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# Two small molecule agonists of glucagon-like peptide-1 receptor modulate the receptor activation response differently

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ABSTRACT

The glucagon-like peptide-1 receptor (GLP-1R) is a target for type 2 diabetes treatment. Due to the inconvenience of peptide therapeutics, small-molecule GLP-1R agonists have been studied. Compound 2 (6,7-dichloro-2-methylsulfonyl-2-*N*-*tert*-butylaminoquinoxaline) and compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine) have been described as small molecule, ago-allosteric modulators of GLP-1R. However, their modes of action at the GLP-1R have not been elucidated. Thus, in this study, we compared the mechanisms of action between these two compounds. When compound 2 was treated with endogenous or exogenous peptide agonists (GLP-1 and exenatide) or fragments of peptide agonists (GLP-1(9–36), Ex3, Ex4, and Ex5), the response curve of these peptide agonists shifted left without a change in maximum efficacy. In contrast, compound B potentiated the response and increased maximum efficacy. However, N-terminal truncated orthosteric antagonists including Ex7, Ex9, and Ex10, augmented the response of compound 2 at the GLP-1R but did not alter compound B activity. Intriguingly, when we co-treated compound 2 with compound B in CHO cells expressing full-length hGLP-1R or N-terminal extracellular domain-truncated GLP-1R, the activation of both types of receptors increased additively, implying that the N-terminus of the receptor is not involved in the modulation by compound agonists. We confirmed that these two compounds increased calcium influx by different patterns in CHO cells expressing GLP-1R. Taken together, our findings suggest that compounds 2 and B have different modes of action to activate GLP-1R. Further study to identify the putative binding sites will help in the discovery of orally available GLP-1R agonists.



## 1. Introduction

Glucagon-like peptide-1 (GLP-1), one of the incretin hormones, is synthesized in L-cells of the small intestine and is rapidly released after food intake [1]. GLP-1 activates its own specific receptor, GLP-1R, thereby stimulating the adenylyl cyclase pathway resulting in an increase in intracellular cAMP, $Ca^{2+}$ influx, and insulin secretion in β-cells [1,2]. In patients with type 2 diabetes, GLP-1 concentration is reduced, and when GLP-1 is administered, insulin secretory function is restored. GLP-1 administration also result in an anti-apoptotic effect in pancreatic β-cells, inhibition of gastric emptying, and lowered food intake leading to decreased body weight gain. [3–6]. Thus, GLP-1 has become a critical therapeutic target for type 2 diabetes.

However, active GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP-4) *in vivo*, which removes the two N-terminal GLP-1 amino acid residues and generates the biologically inactive form, GLP-1 (9–36) [7]. For this reason, GLP-1 has presented considerable difficulties for therapeutic use; thus, longer-acting derivatives of GLP-1, such as exenatide and liraglutide, have been developed [8–10]. Exenatide, a 50% sequence homologue of native GLP-1, activates GLP-1R more potently than GLP-1 and has been approved to treat type 2 diabetes [8,11].

Although GLP-1 and its derivatives have therapeutic potential, these molecules must be administered by injection; thus, many researchers are attempting to discover orally active and small-molecule GLP-1R agonists [12–15]. Compound 2 (6,7-dichloro-2-methylsulfonyl-2-*N*-*tert*-butylaminoquinoxaline) and compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl) pyrimidine) are small molecule ago-allosteric modulators of GLP-1R [12,15,16]. Both compound 2 and compound B increase cAMP by activating GLP-1R and increase insulin secretion *in vivo* or *ex vivo*. Compound 2 increases the affinity of GLP-1 for GLP-1R; however,

*Abbreviations:* GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.

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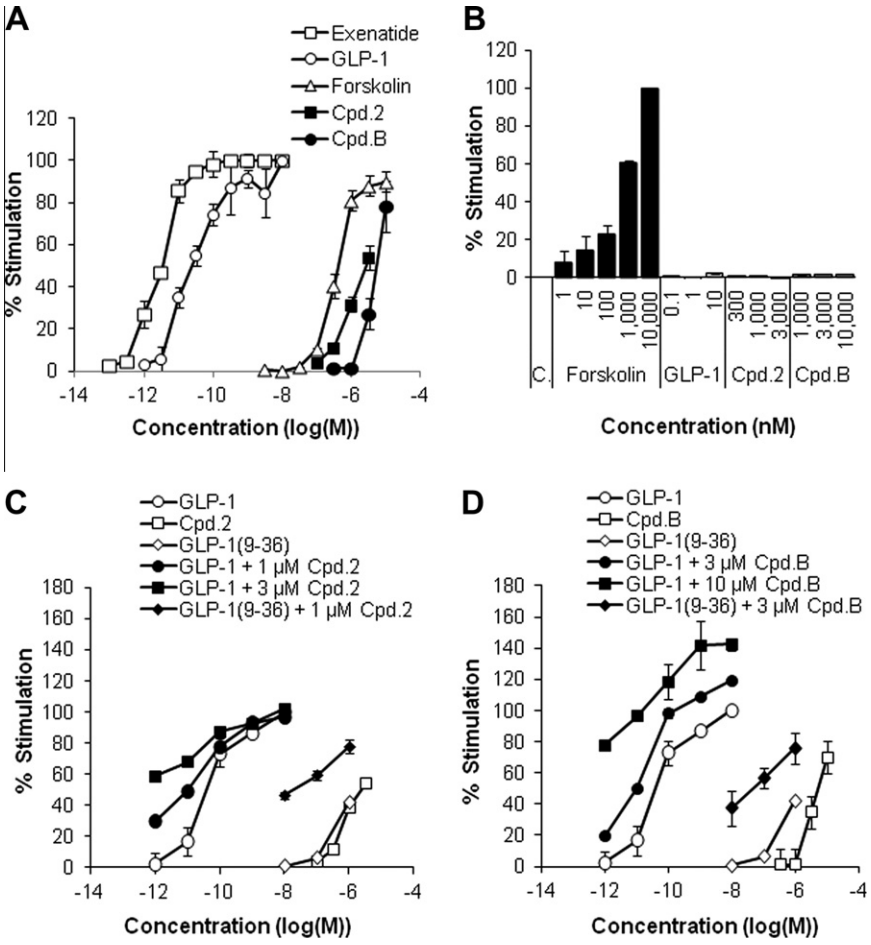


**Fig. 1.** Compound 2 and compound B activate the glucagon-like peptide-1 (GLP-1) receptor and show different receptor activation responses in the presence of GLP-1 or GLP-1 (9–36). CHO cells transfected with hGLP-1R, pCRE-Luc, and pRL-TK (A) or pCRE-Luc and pRL-TK without hGLP-1R were treated with GLP-1, forskolin, exenatide, compound 2, or compound B (B), and luciferase activities were measured. (C and D) CHO cells expressing hGLP-1R were co-treated with compound 2 or compound B with GLP-1 or GLP-1 (9–36), and luciferase activities were measured. Cpd.2, compound 2; Cpd. B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are shown as mean ± SEM.

no reports are available for compound B [12,16]. Furthermore, exendin (9–39) increases the maximum efficacy of compound 2 but does not alter the compound B response [15,16]. Coopman et al. reported that although compound 2 and GLP-1 have comparable GLP-1R activation effects, these molecules have different molecular signaling, such as calcium influx response and receptor internalization [16]. However, the modes of action of compound 2 and compound B have not been compared previously. In this study, we compared the modes of action of these two compounds for activating GLP-1R using CHO cells transiently expressing full-length GLP-1R or the GLP-1R extracellular domain truncated at the N-terminus.

## 2. Materials and methods

### 2.1. Materials

Compound 2 and compound B were synthesized at Dong-A Pharm. Research Center (Yong-In, South Korea). GLP-1 (GLP-1 (7–36) amide) and GLP-1 (9–36) were purchased from Tocris (St. Louis, MO, USA). Exenatide and several of its fragments (Ex3 (Ex (3–39), Ex4 (Ex (4–39), Ex5 (Ex (5–39), Ex7 (Ex (7–39), Ex9 (Ex (9–39), and Ex10 (Ex (10–39) were obtained from Anaspec (Fremont, CA, USA). The human GLP-1R expression vector (pCMV-hGLP-1R) was obtained from Origene (Rockville, MD, USA). pCRE-luciferase was obtained from Stratagene (La Jolla, CA, USA), and the pRL-TK expression vector was obtained from Promega (Madison, WI, USA).

### 2.2. Cell culture

CHO cells (Chinese hamster ovary cell line, passages 10–25) were cultured in α-MEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in a 5% $CO_2$-humidified incubator.

### 2.3. Transient transfection and the dual-luciferase reporter assay

CHO cells were seeded in 96-well plates at a density of 15,000 cells per well the day before transfection. Transient transfections were conducted using Lipofectamine and the Plus Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were transfected with 10 ng/well pCMV6-hGLP1R, 100 ng/well pCRE-luciferase, and 10 ng/well pRL-TK, which acted as a transfection efficiency control. After 24 h, the compounds were solubilized in DMSO and added to the cells for 6 h. After the incubation, luciferase activity was measured using the Dual-Glo Luciferase Assay system (Promega) according to the manufacturer's protocol. Luminescence was measured by Lmax II (Molecular Devices, Sunnyvale, CA, USA). All assays were executed in triplicate, and results are presented as mean ± standard

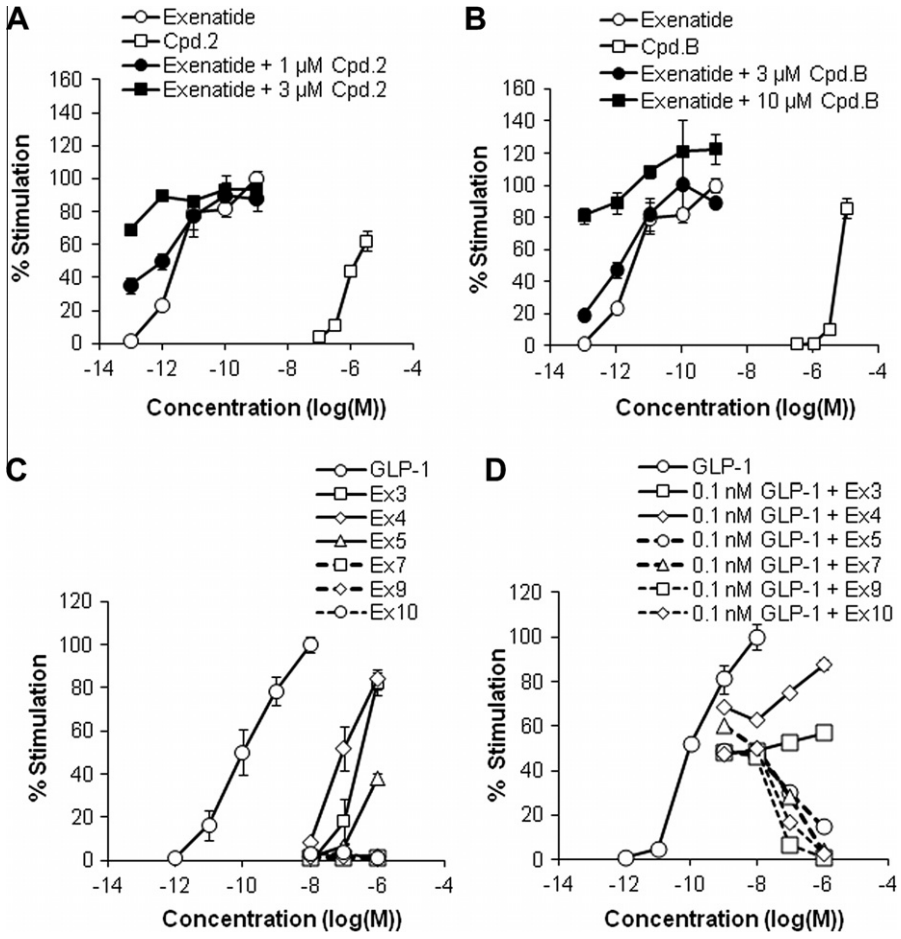


**Fig. 2.** Compound 2 and compound B show different receptor activation responses in the presence of exenatide, and the agonism or antagonism of exenatide fragments were evaluated. (A and B) CHO cells expressing human glucagon-like peptide-1 receptor (hGLP-1R) were co-treated with compound 2 or compound B with exenatide, and luciferase activities were measured. (C) CHO cells expressing hGLP-1R were treated with exenatide fragments (Ex3, Ex4, Ex5, Ex7, Ex9, or Ex10). (D) To evaluate the antagonism to exenatide fragments, CHO cells expressing hGLP-1R were co-treated with a half-maximal concentration of GLP-1 (0.1 nM). Ex3, Ex (3–39), which indicates two N-terminal amino acids truncated on exenatide; Ex4, Ex (4–39); Ex5, Ex (5–39); Ex7, Ex (7–39); Ex9, Ex (9–39); Ex10, Ex (10–39); Cpd. 2, compound 2; Cpd. B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are presented as mean ± SEM.

error of the mean (SEM). Data are expressed as a percentage of maximum stimulation response induced by GLP-1.

### 2.4. Construction of the Δ-ECD-hGLP-1R plasmid

To generate the pCMV6-Δ-ECD-hGLP-1R (deletion of the human GLP-1R N-terminal 1–138 amino acid residues) construct, the Δ-ECD-hGLP-1R coding sequence was obtained from the pCMV6-hGLP-1R expression vector using polymerase chain reaction (PCR; Dyad Peltier Thermal Cycler, Bio-Rad, Hercules, CA, USA) with specific primers. The PCR product was digested with *SalI* and *XhoI* restriction enzymes and ligated into the similarly digested pCMV6 vector. The plasmid was purified and confirmed by enzyme mapping.

### 2.5. Calcium uptake assay

The calcium uptake assay was conducted using the FLIPR Calcium 5 Assay kit (R8185, Molecular Devices) in accordance with the manufacturer's instructions. Briefly, CHO cells (15,000 cells/well) were seeded in a black 96-well plate (Corning, Lowell, MA, USA), and the cells were transfected with 120 ng of pCMV6-hGLP-1R after an overnight incubation. After 24 h, the transfected cells were loaded with 1 × calcium dye in Hank's balanced salt solution (5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, and 137 mM NaCl, pH 7.4) at 37 °C. After 1 h, intracellular calcium changes were recorded at an excitation wavelength of 485 nm and an emission wavelength of 525 nm at 1.3 s intervals for 350 s using a Multireader Flexstation-3 (Molecular Devices). Compounds solubilized in DMSO were prepared in another 96-well plate and automatically applied to the cells after 20 s of recording.

### 2.6. Data analysis

Data are expressed as means ± SEMs. A one-way analysis of variance using SigmaStat 2.0 (SPSS, Inc., Chicago, IL, USA) was used for comparisons among the groups, and multiple comparisons were conducted with the Student–Newman–Keuls test when significant differences were detected among groups. A $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Compound 2 and compound B activated hGLP-1R

We transiently expressed human GLP-1R in CHO cells and evaluated endogenous and exogenous GLP-1R agonists (GLP-1 and exenatide, respectively), an adenylyl cyclase activator (forskolin), and non-peptidic GLP-1R agonists (compound 2 and compound B). The $EC_{50}$ values of exenatide, GLP-1, and forskolin were 2.51 ± 0.52 pM, 34.2 ± 8.3 pM, and 398.3 ± 5.3 nM, respectively. Compounds 2 and B displayed $EC_{50}$ values of 1.01 ± 0.193 and 4.84 ± 0.24 μM,

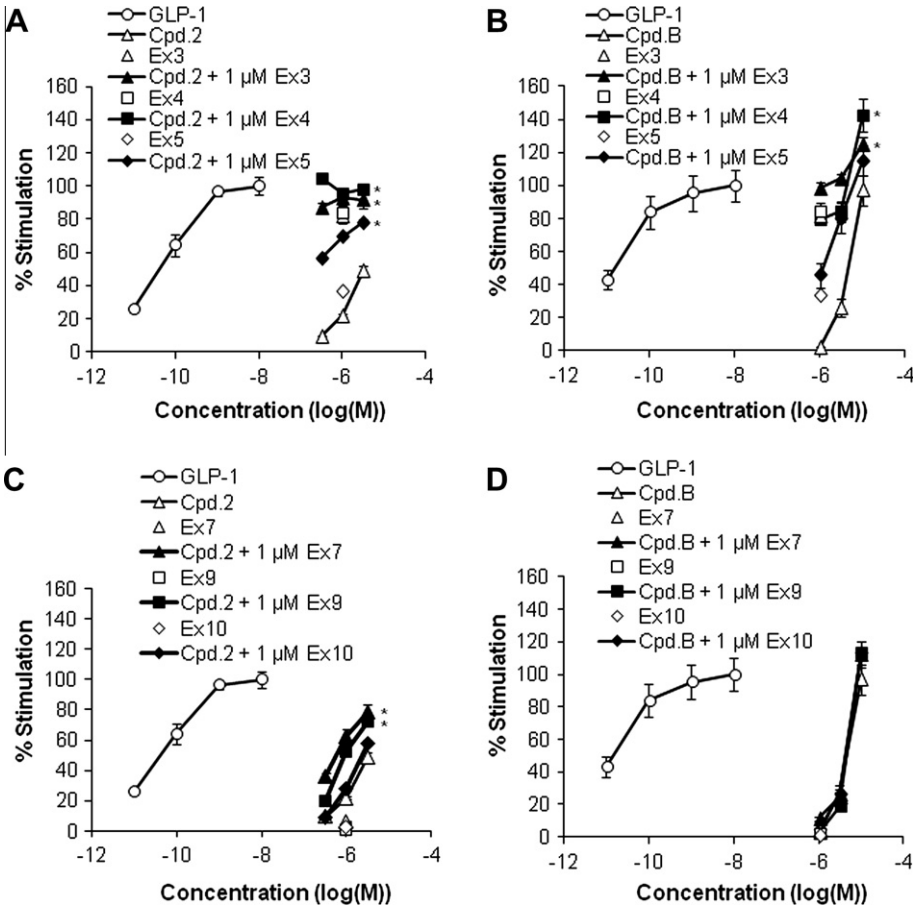


**Fig. 3.** Compound 2 and compound B show different modes of action for glucagon-like peptide-1 receptor (GLP-1R) activation in the presence of exenatide fragments. (A and B) CHO cells expressing hGLP-1R were cotreated with compound 2 or compound B and Ex3, Ex4, or Ex5. (C, D) CHO cells expressing hGLP-1R were cotreated with compound 2 or compound B and Ex7, Ex9, or Ex10. Ex3, Ex (3–39), which indicates two N-terminal amino acids truncated on exenatide; Ex4, Ex (4–39); Ex5, Ex (5–39); Ex7, Ex (7–39); Ex9, Ex (9–39); Ex10, Ex (10–39); Cpd. 2, compound 2; Cpd. B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are presented as mean ± SEM. $^{*}P < 0.05$ vs. the receptor activation response of compound 2 or compound B.

respectively (Fig. 1A). Compound B showed 78% efficacy relative to maximum stimulation by GLP-1, but compound 2 showed a lower efficacy of 53% (Fig 1A) because it had cytotoxic effects at higher concentrations (data not shown). The potency of forskolin in naïve CHO cells is comparable with the potency in GLP-1R-expressing CHO cells; however, the other molecules did not increase luciferase activity (Fig. 1B).

### 3.2. Compound 2 and compound B showed different modes of action in the presence of GLP-1, GLP-1 (9–36), or exenatide

Compound 2 showed an additive effect on GLP-1-induced receptor activation in a concentration-dependent manner but did not increase the maximum efficacy of GLP-1 (Fig. 1C). However, in contrast to compound 2, compound B increased the maximum efficacy of GLP-1 in a concentration-dependent manner (Fig. 1D). Compound 2 and compound B slightly potentiated GLP-1-(9–36)-induced receptor activation, the major metabolite of GLP-1 that is generated by DPP-4 (Fig. 1C and D). In the presence of the exogenous GLP-1R agonist exenatide, these activation patterns were identical (Fig. 2A and B).

### 3.3. Compound 2 and compound B showed different modes of action in the presence of exenatide fragments

To further explore the different modes of action between the two compounds, we performed the reporter gene assay using several exenatide fragments. First, we checked the agonism of these peptides. As shown in Fig. 2C, the more the exenatide N-terminus was truncated, the less agonist activity was observed, and exenatide fragments truncated more than six amino acid residues at the N-terminus, such as Ex7, Ex9, and Ex10, did not show any increase in luciferase activity. Additionally, we assessed the antagonist effects of the various truncated exenatide peptides using a half-maximal concentration of GLP-1 (0.1 nM). Ex7, Ex9, and Ex10 showed antagonism by reducing the luciferase activity induced by GLP-1 (Fig. 2D).

Exenatide fragments that showed agonist effects, including Ex3, Ex4, and Ex5, were treated with compound 2 or compound B. Compound 2 potentiated the GLP-1R activation response of these truncated peptide agonists without increasing maximum efficacy (Fig. 3A). However, compound B increased the maximum efficacy of the peptides (Fig. 3B). Interestingly, orthosteric antagonists, such as Ex7, Ex9, and Ex10, augmented the receptor activation response of compound 2, but did not affect compound B activity (Fig. 3C and D).

### 3.4. Compound 2 increased the receptor activation response of compound B and the two compounds induced calcium influx by different patterns

Next, we co-treated hGLP-1R-expressing CHO cells with compound 2 and compound B. As shown in Fig. 4A, the receptor activation response of compound B was additively increased by compound 2 in a concentration-dependent manner. Moreover, when the extracellular-domain truncated GLP-1R (Δ-ECD-GLP-1

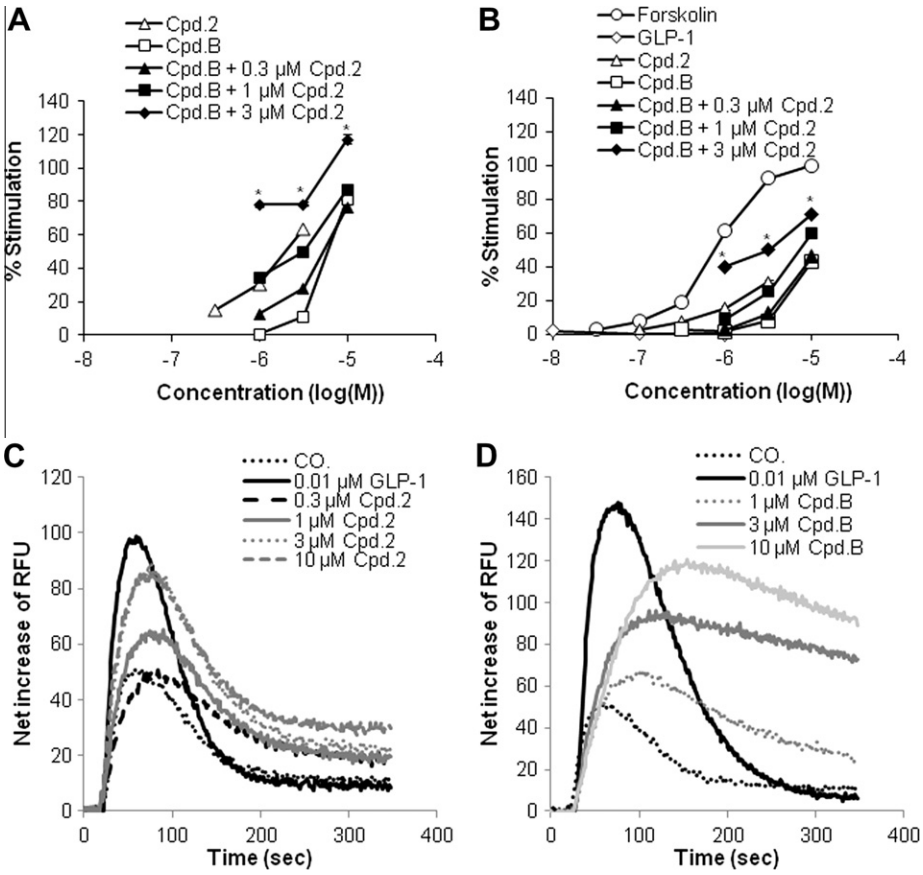


**Fig. 4.** The glucagon-like peptide-1 receptor (GLP-1R) activation effect of compound B additively increased in the presence of compound 2, and the two compounds showed different calcium influx patterns. CHO cells expressing full-length hGLP-1R (A) or N-terminal extracellular domain truncated hGLP-1R were cotreated with compound 2 and compound B (B), and luciferase activities were measured. (C and D) Representative calcium uptake response figures for compound 2 and compound B. CHO cells were treated with each compound, and calcium influx was measured using the Calcium 5 Assay kit. CO., 0.1% DMSO control; Cpd. 2, compound 2; Cpd. B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1. $^{*}P < 0.05$ vs. the receptor activation response of compound 2 or compound B.

receptor) was expressed, both compound 2 and compound B activated the truncated receptor, and the efficacy of compound B was also additively increased by compound 2 (Fig. 4B).

Activation of GLP-1R leads to an influx of intracellular calcium [1,2]. The calcium influx assay was performed in CHO cells expressing hGLP-1R to evaluate whether compound 2 and compound B have different calcium influx patterns. Compound 2 evoked a response in a concentration-dependent manner by slowly increasing calcium influx, which was longer lasting than the response evoked by GLP-1 (Fig 4C). However, compound B showed a different intracellular calcium influx pattern from that of compound 2. Compound B also slowly increased calcium influx in a concentration-dependent manner, but the response lasted longer than that of compound 2 and GLP-1 (Fig. 4D).

## 4. Discussion

In this study, we elucidated the different modes of action of compound 2 and compound B, which are well-known ago-allosteric modulators of GLP-1R. Compound 2 potentiated the GLP-1R and GLP-1 activation response of other full or partial orthosteric agonists without increasing maximum efficacy. However, compound B increased the efficacy of those agonists. The N-terminal amino acid of the peptide agonist binds at the GLP-1R transmembrane domain [11]. While the two amino acids at the N-terminus of the peptide agonists are critical for binding and activation of the receptor, here, we identified that the third to fifth amino acids of exenatide interacted with GLP-1R, and binding was potentiated by both compounds. Furthermore, three GLP-1R orthosteric antagonists augmented compound 2-induced receptor activation, but had no effect on the compound B response, similar to a previous result with exendin (9–39) [12,15]. Although the mechanism that caused this discrepancy was unclear, these findings suggest that small compounds binding at the allosteric site can modulate agonist and antagonist binding at an orthosteric site or vice versa in a compound-dependent manner.

Lin and Wang used homology modeling to discover that compound 2 binds at an allosteric site by hydrophobic interaction [17]. However, no study has investigated the number of allosteric GLP-1R sites. When we co-treated CHO cells transiently expressing full-length or the N-terminal extracellular domain-truncated form of human GLP-1R with both compounds, the receptor activation of compound B was additively increased by compound 2 at both types of GLP-1R. These results indicate that the N-terminal domain of GLP-1R is not involved in receptor activation by the two compound agonists. If the two compounds bind to the same site, maximum efficacy would be saturated, as described for other GPCR studies [18]. Unfortunately, due to their low potency and/or cytotoxicity, we failed to identify the changes in maximum efficacy.

Compound 2 and GLP-1 result in different calcium influx patterns [16]. The different calcium influx patterns observed for compound 2 and compound B in this study might also have been caused by different conformational changes in the GLP-1R, therefore, different molecular signaling pathways might have been induced. Compound 2 and compound B may also have different responses to receptor internalization. Coopman et al. reported that

compound 2 causes less GLP-1R internalization than that of GLP-1 [16]. In contrast, Jensen and Spalding reported that an allosteric activator can act by stimulating receptor dimerization [19]. Therefore, further studies are necessary to evaluate and compare the effect of compound 2 and compound B on GLP-1R internalization and dimerization.

Taken together, our findings suggest that although compound 2 and compound B have comparable modes of GLP-1 reactivation, they have different modes of action in the absence or presence of orthosteric agonists and antagonists. This discrepancy between the two compounds might be caused by different conformational changes in the GLP-1 receptor and subsequent cell signaling, such as the calcium influx pattern.

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