranged PAMs may seem small, it should be noted that GLP-1R PAMs allow less active GLP-1 metabolites to signal at the orthosteric site.\(^{28}\)
known signaling intermediaries in the context of cysteine modification.\[17\] In addition, further studies using recently described conformational FRET sensors,\[18\] or concentration responses to calculate alpha values,\[12, 19\] are required to unambiguously demonstrate the differential actions of the prearranged PAMs at the GLP-1R. It should be noted that the herein-described prearranged PAMs, as well as BETP, exist as racemates, and in the future it will be interesting to study the labeling kinetics of the separate enantiomers before chirality is lost through covalent modification of GLP-1R.

Finally, we provide the first demonstration that GLP-1R PAMs can markedly increase agonist residence time, proposed as a therapeutic strategy to drive sustained responses in vitro and in vivo.\[20\] These molecules can also directly access pre-internalized GLP-1Rs to modulate ligand–receptor binding within endosomes. They could, therefore, be used to prolong non-canonical cAMP signaling from internalized GLP-1Rs\[19\] to delineate effects of membrane versus endosomal GLP-1R signaling, or study the influence of occupancy on post-endocytic receptor trafficking.\[21\]

In summary, we unveil a new class of positive allosteric modulator PAMs that are prearranged. These compounds perform better than their compacted stablemates (i.e. those incorporating a cis-stilbene), as well as benchmark PAM, BETP, and also display signal bias. Thus, prearranged PAMs provide a template for the production of newer and more potent allosteric modulators of the GLP-1R, with broad-applicability to GPCR research and drug discovery.

**Experimental Section**

Experimental details including synthesis, spectroscopic and spec- trometric characterization, and biology can be found in the Supporting Information. CCDC 1530565 and 1530566 contain the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

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Conflict of Interest

The authors declare no conflict of interest.

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