A novel truncated glucagon-like peptide 2 (GLP-2) as a tool for analyzing GLP-2 receptor agonists

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ABSTRACT

Glucagon-like peptide 2 (GLP-2) is an intestinotropic peptide that binds to GLP-2 receptor (GLP-2R), a class-B G protein-coupled receptor. The GLP-2R antagonist GLP-2(3–33) has relatively high partial agonistic activity, and there are as yet no ideal known potent GLP-2R antagonists. We therefore prepared several truncated forms of human GLP-2 and characterized them by binding and reporter assays to find antagonists more potent than GLP-2(3–33). We found that GLP-2(11–33) was the most potent orthosteric GLP-2R antagonist, with binding activity almost equal to those of GLP-2 and GLP-2(3–33) and weaker intrinsic agonistic activity than GLP-2(3–33). GLP-2(11–33) retained weak agonistic activity toward human, cynomolgus monkey, dog, and Syrian hamster GLP-2Rs. However, it had no agonistic activity toward rat GLP-2R. GLP-2(11–33) potentiated the agonistic activity of an ago-allosteric modulator of GLP-2R, compound 1 (N-[1-(2,5-dichlorothiophen-3-yl)-2-(phenylsulfanyl)ethylidene]hydroxylamine), synergistically toward human GLP-2R. In the case of rat GLP-2R, GLP-2(11–33) decreased the agonistic activity of compound 1, although GLP-2 and GLP-2(3–33) increased this activity additively. These findings suggest that the binding sites of the ago-allosteric modulator and GLP-2 overlap, at least in rat GLP-2R. GLP-2(11–33) is a novel, useful tool for analyzing the mode of action of agonists and ago-allosteric modulators of GLP-2R.

Glucagon-like peptide 2 (GLP-2) is a peptide that is encoded, together with glucagon and glucagon-like peptide 1 (GLP-1), by a proglucagon gene (6). GLP-2 is produced in the intestine by tissue-specific post-translational regulation. It exerts its effects through GLP-2 receptor (GLP-2R), which is a Gαs G protein-coupled receptor (GPCR) of class B (19). The peptide is liberated by stimulation of nutrient intake and is rapidly degraded by dipeptidyl peptidase IV (DPPIV) cleaving His-Pro dipeptides from the N-terminus (8, 12). GLP-2 promotes intestinal mucosal growth (7) and is therefore a therapeutic target in intestinal damage. In fact, the DPPIV-resistant peptide GLP-2R agonist, teduglutide, is under clinical development for short bowel syndrome (16, 17, 20) and Crohn’s disease (3).

GPCRs have been central targets in basic pharmacological research and drug discovery. It is considered difficult to develop small-molecule agonists for class B GPCRs, but a few reports on their development have recently appeared. An example is GLP-1 receptor (GLP-1R), which is a class-B GPCR cou-
pled with $G_{\alpha}$. Chen et al. showed that administration of the cyclobutane derivatives SP4 and Boc5 increased intracellular cAMP levels in HEK293 cells expressing rat GLP-1R and cAMP response element (CRE)-driven luciferase (4); the response to these compounds was blocked by exendin(9–39), a peptide orthosteric antagonist of GLP-1R (10). Thus, SP4 and Boc5 act on the orthosteric binding site of GLP-1R and are regarded as orthosteric agonists. In addition, a different type of GLP-1R agonist has been reported: Knudsen et al. have shown that quinoxaline derivatives function both as agonists and as allosteric modulators (14). The quinoxaline derivative (6,7-dichloro-2-methylsulfonyl-2-N-tert-butylaminoquinoxaline) acts on the allosteric site of GLP-1R, because exendin(9–39) does not antagonize the effects of this derivative. Sloop et al. also found pyrimidine derivatives of GLP-1R agonists (23). A pyrimidine derivative (4-(3-(benzoyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyridine) induced CRE-driven luciferase activity in human HEK293 cells expressing GLP-1R. However, this reporter activity was not suppressed by exendin(9–39). From these observations, the quinoxaline and pyrimidine derivatives were categorized as ago-allosteric modulators of GLP-1R. Therefore, potent orthosteric antagonists are powerful tools for analyzing and classifying the modes of agonism of such agonists.

GLP-2(3–33) has been used as an antagonist of GLP-2R and is generated naturally by DPP IV in vivo (8, 12). However, this peptide is also a partial agonist with relatively high activity (24). To search for more potent antagonists to help investigate the binding mode of action of agonists and ago-allosteric modulators of GLP-2R, we examined several truncated GLP-2 peptides. In this report, we present a novel orthosteric GLP-2R antagonist, GLP-2(11–33), and the interaction between an ago-allosteric modulator of GLP-2R (25) and this antagonist.

MATERIALS AND METHODS

Reagents. Forskolin and human GLP-2 were obtained from Sigma-Aldrich (St. Louis, MO) and Peptide Institute, Inc. (Osaka, Japan), respectively. The following human truncated GLP-2 peptides were custom-synthesized by Sigma Genosys (Ishikari, Japan) and stored at −20°C until use: GLP-2(3–33), GLP-2(6–33), GLP-2(11–33), GLP-2(12–33), GLP-2(13–33), GLP-2(14–33), GLP-2(15–33), GLP-2(16–33), GLP-2(1–25), and GLP-2(1–29).

We synthesized N-[1-(2,5-dichlorothiophen-3-yl)-2-(phenylsulfonyl)ethylidene]hydroxylamine at Eisai Co., Ltd. (Tsukuba, Japan). In this synthesis, both low- and high-polarity compounds were generated and separated by using silica gel column chromatography. The low-polarity compound was active (25) and was used in this study (compound 1).

Cloning of receptors. Human GLP-2R cDNA (GenBank accession number AF105367) was amplified with primers (5'-AGAATTCGCCATGAAGCTGGGATCGAGCAG-3' and 5'-CCCTCGAGCTAGATCTCACTCTCTTCCAGA-3') by using Marathon-Ready cDNA library human whole brain (Clontech, Mountain View, CA) as a template. Rat (GenBank accession number AF105368), Syrian hamster (GenBank accession number AB694745), dog (GenBank accession number XM_546617), and crab-eating (cyromolgus) monkey (GenBank accession number JN039378) GLP-2R cDNAs were amplified by using the following forward and reverse primers and small intestine cDNA of each species as a template: 5'-ACACACTCGAGACCATGAGGCCCCAACCAGCCCGAGCAGTGC-3' and 5'-TTGGATCCTTAGATCTCACTCTCTTCCAGA-3' for rat GLP-2R; 5'-TTTGAATTCGCCGCCACCATGAGGCCCCAGTCAAGC-3' and 5'-CCCCGTCGACTTAGATCTCACTCTCTTCCAGAATCTCCTCATC-3' for Syrian hamster GLP-2R; 5'-TTTGAATTCGCCGCCACCATGAGGCCCCAGTCAAGC-3' and 5'-CCCCGTCGACTTAGATCTCACTCTCTTCCAGAATCTCCTCATC-3' for dog; and 5'-TTTGAATTCGCCGCCACCATGAGGCCCCAGTCAAGC-3' and 5'-CCCCGTCGACTTAGATCTCACTCTCTTCCAGAATCTCCTCATC-3' for monkey GLP-2R. Amplified cDNA fragments were subcloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). Subcloned fragments were sequenced with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

Construction of a reporter vector. Because activities of truncated GLP-2 peptides and compound 1 were weaker than GLP-2, we used a sensitive reporter assay to catch the activities in which the secreted form of human placental alkaline phosphatase (PLAP) was produced (11). We constructed cDNA of PLAP regulated by CRE in the vasoactive intestinal peptide promoter (5, 9); the cDNA was incorporated into a retrovirus vector (18).

Generation of cell lines stably expressing CRE-PLAP and of those stably expressing a receptor. The CRE-PLAP gene was transduced by murine leukemia-based retrovirus into the HEK293 cell line (17). After the transduction, a cell clone was selected by
responsiveness to forskolin, and the established cell line was named SE302. By using the same method, human, crab-eating monkey, dog, Syrian hamster, or rat GLP-2R cDNA was transduced into the SE302 cell line and selected by using G418 (Invitrogen) or hygromycin B (Invitrogen) to obtain stably GLP-2R-overexpressing cell lines.

Cell culture. For maintenance, SE302 cells and SE302 cells overexpressing GLP-2R were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 1× penicillin/streptomycin and 10% FBS. The FBS was treated at 65°C for 30 min to minimize the likelihood of endogenous alkaline phosphatase activity interfering with that of heat-resistant PLAP. The cells were then seeded at 2 × 10^4/well on to gelatin-coated 96-well plates (Asahi Glass Co., Ltd., Tokyo, Japan) and incubated at 37°C overnight. On day 2, a peptide or compound, or both, was added to the culture, which was then incubated at 37°C overnight. The supernatants were collected and mixed with CDP-Star Ready-to-Use substrate/enhancer with Emerald II (Applied Biosystems) in wells of 96-well plates (Sumitomo Bakelite, Tokyo, Japan); the plates were incubated for 1 h at room temperature on day 3. PLAP activity was determined with an EnVision2101 Multilabel Reader (PerkinElmer, Waltham, MA). In addition, cell viability was checked by using alamarBlue (Invitrogen).

Binding assay. Membranes for binding experiments were prepared from confluent SE302 cells overexpressing human GLP-2R. The cells were washed with Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich), collected with cell scrapers into 10 mL of ice-cold DPBS, and centrifuged at 160 g at 4°C for 5 min. The collected cells were homogenized with a Teflon-glass homogenizer (10 strokes) in ice-cold 0.25 M sucrose containing 5 mM Tris-HCl (pH 7.4), and the homogenate was centrifuged at 1,000 g at 4°C for 10 min. The resulting supernatant was collected and centrifuged at 50,000 g at 4°C for 20 min. The supernatant was discarded and the pellet was homogenized in 50 mM Tris-HCl (pH 7.4) for 10 s with a Polytron PT 3100 homogenizer (Kinematica AB, Lucerne, Switzerland) set at 4. The homogenate was centrifuged and homogenized two more times. An aliquot of the homogenate was used for protein quantification and the remaining membranes (divided into 1-mL aliquots) were frozen at −80°C as a membrane source for the binding assay. Receptor binding studies were conducted with 100 pM [125I]-Bolton Hunter labeled human GLP-2 (PerkinElmer) as a ligand. On the day of the experiments, the membranes were reconstituted at 100 μg protein in 0.5 mL of binding buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% bovine serum albumin (BSA; Sigma-Aldrich)). Binding was equilibrated by incubation at room temperature for 2 h. The ligand-receptor complexes were separated by filtration overnight at 4°C in a Whatman GF/C glass filter presoaked with 50 mM Tris-HCl (pH 7.4) containing 1% BSA. Non-specific binding was determined in the presence of 100 nM GLP-2. The filters were washed three times with 4 mL of ice-cold 50 mM Tris-HCl containing 1% BSA. Each filter was collected at the bottom of a polypropylene tube, and the radioactivity remaining was quantified with a gamma counter (Aloka Co., Ltd., Tokyo, Japan).

Statistics. Regression curves of the reporter activity were generated by using GraphPad Prism 4.0E for Windows (GraphPad Software, Inc., San Diego, CA), and their top and bottom values and EC50 and IC50 values were calculated. The top value of the activity of each peptide was regarded as its intrinsic agonistic activity.

RESULTS AND DISCUSSION

Orthosteric GLP-2R antagonists are needed to analyze the binding and functioning modes of GLP-2R agonists and ago-allosteric modulators. GLP-2(3–33), which is generated by DPPIV in vivo (8, 12), exhibits partial agonist and antagonist properties (24). This peptide has been used as a GLP-2R antagonist in both in vitro and in vivo experiments (1, 2, 14, 22). However, we considered GLP-2(3–33) to be insufficient as a GLP-2R antagonist in our study because of its comparatively high partial agonism (24), so we searched for more potent novel GLP-2R antagonists.

We evaluated the binding and reporter activities of GLP-2 and several GLP-2 truncated peptides. Fig. 1A shows the amino acid sequences of human GLP-2 and its truncated peptides, and Fig. 1B shows their concentration-reporter activity curve. Table 1
summarizes their binding and intrinsic agonistic activities. GLP-2(3–33) had 1/300 the EC\textsubscript{50} value of GLP-2 but retained about 60% of its intrinsic activity, as reported by Thulesen \textit{et al.} (24). GLP-2(6–33) exhibited similar activity to GLP-2(3–33). Deleting 10 amino acid residues from the N-terminus caused an extreme reduction in intrinsic activity and EC\textsubscript{50} value. GLP-2(11–33) had 1/10 the intrinsic activity and 1/13,400 the EC\textsubscript{50} value of GLP-2, but it retained binding activities of 88% at 1 μM and 100% at 10 μM. GLP-2(12–33) and GLP-2(13–33) had dramatically decreased binding activity. With four amino acid residues deleted from the C-terminus, GLP-2(1–29) manifested full native intrinsic activity despite a reduced EC\textsubscript{50} value. We therefore judged that GLP-2(11–33) was a novel, potent, and specific peptide-derived orthosteric antagonist of human GLP-2R. Our findings suggest that the N- and C-termini of GLP-2 are needed for maintaining the intrinsic and binding activities, respectively, of human GLP-2R.
GLP-2 receptor antagonist

GLP-2 receptor antagonist

We consider that the amino acid sequence between the sixth and 10th amino acid residues from the N-terminus of GLP-2 is crucial for retaining agonistic activity. It is likely that the 11th amino acid residue from the N-terminus is critical for the binding to human GLP-2R. In addition, we found that GLP-2(1–25) had no reporter activity until it reached a concentration of 10 μM (data not shown). Thus, it is speculated that the amino acid sequence between the 26th and 29th amino acid residues from the N-terminus is also important for the agonistic activity.

Table 2  Summary of species-specific differences of responsiveness of glucagon-like peptide 2 (GLP-2) receptors (GLP-2Rs) to human GLP-2, GLP-2(11–33), or GLP-2(11–33) with compound 1, as determined by using a reporter system in which HEK293 cells stably overexpressing GLP-2R and placental alkaline phosphatase gene regulated by the cAMP response element were used.

<table>
<thead>
<tr>
<th>Species of GLP-2R</th>
<th>Human GLP-2</th>
<th>Human GLP-2(11–33)</th>
<th>Human GLP-2(11–33) plus compound 1 (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (pM)</td>
<td>Intrinsic activity</td>
<td>EC₅₀ (nM)</td>
</tr>
<tr>
<td>Human</td>
<td>32</td>
<td>19</td>
<td>2,100</td>
</tr>
<tr>
<td>Crab-eating monkey</td>
<td>22</td>
<td>47</td>
<td>270</td>
</tr>
<tr>
<td>Dog</td>
<td>24</td>
<td>19</td>
<td>340</td>
</tr>
<tr>
<td>Syrian hamster</td>
<td>40</td>
<td>12</td>
<td>210</td>
</tr>
<tr>
<td>Rat</td>
<td>25</td>
<td>No activity</td>
<td>–</td>
</tr>
</tbody>
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Means of triplicate samples.
–, Not applicable.

Fig. 2  A. Chemical structure of compound 1 (N-[1-(2,5-dichlorothiophen-3-yl)-2-(phenylsulfanyl)ethyldiene]hydroxylamine).
B–E. Effects of a truncated glucagon-like peptide 2, GLP-2(11–33), on placental alkaline phosphatase (PLAP) activity induced by compound 1 in HEK293 cells stably overexpressing human GLP-2 receptor (GLP-2R) (B), crab-eating monkey GLP-2R (C), dog GLP-2R (D), or Syrian hamster GLP-2R (E), and PLAP regulated by cAMP response element. Regression curves were produced by using GraphPad Prism (GraphPad Software, Inc.). Open circles, without compound 1; black circles, with compound 1 at 10 μM. Each value is the mean of three samples.
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GLP-2 amino acid sequences are highly conserved among species: the homology is 97%, 94% and 97% between humans and rats, between humans and mice and between rats and mice, respectively (22). Human GLP-2(3–33) lowered small intestinal weights in rats as rat GLP-2(3–33) did (22). In a similar way, human GLP-2(3–33) decreased small intestinal weights in mice (14). Therefore, it is likely that human GLP-2(11–33) also bind to at least on rat and mouse GLP-2Rs, and compete with rat GLP-2 or mouse GLP-2.

We investigated the intrinsic activity of human GLP-2 and GLP-2(11–33) on GLP-2Rs in humans, crab-eating monkeys, dogs, Syrian hamsters, and rats to examine whether there were species-specific differences. Human GLP-2 induced PLAP reporter activity in GLP-2Rs of all species, with similar EC$_{50}$ values between 22 and 40 pM (Table 2). Partial agonism by GLP-2(11–33) was observed on human, monkey, dog and Syrian hamster GLP-2Rs, but their intrinsic activities and EC$_{50}$ values varied. No agonistic activity was recognized on rat GLP-2R.

As a next step, we studied the effects of GLP-2(11–33) on the PLAP activity of compound 1 in the same cells. Regression curves were produced by using GraphPad Prism (GraphPad Software, Inc.). Open circles, without compound 1; black circles, with compound 1 at 10 μM. Each value is the mean of three samples.

**Fig. 3** Effects of a truncated glucagon-like peptide 2, GLP-2(11–33), on placental alkaline phosphatase (PLAP) activity induced by compound 1 at 10 μM (A) in HEK293 cells stably overexpressing rat GLP-2 receptor (GLP-2R) and PLAP driven by cAMP response element. B and C show effects of GLP-2 and GLP-2(3–33), respectively, on the PLAP activity of compound 1 in the same cells. Regression curves were produced by using GraphPad Prism (GraphPad Software, Inc.). Open circles, without compound 1; black circles, with compound 1 at 10 μM. Each value is the mean of three samples.
(Fig. 2D), and Syrian hamster GLP-2Rs (Fig. 2E). Changes in maximum reporter activities and EC₅₀ values with the addition of GLP-2(11–33) and compound 1 are shown in Table 2. In the case of rat GLP-2R there was a striking contrast. GLP-2(11–33) suppressed the PLAP activity of compound 1 in a concentration-dependent fashion (Fig. 3A). The IC₅₀ value was calculated as 1.3 μM. Calculation of the bottom value revealed that GLP-2(11–33) did not inhibit the activity of compound 1 completely; 20% activity of 10 μM compound 1 remained at 10 μM of GLP-2(11–33). We also investigated the effects of GLP-2 and GLP-2(3–33) on the activity of compound 1 toward rat GLP-2R. There were additional increases in reporter activity with a combination of compound 1 with GLP-2 (Fig. 3B) or GLP-2(3–33) (Fig. 3C).

We previously discovered oxime-based compounds as ago-allosteric modulators of human GLP-2R, working both as GLP-2R agonists alone and as allosteric modulators (25); a combination of compound 1 and GLP-2 or GLP-2(3–33) resulted in an additive effect on reporter activity toward human GLP-2R. In combination with GLP-2(11–33), the reporter activity of compound 1 was not inhibited, but potentiated synergistically. The binding assay showed that an active oxime derivative, compound 17 (N-[1-(2,5-dichlorothiophen-3-yl)-2-(pyridin-4-ylsulfanyl) ethylidene]hydroxylamine) did not inhibit the specific binding of [¹³⁵I]human GLP-2 to human GLP-2R until the concentration of the compound reached 100 μM (25). Together, the results from the reporter and binding assays suggested that the oxime derivatives were bound to the allosteric site of human GLP-2R.

It has been speculated that the binding sites of several ago-allosteric modulators overlap with those of the endogenous ligands, for example, ghrelin (13, 21). However, it has not been clarified how an ago-allosteric modulator binds when its binding site overlaps with that of an orthosteric ligand, although some scenarios have been presented to explain the phenomenon (21). We observed additional increases in the agonistic activity of compound 1 with the addition of GLP-2 or GLP-2(3–33), indicating that compound 1 functioned as an ago-allosteric modulator of rat GLP-2R, as is the case with human GLP-2R. Moreover, inhibition of the activity of compound 1 by GLP-2(11–33) showed that compound 1 was bound to the orthosteric site of rat GLP-2R.

We found a novel orthosteric antagonist of GLP-2R, GLP-2(11–33), and employed it in a reporter assay expressing GLP-2R in several species. Use of this peptide shows that, in the reporter assay, it is possible that the binding sites of the ago-allosteric modulator and GLP-2 overlap, at least in the case of rat GLP-2R. This cannot be deduced by using GLP-2(3–33)—the previously reported classic orthosteric antagonist of GLP-2R—alone. Therefore, GLP-2(11–33) is a useful tool for analyzing the binding modes of GLP-2R agonists and ago-allosteric modulators.

REFERENCES

binding site for the endogenous agonist, small-molecule agonists, and ago-allosteric modulators on the ghrelin receptor. *Mol Pharmacol* 75, 44–49.


