Allosteric modulation of endogenous metabolites as an avenue for drug discovery


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Running Title: Allosteric modulation of metabolites at GPCRs

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Abbreviations: A1-AR – Adenosine receptor subtype 1, ACh – Acetylcholine, BSA - bovine serum albumin, Ch – Choline, CHO - Chinese hamster ovary, DM - diabetes mellitus, DMEM - Dulbeccos modified eagles medium, DPPIV - dipeptidyl peptidase IV, ERK1/2 - extracellular signal-regulated kinases 1 and 2, FBS - fetal bovine serum, Gα - α subunit of G protein, GLP-1R - glucagon-like peptide 1 receptor, GPCR - G protein-coupled receptor, IBMX - 3-Isobutyl-1-methylxanthine, M2 mAChR – muscarinic acetylcholine receptor subtype 2, MAPK - mitogen activated protein kinases.
Abstract

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors and a key drug target class. Recently, allosteric drugs that can co-bind with, and modulate the activity of, the endogenous ligand(s) for the receptor have become a major focus of the pharmaceutical and biotechnology industry for the development of novel GPCR therapeutics. This class of drugs has distinct properties when compared to drugs targeting the endogenous (orthosteric) ligand binding site that include the ability to sculpt cellular signalling, and to respond differently in the presence of discrete orthosteric ligands; a behaviour termed “probe dependence”. Here, using cell signalling assays combined with ex vivo and in vivo studies of insulin secretion, we demonstrate that allosteric ligands can cause marked potentiation of previously “inert” metabolic products of neurotransmitters and peptide hormones, a novel consequence of the phenomenon of probe dependence. Indeed, at the muscarinic M2 receptor and glucagon-like peptide-1 receptor, allosteric potentiation of the metabolites, choline and GLP-1(9-36)NH2, respectively, was ~100-fold and up to 200-fold greater than that seen with the physiological signalling molecules acetylcholine and GLP-1(7-36)NH2. Modulation of GLP-1(9-36)NH2 was also demonstrated in ex vivo and in vivo assays of insulin secretion. This work opens up new avenues for allosteric drug discovery by directly targeting modulation of metabolites, but it also identifies a behaviour that could contribute to unexpected clinical outcomes if interaction of allosteric drugs with metabolites is not part of their preclinical assessment.
Introduction

G protein-coupled receptors (GPCRs) are the largest superfamily of cell surface proteins, and play crucial roles in virtually every physiological process. Their widespread abundance and ability to couple to a variety of signalling and effector systems makes them extremely attractive targets for drug development (Christopoulos, 2002). Traditionally, GPCR agonist drug discovery efforts have focused on either increasing the endogenous orthosteric agonist concentration by inhibiting its breakdown, or targeting the orthosteric binding site of the receptor with surrogate agonists. However, in recent years there has been a significant increase in the identification of small molecules that target topographically distinct allosteric sites on GPCRs (May et al., 2007b). Binding of allosteric ligands can elicit a conformational change in the receptor while still allowing the orthosteric ligand to bind, thus modulating the pharmacological properties (affinity and/or efficacy) of the orthosteric ligand, in addition to potentially activating the receptor in the absence of orthosteric ligand. Allosteric drugs have substantial potential as therapeutics, as they can provide novel receptor selectivity, in addition to offering the possibility to ‘fine tune’ existing physiological responses while maintaining the spatial and temporal characteristics of innate endogenous signalling (Christopoulos and Kenakin, 2002).

One characteristic of allostery is the phenomenon of ‘probe-dependence’, whereby the extent and direction of an allosteric interaction varies with the nature of the orthosteric ligand occupying the receptor (Kenakin, 2005). Furthermore, biased signalling leading to pathway-selective allosteric modulation can also result (Leach et al., 2007). These concepts are particularly relevant to receptor systems that have multiple endogenous ligands, such as the glucagon-like peptide-1 receptor (GLP-1R) system (Baggio and Drucker, 2007), because probe-dependence can lead to different endogenous agonists of the same GPCR being
allosterically modulated in strikingly different ways (Koole et al., 2010). However, a hitherto-unappreciated extension of this phenomenon is the possibility that endogenous metabolites of GPCR agonists, which may normally be minimally active in their own right, can also be influenced by allosteric modulators. Indeed, a recent study reported that the allosteric compound LUF6000 can enhance signalling by inosine (the metabolite of adenosine) at the adenosine A₃ receptor (A₃-AR) (Gao et al., 2011). Although drug discovery programs focusing on developing small molecule allosteric drugs invariably screen for compounds that modulate responses mediated by the predominant orthosteric receptor agonist, it is currently not routine to incorporate similar studies on endogenous metabolites. However augmentation of metabolite signalling could offer a new therapeutic avenue for development of novel drugs, especially in systems where the endogenous ligand is rapidly degraded to its (ostensibly) inactive metabolite (Fig. 1).

In this study, we investigated the potential to allosterically modulate the activity of the predominant, inactive, metabolite of the physiological ligand at three different GPCRs for which small molecule allosteric modulators have been described; the GLP-1R (Knudsen et al., 2007; Koole et al., 2010; Sloop et al., 2010), the M₂ muscarinic acetylcholine receptor (mAChR; (Valant et al., 2012)) and the adenosine A₁ receptor (A₁-AR; (Bruns and Fergus, 1990)) (Supplemental Fig. 1). In each instance, we find a significant degree of allosteric potentiation of the endogenous metabolite by the allosteric modulator. Moreover, for the GLP-1R, we also provide evidence of the allosteric modulator engendering biased signalling in terms of enhancing cAMP signalling mediated by the metabolite, whilst having little effect on ERK1/2 phosphorylation or intracellular Ca²⁺ mobilisation. Ex vivo studies using static cultures of rat pancreatic islets, as well as in vivo experiments also revealed that allosteric modulation of the GLP-1 metabolite resulted in glucose-dependent insulin secretion. To our
knowledge, this is the first study to explore the potential to allosterically modulate endogenous metabolites of multiple GPCR ligands at their respective receptors. The outcomes could have significant implications in development and screening of novel therapeutics in drug discovery programs.
Materials and Methods

Materials. Dulbecco’s modified Eagle’s medium (DMEM), hygromycin-B and Fluo-4 acetoxymethyl ester were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia). AlphaScreen reagents, Bolton-Hunter reagent [\(^{125}\)I] and 384-well ProxiPlates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). SureFire extracellular signal-regulated kinases 1 and 2 (ERK1/2) reagents were generously provided by TGR Biosciences (Adelaide, SA, Australia). Compound 2 was generated according to a method published previously (Teng et al., 2007) to a purity of > 95 %, and compound integrity was confirmed by nuclear magnetic resonance (NMR). BETP and LY2033298 were provided by Eli Lilly and GLP-1 peptides were purchased from American Peptide (Sunnyvale, CA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or BDH Merck (Melbourne, VIC, Australia) and were of an analytical grade.

Transfections and Cell Culture. GLP-1R, M\(_2\) mAChR and A\(_1\)-AR were isogenically integrated into FlpIn-Chinese hamster ovary (FlpInCHO) cells (Invitrogen) and selection of receptor-expressing cells accomplished by treatment with 600 \(\mu\)g/ml hygromycin-B as previously described. Transfected and parental FlpInCHO cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and incubated in a humidified environment at 37\(^\circ\)C in 5% CO\(_2\). For all whole cell assays, cells were seeded at a density of 30,000 cells/well into 96-well culture plates and incubated overnight at 37\(^\circ\)C in 5% CO\(_2\) prior to assaying.

Radioligand Binding Assay. GLP-1R experiments: Growth media was replaced with binding buffer [DMEM containing 25 mM HEPES and 0.1% (w/v) BSA] containing 0.5 nM \(^{125}\)I-exendin(9–39) and increasing concentrations of unlabeled peptide in the presence and
absence of increasing concentrations of allosteric ligand. Cells were incubated overnight at 4 °C, followed by three washes in ice cold PBS to remove unbound radioligand. 0.1 M NaOH was added, and radioactivity determined by gamma counting. For GLP-1R experiments, nonspecific binding was defined by 1 μM exendin(9–39).

M2 mAChR experiments: M2 mAChR FlpIn-CHO membrane homogenates (5–20 μg) were incubated in a 500-μl total volume of assay buffer containing [3H]NMS (0.5 nM) with a range of concentrations of choline in the absence and presence of LY2033298 (1 μM and 10 μM) at 30°C for 90 min. All assays were performed in the presence of guanosine-5'-[(βγ-imino)triphosphate. For all experiments, nonspecific binding was defined by 10 μM atropine, and the effects of vehicle were also determined. Incubation was terminated by rapid filtration through Whatman GF/B filters using a cell harvester (Brandel Inc., Gaithersburg, MD). Filters were washed three times with 3-ml aliquots of ice-cold 0.9% NaCl buffer and dried before the addition of 4 ml of scintillation mixture (UltimaGold; PerkinElmer Life and Analytical Sciences). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined in disintegrations per minute using scintillation counting.

cAMP Assays. cAMP accumulation assays were carried out using the AlphaScreen SureFire kit as previously described (Koole et al., 2010). Cells were stimulated with peptide ligand and/or allosteric ligand and incubated for 30 min at 37 °C in 5 % CO2. cAMP accumulation was measured after 30 min of cell stimulation. All values were converted to concentration of cAMP, and data were subsequently normalised to the maximum response elicited by GLP-1(7-36)NH2.
ERK1/2 Phosphorylation Assay. Receptor-mediated ERK1/2 phosphorylation was determined by using the AlphaScreen ERK1/2 SureFire protocol as described previously (May et al., 2007a). Initial ERK1/2 phosphorylation time course experiments were performed over 1 h to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by agonists. For GLP-1R all responses peaked at 7 min, for M₂ mAChR, ACh and Ch responses peaked at 5 min and LY2033298 peaked at 8 min. For A₁-AR, adenosine and inosine peaked at 5 min, PD81723 and VCP171 peaked at 7 min. Subsequent concentration-response curves were constructed at the peak time point for each receptor/ligand combination.

Intracellular Ca²⁺ Mobilization Assay. Intracellular Ca²⁺ mobilization determined as described previously (Werry et al., 2005). Fluorescence was determined immediately after drug addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings were taken every 1.36 s for 120 s. Concentration-response curves were constructed from the peak response, calculated using five-point smoothing, followed by correction against basal fluorescence.

[^35S]GTPγS Binding Assay. [^35S]GTPγS binding was determined as described previously (Valant et al., 2012). M₂ mAChR FlpIn-CHO cell membranes (5-25 µg) were equilibrated in a 500 µl total volume of assay buffer containing 10 µM guanosine 5’-diphosphate and a range of concentrations of ligands (ACh or Ch) in the absence or presence of LY2033298 (0.1 to 10 µM) at 30 °C for 60 min. After this time, 50 µl of [^35S]GTPγS (1 nM) was added, and incubation continued for 30 min at 30 °C. Incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandell cell harvester (Gaithersburg, MD). Filters were washed three times with 3 ml aliquots of ice-cold 0.9 % NaCl buffer and dried before the addition of 4 ml of scintillation mixture (Ultima Gold, PerkinElmer Life Sciences).
were then left to stand until the filters became uniformly translucent before radioactivity was
determined in dpm using scintillation counting.

**Ex vivo pancreatic islet assays and in vivo IVGTT studies.** Animals were maintained in
accordance with the Institutional Animal Use and Care Committee of Eli Lilly and Company
and the NIH “Guide for the Use and Care of Laboratory Animals”.

**Ex vivo pancreatic islet assays.** The procedures for isolating islets and performing the
insulin secretion assays were previously described (Sloop et al., 2010). Islets were isolated
from pancreases of male Sprague Dawley rats using Hank’s balanced salt solution buffer
(HBSS, Sigma, St. Louis, Missouri, USA) containing 2 % bovine serum albumin (BSA,
Applichem, Boca Raton, Florida, USA) and 1 mg/ml collagenase (Sigma, St. Louis,
Missouri, USA). Islets were purified using Histopaque (Histopaque-1077: Histopaque-11991
mixture, Sigma, St. Louis, Missouri, USA) gradients and cultured overnight in RPMI-1640
medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen,
Carlsbad, California, USA). For the insulin secretion assays, islets were cultured at 37 °C for
90 min in Earl’s balanced salt solution (EBSS, Invitrogen, Carlsbad, California, USA)
containing the indicated concentrations of glucose and treatment conditions. Insulin that was
released into the medium was measured using HTRF technology (Cisbio Bioassays, Bedford,
Massachusetts, USA).

**In vivo IVGTT studies.** Male Wistar rats were purchased from Harlan (Indianapolis,
Indiana, USA) and maintained on a 12:12 hour light-dark cycle at 21°C. For the studies, rats
were fasted overnight and anaesthetized the next morning with 60 mg/kg Nembutal
(Lundbeck, Deerfield, Illinois, USA). Catheters were then surgically inserted into the jugular
vein and carotid artery for compound and/or peptide infusions and blood collection, respectively. For animal treatment, BETP was solubilized in a dosing solution containing 10% ethanol/solutol, 20% polyethylene glycol-400, and 70% phosphate-buffered saline (pH 7.4) and infused intravenously alone or in combination with GLP-1(9-36)-NH₂ (Bachem, Torrance, California, USA) formulated in saline containing 0.1% albumin. Blood was collected to determine glucose, insulin and total GLP-1 levels following administration of an intravenous glucose bolus of 0.5 g/kg. Plasma levels of glucose were measured using a Hitachi 912 clinical chemistry analyzer (Roche, Indianapolis, Indiana, USA), and insulin and total GLP-1 levels were determined using electrochemiluminescence assays for each (Meso Scale, Gaithersburg, Maryland, USA).

**Data Analysis.** All data obtained were analysed in GraphPad Prism 5.0.2 (GraphPad Software Inc.; San Diego, CA, USA). Radioligand inhibition binding data were fitted to a one site inhibition mass action curve. Where possible, in whole cell ligand interaction studies, data were fitted to the following two forms of an operational model of allosterism and agonism (27, 28) to derive functional estimates of modulator affinity and cooperativity.

\[
E = \frac{E_m \left( \tau_A [A] (K_B + \alpha \beta [B]) + \tau_B [B] K_A \right)^n}{\left( [A] K_B + K_A K_B + [B] K_A + \alpha [A] [B] \right)^n + \left( \tau_A [A] (K_B + \alpha \beta [B]) + \tau_B [B] K_A \right)^n} \tag{1}
\]

\[
E = \frac{E_m \left( \tau_A [A] (K_B + \alpha \beta [B]) + \tau_B [B] EC_{50} \right)^n}{EC_{50}^n \left( K_B + [B] \right)^n + \left( \tau_A [A] (K_B + \alpha \beta [B]) + \tau_B [B] EC_{50} \right)^n} \tag{2}
\]
where $E_m$ is the maximum attainable system response for the pathway under investigation, 
[A] and [B] are the concentrations of orthosteric agonist and allosteric modulator/agonist, respectively, $K_B$ is the dissociation constant of the allosteric modulator, $EC_{50}$ is the concentration of orthosteric (full) agonist yielding 50% of the response between minimal and maximal receptor activation in the absence of allosteric ligand, $n$ is a transducer slope factor linking occupancy to response, $\alpha$ is the binding cooperativity factor, $\beta$ is an empirical scaling factor describing the allosteric effect of the modulator on orthosteric agonist signaling efficacy, respectively, and $\tau_A$ and $\tau_B$ are operational measure of the ligands’ respective signaling efficacies that incorporate receptor expression levels and efficiency of stimulus-response coupling. Equation 1 was used in interaction studies performed between allosteirc ligand and a partial agonist, while equation 2 was used when the modulator was interacted with full agonists, depending on the pathway investigated. This is so because equation 2 is only valid in cases where the orthosteric agonist has high efficacy ($\tau >> 1$) such that $K_A$ is $>>$ [A]. For all other data, concentration-response curves were fitted with a three-parameter logistic equation.
Results

Allosteric modulation of GPCR agonist metabolites is potentially a widespread phenomenon.

To validate our hypothesis that metabolites of endogenous ligands can be allosterically modulated at the GPCR of the parental ligand, we performed an initial screen using a representative allosteric ligand for three different model systems; the M2 mAChR, the A1-AR and the GLP-1R. Recently, we characterized LY2033298 as an allosteric modulator of the M2 mAChR (Valant et al., 2012). PD81723 is a well accepted allosteric modulator of the A1-AR (Bruns and Fergus, 1990) and we have also recently identified a series of low molecular weight pyrimidine-based compounds that activate the GLP-1R allosterically, the most potent representative being BETP (previously described as Compound B) (Sloop et al., 2010). These three ligands (Supplemental Fig. 1D) were selected as representative modulators for each receptor respectively. Both the M2 mAChR and the A1-AR are predominantly coupled to G\(_{\alpha_i}\)–proteins, whereas the GLP-1R is primarily coupled to G\(_{\alpha_s}\). Therefore, in the initial screen ERK1/2 phosphorylation was assessed for both the M2 mAChR and the A1-AR, whereas cAMP accumulation assays were performed for the GLP-1R. All data were analysed using an operational model of allosterism to derive global cooperativity estimates (\(\alpha\beta\); a composite cooperativity factor quantifying allosteric modulation of the orthosteric ligand affinity (\(\alpha\)) and efficacy (\(\beta\))) (Table. 1).

The cognate agonist for the M2 mAChR, ACh, is rapidly converted to its inactive metabolites, choline (Ch) and acetate, in the synaptic cleft by acetylcholinesterase (Birks and Macintosh, 1957) (Supplemental Fig. 1A). In this study, Ch exhibited greater than 1000-fold lower potency in ERK1/2 phosphorylation compared to the parent agonist ACh (Fig. 2A-B). However, LY2033298 strongly potentiated the ERK1/2 response of Ch, to a greater extent.
(112-fold) than that of ACh itself (Fig. 2A and 2B, Table 1). In addition, assessment using a more proximal assay of M2 mAChR activation (GTPγS binding) revealed LY2033298 potentiated the response to both ACh and Ch, but this effect was much greater for the metabolite (Supplemental Fig. 2, Table 1). Competition binding assays revealed weak positive modulation of Ch affinity (13-fold) (Supplemental Fig. 3) indicating that potentiation of Ch in functional assays is principally via efficacy modulation. In our earlier study, we showed that LY2033298 also displayed positive cooperativity with ACh in binding affinity (16-fold) indicating that there is no efficacy modulation by LY2033298 with the parent agonist (Valant et al., 2012). Similarly to ACh, adenosine is also rapidly metabolised (by adenosine deaminase) to inosine (Plagemann et al., 1985) (Supplemental Fig. 1B); Inosine displayed greater than 1000-fold lower potency at the A1-AR in ERK1/2 phosphorylation compared to its parent ligand, adenosine. In addition, this response was significantly potentiated by the allosteric modulator, PD81723 (Fig. 2C and D); although in this instance the degree of potentiation was no greater than that observed with adenosine (Table 1). At the GLP-1R, BETP displayed very weak partial agonism in cAMP accumulation (Fig. 2E and F), but had no effect on cAMP responses mediated by GLP-1(7-36)NH2 in interaction assays (Fig. 2E). In the absence of allosteric modulation, the metabolite GLP-1(9-36)NH2 only exhibited very weak partial agonism for cAMP, with 1000-fold lower potency and only approximately 15% of the maximal signal compared to GLP-1(7-36)NH2 (Fig. 2F). However, this weak response was strongly potentiated in a concentration-dependent manner by BETP (Fig. 2F). Cooperativity estimates could not be derived for this data set as the operational model of allosterism could not adequately describe the data. Regardless, there is a strikingly strong positive allosteric effect with both an increase in potency (pEC50 shift from 6.4 ± 0.08 to 7.5 ± 0.07) and maximal agonist effect (Emax shift from 15 ± 3 % to 99 ± 4 %) of GLP-1(9-36)NH2-mediated response (Fig. 2F). For all ligands studied, no response was seen in
untransfected cells. Collectively these data identify a novel consequence of allosteric drug action, specifically, the augmentation of metabolite signalling that in two of the three cases studied (the M2 mAChR and GLP-1R) cannot be predicted from assessment of the parent ligand.

*Activation of the GLP-1R by the major metabolite of GLP-1(7-36)NH$_2$ (GLP-1(9-36)NH$_2$) is augmented by structurally distinct small molecule allosteric ligands in a pathway selective manner.*

To further explore this phenomenon, we performed additional studies using the GLP-1R as a model system. In addition to BETP, we characterised the quinoxaline-based Novo Nordisk Compound 2 (Supplemental Fig. 1D) for its ability to modulate the metabolite in cAMP accumulation assays. Previously, we showed that Compound 2 has a limited ability to augment the actions of GLP-1 or its endogenous peptide variants at the GLP-1R in cAMP signalling, despite showing direct allosteric agonism in its own right (Koole et al., 2010) (Fig. 2G). However, similar to that observed with BETP (Fig. 2E-2F), there was a large potentiation of GLP-1(9-36)NH$_2$-mediated cAMP signalling (Fig. 2H). Derivation of global cooperativity estimates ($\alpha\beta$) revealed a greater than 400-fold potentiation of the metabolite response and an ~180-fold greater magnitude of positive cooperativity between Compound 2 and GLP-1(9-36)NH$_2$ compared to GLP-1(7-36)NH$_2$ (Table 1). Both Compound 2 and BETP exhibited almost neutral cooperativity with GLP-1(7-36)NH$_2$ and GLP-1(9-36)NH$_2$ peptides in whole cell competition binding assays (Supplemental Fig. 4) indicating that the allosteric effects of these compounds on GLP-1(9-36)NH$_2$-mediated cAMP signalling are principally driven by changes in orthosteric agonist efficacy.
Despite the critical role of GLP-1R-mediated cAMP production in insulin secretion, there is also a role for other signalling components/pathways such as β-arrestin signalling, mobilisation of intracellular Ca^{2+} and activation of mitogen activated kinases such as ERK1/2 in the augmentation of insulin response and β-cell survival (Baggio and Drucker, 2007; Sonoda et al., 2008). We therefore extended the study to explore allosteric effects of BETP and Compound 2 on GLP-1(9-36)NH₂ in ERK1/2 phosphorylation and Ca^{2+} mobilisation (Fig. 3) and compared the effects with the parent peptide. In agreement with our previous findings, GLP-1(7-36)NH₂ displayed robust agonism in ERK phosphorylation and Ca^{2+} mobilisation (Fig. 3A, C, E, G). Significantly, GLP-1(9-36)NH₂ also displayed agonism in pERK1/2 phosphorylation in a concentration-dependent manner, but only a very weak Ca^{2+} response was observed (at 3 µM of peptide). In interaction studies, BETP exhibited negative cooperativity with GLP-1(7-36)NH₂ in ERK phosphorylation but an augmentation in Ca^{2+} signalling at the highest concentration tested (30 µM), with a small increase in pEC_{50} and E_{max} (Fig. 3A and C, Table 1). In contrast, GLP-1(9-36)NH₂ displayed neutral cooperativity with BETP in ERK1/2 phosphorylation (Fig. 3B) and there was also no apparent change in Ca^{2+} response mediated by GLP-1(9-36)NH₂ in the presence of 30 µM BETP (the small change in response can be attributed to agonism from BETP alone) (Fig. 3D.). Compound 2 displayed neutral cooperativity in both ERK1/2 phosphorylation and intracellular Ca^{2+} mobilisation when interacted with either GLP-1(7-36)NH₂ or GLP-1(9-36)NH₂ (Fig. 3E-H, Table 1). Collectively, these results suggest that allosteric modulation can engender functional selectivity in the actions of both the metabolite and the parent ligand when acting at the GLP-1R. However, the differential modulation between the metabolite and the cognate ligand on the different signalling pathways highlights a novel use of allosteric ligands to engender pathway selective modulation of response of the metabolite, even if no modulation is observed from the cognate agonist of the system.
**Allosteric modulation of the metabolite GLP-1(9-36)NH₂ via the GLP-1R results in glucose-dependent insulin secretion ex vivo in rat islets and in vivo.**

Activation of the GLP-1R by GLP-1 only increases insulin secretion in conditions of elevated glucose (Goke et al., 1993; Sloop et al., 2010). To evaluate the ability of the metabolite to activate glucose-dependent insulin secretion, pancreatic islets isolated from SD rats were used. Previously, we showed that GLP-1(7-36)NH₂ had insulinotropic activity in islet experiments using high glucose conditions, and BETP also caused a robust concentration-dependent increase in insulin secretion (Sloop et al., 2010). Here we show that in high glucose conditions, GLP-1(9-36)NH₂ does not induce insulin secretion at concentrations of up to 10 µM (Fig. 4, Supplemental Fig. 5A). However, in the presence of 1 µM BETP (which only minimally increases insulin levels by itself), a dose-dependent increase in GLP-1(9-36)NH₂-mediated insulin-secretion was observed, with a pEC₅₀ of 7.4 ± 0.3 (EC₅₀ 38 nM), and a maximal response achieved at 100 nM (Fig. 4A). Maximum insulin levels in islet cultures treated with the combination of BETP and 1 µM of the metabolite were similar to those induced by 100 nM GLP-1(7-36)NH₂ (Supplemental Fig. 5A).

To explore the in vivo insulinotropic effects, glucose-stimulated insulin secretion was measured in compound treated male Wistar rats undergoing an intravenous glucose tolerance test (IVGTT). Similar to our previous study (Sloop et al., 2010), GLP-1(7-36)NH₂ displayed insulin secretagogue activity during the 20 min time course, however compared with vehicle, animals dosed with 150 nmol/kg of GLP-1(9-36)NH₂ had lower levels of plasma insulin than those treated with GLP-1(7-36)NH₂ (Fig. 4B). BETP had no insulinotropic activity at the dose administered (Fig. 4B). However, co-administration of GLP-1(9-36)NH₂ and BETP
elicited a similar elevation in plasma insulin to animals dosed with GLP-1(7-36)NH₂ although insulin levels remained elevated over the 20 min time period for GLP-1(7-36)NH₂ whereas in the animals dosed with BETP and GLP-1(9-36)NH₂ plasma insulin levels dropped to the level of vehicle after 10 min (Fig. 4B). Determination of total GLP-1 levels throughout the time course showed that GLP-1(9-36)NH₂ was cleared from the plasma within this same 10 min time period, and additionally revealed that BETP did not alter the pharmacokinetics of GLP-1(9-36)NH₂ (Supplemental Fig. 6). In addition, plasma insulin levels remained elevated (similar to GLP-1(7-36)NH₂) when animals were administered with higher doses of GLP-1(9-36)NH₂ (400 nmol/kg) in the presence of BETP (10mg/kg) (Supplemental Fig. 5B). Collectively the *ex vivo* and *in vitro* studies support a model whereby BETP allosterically potentiates GLP-1(9-36)NH₂-mediated cAMP signalling resulting in insulin release. The ability of BETP to specifically augment GLP-1(9-36)NH₂-mediated cAMP signalling in GLP-1R expressing cells, in combination with the ability to modulate glucose-dependent insulin secretion, provides compelling proof-of-concept that allosteric potentiation of metabolites is a viable approach for the development of GLP-1R based therapeutics.
Discussion

In this study, we demonstrate the ability of allosteric ligands to modulate signalling mediated by an inactive metabolite of the primary endogenous ligand. To determine the generality of this hypothesis, three receptors (the GLP-1R, M2 mAChR and the A1-AR) from two different subclasses of GPCRs were selected, each of which is a therapeutic target and also has identified allosteric modulators. The GLP-1R is a family B GPCR and is a promising target in the development of treatments for type 2 diabetes mellitus (DM). Both the M2 mAChR and A1-AR are prototypical family A GPCRs, with separate mechanisms of activation compared with family B GPCRs, and are therapeutic targets for treatment of Alzheimers disease/asthma and neuropathic pain, respectively.

For all three receptors, the potency of the metabolite alone at the selected signaling pathway (ERK1/2 phosphorylation for Gαi-coupled M2 mAChR and A1-AR, cAMP for Gαs-coupled GLP-1R) was markedly lower than the cognate agonist (greater than 1000-fold). However, in each case, an allosteric ligand markedly potentiated signalling by the metabolite. In two of the cases (the GLP-1R and M2 mAChR) the allosteric effect on the metabolite was much more pronounced than the effect on the parent ligand. Collectively, these findings highlight the virtually-untapped potential for metabolic products of GPCR endogenous agonists to recruit signalling pathways that would otherwise remain quiescent after inactivation of the parent agonist. The ability to activate responses from convergent and divergent signalling cascades could therefore have the potential to generate a more tuneable response from the metabolite than that of the parent compound.

The ability of each of these allosteric ligands to promote strong potentiation on the actions of the respective metabolite may be therapeutically relevant. Choline levels in the brain have been reported to range between 10-15 µM depending on the species (Tucek, 1985). Choline
affinity for the M₂ mAChR is low (in the mM range), however both affinity (>10-fold) and potency (>200-fold) can be enhanced by the allosteric ligand LY2033298. This suggests that it is very likely that allosteric potentiation of these responses may be possible in a physiological setting. Similarly, resting inosine levels in the brain and the heart can reach concentrations as high as 10 µM and at least 30-fold higher in ischemic conditions (Backstrom et al., 2003). Evidence for modulation of inosine at the A₁-AR (in addition to previous evidence for modulation at the A₃-AR (Gao et al., 2011) provides additional evidence that targeting metabolites is viable. Furthermore, circulating GLP-1(9-36)NH₂ concentrations are >10-fold higher than that of the GLP-1(7-36)NH₂ (Goke et al., 1993). However this metabolite exhibits a ~1000-fold lower binding affinity for the GLP-1R and equally low efficacy and potency for cAMP accumulation. This indicates that at least 100-fold potentiation of the metabolite response would be required for a therapeutically beneficial effect. The in vitro experiments show that Compound 2 can produce this degree of potentiation (>250-fold), consistent with modulation of metabolites as a therapeutically relevant approach.

ACh and adenosine both act at several subtypes of the mAChR and adenosine receptor respectively. Therefore it is plausible that the metabolites investigated in this study could also have effects at these other subtypes. Certainly this is true for inosine, for which allosteric potentiation of cAMP signalling at the A₃-AR has been reported (Gao et al., 2011). One advantage of allosteric ligands is their ability to provide selectivity and therefore use of a selective modulator should, in theory, only modulate the metabolite at the subtype where the allosteric ligand binds.

As an extension of our initial screen, the GLP-1R was used as a model system to further explore the phenomenon. The GLP-1R has actions that address key symptoms associated
with DM, including glucose-dependent increases in insulin synthesis and release, decreases in β-cell apoptosis, body mass and gastric emptying (Drucker and Nauck, 2006; Vahl and D'Alessio, 2004). GLP-1 is principally released from intestinal L cells in its amidated form (GLP-1(7-36)NH₂) in response to meal ingestion resulting in insulin release (Drucker, 2006). It is very rapidly degraded by dipeptidyl peptidase IV (within 1-2 min) to GLP-1(9-36)NH₂, with only ~10% reaching the systemic circulation and even lower levels reaching the pancreatic β cells (Deacon et al., 1995); the metabolite is thus the major circulating form of GLP-1, however it does not stimulate insulin secretion (Deacon et al., 1995; Tomas et al., 2010), presumably due to the lack of GLP-1R mediated increases in cAMP, which is thought to be a major contributor to insulin secretion.

A novel treatment for type 2 DM would therefore be to potentiate the actions of GLP-1(9-36)NH₂ mediated through cAMP to elicit insulin secretion. Our results show that this is indeed possible, with two structurally distinct allosteric ligands, BETP and Compound 2, able to strongly potentiate cAMP signalling in heterologous cell systems. A key finding in our study was the demonstration that one of these compounds, BETP, could also strongly potentiate the ability of the GLP-1 metabolite to promote insulin secretion in both ex vivo and in vivo rat models. Relatively high concentrations of GLP-1(9-36)NH₂ were required to elicit an insulin response (even in the presence of BETP) in the isolated islets (30 nM and above) compared to circulating levels of GLP-1(9-36)NH₂ in normal physiology (around 100 pM). However, it is not uncommon to require much larger doses of hormones in ex vivo experiments compared to in vivo, for example the EC₅₀ for GLP-1(7-36)NH₂ stimulation of islet culture insulin release is 1-10nM (Goke et al., 1993; Sloop et al., 2010; Tomas et al., 2010), only ~10-fold lower than the augmented metabolite response. As the metabolite circulates at >10-fold higher concentrations than the parental peptide, these data suggest that
regulation of physiological levels of GLP-1(9-36)NH₂ may be possible, even with compounds that have not been optimised for allosteric activity. Further exploration of this phenomenon in vivo at physiological levels of metabolite is currently limited due to the poor pharmacokinetic properties of the allosteric ligands available. However, \textit{in vivo} effects on insulin secretion and blood glucose elicited by GLP-1(9-36)NH₂ are modulated by BETP, albeit with pharmacological dosing with the metabolite. This provides the proof-of-concept that modulating metabolites is possible for physiologically relevant endpoints. It is likely that specific screening programs to identify modulators optimised for potentiation of metabolites are required to conclusively show allosteric modulation of metabolites can occur in an endogenous system.

To date, the level of modulation seen with the metabolites for existing compounds is purely serendipitous, however, the ability to develop allosteric ligands that induce very strong potentiation certainly exists. Screening programs using the endogenous ligand ACh identified an M₄ mAChR allosteric ligand that enhanced the response mediated by ACh 780-fold (Leach et al., 2010). Similarly, BQCA, an M₁ mAChR allosteric ligand can potentiate the actions of ACh by up to 10,000-fold (Canals et al., 2012). So there is clear precedent for the ability to develop compounds that will be effective even where metabolite activity is only 1,000\textsuperscript{th} that of the parent ligand (assuming the metabolite levels do not reach higher levels than the parent). Thus, these data provide compelling evidence for proof-of-concept that allosteric modulation of metabolites could lead to physiologically relevant responses that are therapeutically beneficial.

Currently, for the therapeutically relevant effects of GLP-1R activation, the underlying signalling is not fully understood, but it is clear that physiological responses are a composite of multiple pathways. In our \textit{in vitro} assays, we showed that allosteric ligands can engender
functional selectivity in the actions of the metabolite when acting at the GLP-1R whereby cAMP signalling was strongly potentiated but no change was observed in ERK phosphorylation or Ca$^{2+}$ mobilisation; together with the islet experiments and in vivo studies, this suggests that modulation of cAMP without altering pERK1/2 and calcium signalling is sufficient to promote insulin secretion. Nonetheless, the ideal signalling profiles for other therapeutically relevant effects of GLP-1R activation, such as beta-cell survival, still remain to be determined. As more information becomes available, a more detailed understanding of the required combination of collateral efficacies required to therapeutically target different disease states will become apparent. Therefore, information characterising functional selectivity of all classes of ligands and behaviour will become increasingly important in drug discovery programs.

Probe-dependence of allosteric drugs has multiple implications in drug discovery and the ability to modulate the action of normally inactive endogenous metabolites could be exploited to develop novel therapeutics. In addition, metabolites are often further metabolised offering additional scope for drug discovery. However, in some cases, modulation of metabolites could also contribute towards unwanted or unanticipated side-effects of drugs. This study thus highlights the need to understand allosteric effects on all ligands, including metabolites normally considered to be inactive as part of the profile of modulator action. This concept is also relevant for other non-GPCR drug targets, such as ligand-gated ion channels. As a further layer of complexity, the breakdown product of one ligand could activate a different receptor with desirable properties, offering the potential to develop allosteric ligands with properties for modulating that specific receptor target. The findings of pronounced potentiation (in some cases) compared to the endogenous agonist has substantial, previously unappreciated, implications for therapeutic development of small molecule modulators.
Authorship contributions

*Participated in research design:* Wootten, Sloop, Willard, Christopoulos, Sexton

*Conducted experiments:* Wootten, Savage, Valant, May, Ficorilli, Showalter

*Contributed new reagents or analytic tools:* Sloop, Willard

*Performed data analysis:* Wootten, Savage, Valant, May Sloop, Willard, Christopoulos, Sexton

*Wrote or contributed to writing of the manuscript:* Wootten, Sloop, Christopoulos, Sexton
References


Footnotes

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Figure Legends

Figure 1. Allosteric enhancement of metabolite activity as a novel mechanism of drug action. (A) Schematic illustration of neurotransmitter release and activation of post-synaptic receptors to elicit physiological signalling. (B) The neurotransmitter is rapidly degraded by metabolising enzymes leading to decay of neurotransmitter signalling. (C) An allosteric enhancer of metabolite activity (orange triangles) co-binds with the metabolite engendering and/or enhancing signalling to extend the activation of the receptor. A similar process can be envisaged for rapidly metabolised hormones or paracrine regulators.

Figure 2. Small molecule ligands of three different GPCRs display a high degree of positive allosteric modulation of the metabolite of the cognate ligand in intact cells. Interaction studies were performed in ERK phosphorylation between LY2033298 and ACh (A) or Ch (B) in FlpInCHO cells stably expressing the human M2 mAChR. Interaction studies between PD81723 and (C) adenosine or (D) inosine were performed in ERK phosphorylation assays in FlpInCHO cells expressing the A1-AR. cAMP accumulation interaction studies were performed between BETP and (E) GLP-1(7-36)NH₂ or (F) GLP-1(9-36)NH₂ or between Compound 2 and (G) GLP-1(7-36)NH₂ or (H) GLP-1(9-36)NH₂ in FlpInCHO cells stably expressing the human GLP-1R. All values are mean ± SEM of three to six independent experiments performed in duplicate.

Figure 3. Differing degrees of allosteric modulation of GLP-1(7-36)NH₂ and GLP-1(9-36)NH₂ by BETP or compound 2 at the GLP-1R in ERK1/2 phosphorylation and intracellular calcium mobilisation in intact cells. Interaction studies between BETP (A-D) and GLP-1(7-36)NH₂ (A,C) or GLP-1(9-36)NH₂ (B,D) in ERK1/2 phosphorylation (A,B) or
intracellular calcium mobilisation (C,D), respectively. Interaction studies between Compound 2 (E-H) and GLP-1(7-36)NH₂ (E,G) or GLP-1(9-36)NH₂ (F,H) in ERK1/2 phosphorylation (E,F) or intracellular calcium mobilisation (G,H), respectively. All values are mean ± SEM of three to four independent experiments performed in duplicate.

Figure 4. Ex vivo and in vivo studies reveal allosteric modulation of the GLP-1 metabolite at the GLP-1R leads to insulin secretion. (A) Insulin concentrations from cultures of SD rat islets incubated in media containing high glucose (11.2 mM) and BETP with increasing concentrations of GLP-1(9-36)NH₂. Islet treatments were performed for 90 min. (B) Time course of plasma insulin concentrations in fasted, anaesthetised animals treated with either vehicle, GLP-1(7-36)NH₂ (3 nmol/kg), GLP-1(9-36)NH₂ (150 nmol/kg), BETP (5 mg/kg) or co-administration of BETP and GLP-1(9-36)NH₂, immediately prior to intravenous administration of a glucose bolus (0.5 g/kg). Inset, AUC₀-10min of the insulin secretion for the various treatment groups. All results are expressed as mean ± SEM of five experiments, (* = p < 0.05 as determined using a one way anova followed by Dunnett’s comparison to vehicle group).
Table Legends

Table 1: Allosteric parameters determining the cooperativity for the interaction between the allosteric modulators and agonist/metabolite at the three different GPCRs, using various signal outputs. Data were analysed with an operational model of allosterism as defined in materials and methods. Logαβ values represent the composite cooperativity between the allosteric modulator and the orthosteric ligand. Antilogarithms are shown in parenthesis.

<table>
<thead>
<tr>
<th>Allosteric Ligand</th>
<th>Signalling Pathway</th>
<th>Log αβ (αβ)</th>
<th>Orthosteric Ligand</th>
<th>Orthosteric Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1R</td>
<td>BETP</td>
<td>GLP-1(7-36)NH₂</td>
<td>0.18 ± 0.15 (1.5)</td>
<td>GLP-1(9-36)NH₂</td>
</tr>
<tr>
<td></td>
<td>cAMP</td>
<td></td>
<td>0.36 ± 0.14 (2.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pERK1/2</td>
<td>-0.97 ± 0.39 (0.1)</td>
<td>ND*</td>
<td>-0.01 ± 0.11 (1.0)</td>
</tr>
<tr>
<td></td>
<td>Ca²⁺</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Compound 2</td>
<td>cAMP</td>
<td>GLP-1(7-36)NH₂</td>
<td>2.63 ± 0.43 (426)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pERK1/2</td>
<td>-0.27 ± 0.26 (0.53)</td>
<td>0.25 ± 0.31 (1.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca²⁺</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>M₂ mAChR</td>
<td>LY2033298</td>
<td>ACh</td>
<td>0.31 ± 0.07 (2.0)</td>
<td>2.35 ± 0.16 (224)</td>
</tr>
<tr>
<td></td>
<td>pERK1/2</td>
<td></td>
<td>1.20 ± 0.08 (16)</td>
<td>1.85 ± 0.10 (71)</td>
</tr>
<tr>
<td></td>
<td>GTPγS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₁-AR</td>
<td>PD81723</td>
<td>Adenosine</td>
<td>1.31 ± 0.12 (20)</td>
<td>1.08 ± 0.12 (12)</td>
</tr>
</tbody>
</table>

ND, Data not able to be experimentally defined.
aβ is the cooperativity factor that defines the fold-change in receptor signalling by the allosteric modulator.
*Cooperativity factors could not be defined, but positive allosteric modulation was observed.
*pKB values (the negative logarithm of the affinity) for the allosteric ligands derived from application of the operational model of allosterism were 5.01±0.23 for BETP, 5.14±0.16 for Compound 2 and 4.58±0.32 for PD81723. For LY2033298 the pKB was fixed to the equilibrium dissociation constant (4.74) previously determined in radioligand binding assays (Valant et al., 2012).
**Figure 1.**

**A**
- Endogenous neurotransmitter

**B**
- Neurotransmitter metabolites

**C**
- Allosteric enhancer of metabolite function
Figure 3.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

[BETP] (A-D) or [Compound 2] (E-H)

- ● 0
- ▲ 1 μM
- ○ 0.1 μM
- ▼ 3 μM
- ◆ 10 μM
- □ 30 μM
Figure 4.

A. [BETP]

![Graph showing the effect of BETP on insulin levels.](image)

**Insulin (ng/ml)**

- **0**
- **1 μM**

**Log [GLP-1(9-36)NH₂ + 11.2 mM Glucose**

B.

**Plasma Insulin (ng/ml)**

- **Vehicle + Vehicle**
- **Vehicle + GLP-1(7-36)NH₂ (3 nmol/kg)**
- **Vehicle + GLP-1(9-36)NH₂ (150 nmol/kg)**
- **BETP (5 mg/kg) + Vehicle**
- **BETP (5 mg/kg) + GLP-1(9-36)NH₂ (150 nmol/kg)**

**Time (mins)**

- **0**
- **5**
- **10**
- **15**
- **20**
Supplementary Information

Article Title
Allosteric modulation of endogenous metabolites as an avenue for drug discovery

Authors

Journal Title
Molecular Pharmacology
Supplemental Fig. 1. Metabolic breakdown of endogenous ligands to their metabolites and structures of the allosteric ligands used in this study. (A) Acetylcholine is metabolised by acetylcholinesterases to choline and acetate. (B) Adenosine is metabolised by adenosine deaminase to inosine. (C) The peptide GLP-1(7-36)NH₂ is degraded by dipeptidyl peptidase IV to the inert metabolite GLP-1(9-36)NH₂ (the primary amino acid sequences are shown). (D) Structures of the four allosteric ligands used in this study.

Supplemental Fig. 2. The allosteric agonist LY2033298 displays positive allosteric modulation of the metabolite choline in GTPγS binding in membranes expressing M2 mAChR. Interaction studies between LY2033298 and ACh (A) or Ch (B) GTPγS binding assays. All values are mean ± SEM of three independent experiments performed in duplicate.

Supplemental Fig. 3. The allosteric agonist LY2033298 shows weak positive allosteric modulation of the metabolite choline in competition binding assays in membranes expressing M2 mAChR. Interaction studies between LY2033298 and Ch in a competition radioligand binding assay using the radioligand [³H]NMS. Curves were fitted using a one site modulator plus allosteric ligand model. The logα for NMS was fitted to 0.5 as determined in Valant et al 2012. All values are mean ± SEM of three independent experiments performed in duplicate.

Supplemental Fig. 4. Small molecule ligands of the GLP-1R do not modulate binding affinity of the GLP-1(7-36)NH₂ or its metabolite GLP-1R(9-36)NH₂ in competition binding experiments in intact cells expressing human GLP-1R. Effects of increasing concentrations of
either Compound 2 (A and B) or BETP (C and D) on the inhibition of $^{125}$I-exendin(9-39) binding by GLP-1(7-36)NH$_2$ (A and C) or GLP-1(9-36)NH$_2$ (B and D). Data are normalised to specific radioligand binding. Nonspecific binding was determined by inhibition of $^{125}$I-exendin(9-39) by 1 µM exendin(9-39). All values are mean ± SEM of four independent experiments performed in duplicate.

**Supplemental Fig. 5. Ex vivo and in vivo studies reveal allosteric modulation of the GLP-1 metabolite at the GLP-1R leads to insulin secretion.** (A) Insulin concentrations from cultures of SD rat islets incubated in media containing low glucose (2.8mM), high glucose (11.2 mM), GLP-1(7-36)NH$_2$ (100 nM), BETP (1 µM) and GLP-1(9-36)NH$_2$ (1 and 10 µM) in the presence and absence of BETP (1 µM). Islet treatments were performed for 90 min. (B) Timecourse of plasma insulin concentrations in fasted, anaesthetised animals treated with either vehicle, GLP-1(7-36)NH$_2$ (3 nmol/kg), GLP-1(9-36)NH$_2$ (400 nmol/kg), BETP (10 mg/kg) or co-administration of BETP and GLP-1(9-36)NH$_2$, immediately prior to intravenous administration of a glucose bolus (0.5 g/kg). Inset, AUC$^{0-20}$min of the insulin secretion for the various treatment groups. All results are expressed as mean ± SEM of five experiments, (* = p < 0.05 as determined using a one way anova followed by Dunnett’s comparison to vehicle group).

**Supplemental Fig. 6. In vivo studies reveal BETP does not alter the pharmacokinetics of GLP-1(9-36)NH$_2$.** Time course of total plasma GLP-1 levels in fasted anaesthetized animals treated with either vehicle, GLP-1(7-36)NH$_2$ (3 nmol/kg), GLP-1(9-36)NH$_2$ (150 nmol/kg), BETP (5 mg/kg) or GLP-1(9-36)NH$_2$ (150 nmol/kg) in the presence of BETP (5 mg/kg) immediately prior to intravenous administration of a glucose bolus (0.5 g/kg). Inset. Same data set with smaller y axis. Results are expressed as mean ± SEM of six experiments (* = p < 0.05 as determined using a one way anova followed by Dunnett’s comparison to vehicle group).
Supplementary Figure 1.

A

Acetylcholinesterase

\[
\begin{align*}
\text{Acetylcholine} & \rightarrow \text{Choline} + \text{Acetate} \\
& \quad \text{NH}_2
\end{align*}
\]

B

Adenosine deaminase

\[
\begin{align*}
\text{Adenosine} & \rightarrow \text{Inosine} \\
& \quad \text{NH}_2
\end{align*}
\]

C

Dipeptidyl peptidase IV

\[
\begin{align*}
\text{GLP-1(7-36)NH}_2 & \rightarrow \text{GLP-1(9-36)NH}_2 \\
& \quad \text{NH}_2
\end{align*}
\]

D

LY2033298

PD81372

BETP

Compound 2
Supplemental Fig. 3

\[ \text{pKb} = 4.36 \]
\[ \log \alpha_{\text{NMS}} = 0.50 \]
\[ \log \alpha_{\text{choline}} = 1.12 \]
Supplemental Figure 4.

A.

B.

C.

D.

Log [GLP-1(7-36)NH₂] M

% Specific Binding

Log [GLP-1(9-36)NH₂] M

Log [GLP-1(7-36)NH₂] M

% Specific Binding

Log [GLP-1(9-36)NH₂] M

% Specific Binding

[Compound 2]

- 0
- 0.1 μM
- 1 μM
- 3 μM
- 10 μM
- 30 μM

[Compound 2]

- 0
- 0.1 μM
- 1 μM
- 3 μM
- 10 μM
- 30 μM

[BETP]

- 0
- 0.1 μM
- 1 μM
- 3 μM
- 10 μM
- 30 μM

[BETP]
Supplemental Figure 5.

A.

![Graph showing plasma insulin levels with different treatments](image)

- **Vehicle**
- **10 nmol/kg GLP-1(7-36)NH₂**
- **1 μM BETP**
- **1 μM GLP-1(7-36)NH₂**
- **10 μM GLP-1(7-36)NH₂**
- **1 μM BETP + 10 μM GLP-1(7-36)NH₂**
- **1 μM BETP + 10 μM GLP-1(7-36)NH₂ + 10 μM GLP-1(9-36)NH₂**

B.

![Graph showing plasma insulin AUC (area under curve) with different treatments](image)

- **Vehicle**
- **GLP-1(7-36)NH₂ (3 nmol/kg)**
- **GLP-1(9-36)NH₂ (400 nmol/kg)**
- **BETP (10 mg/kg) + GLP-1(9-36)NH₂ (400 nmol/kg)**

**Legend:**
- **Vehicle**
- **GLP-1(7-36)NH₂ (3 nmol/kg)**
- **GLP-1(9-36)NH₂ (400 nmol/kg)**
- **BETP (10 mg/kg) + GLP-1(9-36)NH₂ (400 nmol/kg)**

**11.2 mM glucose**