Biased GLP-2 agonist with strong G protein-coupling but impaired arrestin recruitment and receptor desensitization enhances intestinal growth in mice

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Abstract

Background and Purpose: Glucagon-like peptide-2 (GLP-2) is secreted postprandially by enteroendocrine L-cells and stimulates growth of the gut and bone. One GLP-2 analogue is approved for short bowel syndrome (SBS). To improve therapeutic efficacy, we developed biased GLP-2 receptor (GLP-2R) agonists through N-terminal modifications.

Experimental Approach: Variants with Ala and Trp substitutions of the first seven positions of GLP-2(1-33) were studied in vitro for affinity, G protein activation (cAMP accumulation), recruitment of β-arrestin 1 and 2, and internalization of the human and mouse GLP-2R. The intestinotrophic actions of the most efficacious (cAMP) biased variant were examined in mice.

Key Results: Ala substitutions had more profound effects than Trp substitutions. For both, alterations at positions 1, 3 and 6 most severely impaired activity. β-arrestin recruitment was more affected than cAMP accumulation. Among Ala substitutions, [H1A], [D3A] and [F6A] impaired potency (EC50) for cAMP-accumulation >20-fold and efficacy (Emax) to 48%–87%, and were unable to recruit arrestins. The Trp substitutions, [A2W], [D3W] and [G4W] were partial agonists (Emax of 46%–59%) with 1.7–12-fold decreased potencies in cAMP and diminished β-arrestin recruitment. The biased variants, [F6A], [F6W] and [S7W] induced less GLP-2R internalization compared with GLP-2, which induced internalization in a partly arrestin-independent manner. In mice, [S7W] enhanced gut trophic actions with increased weight of the small intestine, increased villus height and crypt depth compared with GLP-2.

Abbreviations: DPP-4, dipeptidyl peptidase-4; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, GIP receptor; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GLP-2, glucagon-like peptide-2; GLP-2R, GLP-2 receptor; SBS, short bowel syndrome.
1 | INTRODUCTION

Glucagon-like peptide-2 (GLP-2) is a 33 amino acid long intestinal hormone secreted postprandially by enteroendocrine L cells. The hormone acts mainly through the GLP-2 receptor (GLP-2R), which belongs to class B1 of the G protein-coupled receptors (GPCRs) (Fredriksson et al., 2003), but GLP-2 also causes some activation of the glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) (Gadgaard et al., 2022). GLP-2 plays an important role in the intestines, where it stimulates mucosal cell proliferation and inhibits apoptosis (Boushey et al., 2001; Burrin et al., 2007; Drucker et al., 1996). It furthermore enhances nutrient absorption, stimulates intestinal blood flow, has anti-inflammatory activity, and helps to maintain the integrity of the intestinal barrier (Bremholm et al., 2009; Brubaker, 2018; Drucker et al., 1996; Estall & Drucker, 2006; Guan et al., 2006; Moore et al., 2010; Nagell et al., 2004; Orskov et al., 1986). GLP-2 also appears to play a role in bone turnover, as evident from studies where exogenous GLP-2 acutely reduces bone resorption to 50%-60% of basal activity in humans (Askov-Hansen et al., 2013; Gottschalck et al., 2008; Henriksen et al., 2003; Skov-Jejepesen et al., 2019). Moreover, GLP-2 dose-dependently increases the hip bone mineral density in a clinical study of postmenopausal women receiving daily subcutaneous GLP-2 injections for 4 months (Henriksen et al., 2009). In addition, mice with deletion of the GLP-2R show marked skeletal deficits (Undale et al., 2006). Thus, GLP-2 also seems to have potential in the treatment of bone-related diseases, as reviewed recently (Schillerup et al., 2019). However, the native form of GLP-2 is of limited use as a therapeutic agent due to its short half-life in humans of approximately 7 min by degradation mediated by the protease, dipeptidyl peptidase-4 (DPP-4), as well as by a high rate of renal clearance (Drucker, Shi, et al., 1997; Hartmann et al., 2000).

Currently, teduglutide is the only GLP-2 analogue that has been approved for medical treatment. Teduglutide is a DPP-4 resistant GLP-2 analogue, which is used to treat short bowel syndrome (SBS); a severe and chronic disease characterized by malabsorption due to physical or functional loss of significant portions of the small intestine (Jeppesen, 2015; Jeppesen et al., 2001). The compound has a half-life of 3.0-5.5 h when injected subcutaneously (Marier et al., 2008) and is therefore given once-daily to patients suffering from SBS, but this still does not provide 24-h exposure.

Biased agonism is the ability of a ligand to selectively activate one intracellular pathway over another, relative to a reference ligand (Wootten et al., 2018). In the field of GPCRs, this could be G protein activation over β-arrestin recruitment—representing G protein-biased agonism. The major benefit of selectively activating one pathway over another is the ability to improve efficacy and/or reduce side-effect profiles. This has, for instance, been shown for ligands of the μ-opioid receptor, which belongs to the class A GPCRs, where agonists biased towards G protein signalling show stronger analgesic effects compared with unselective ligands (Manglik et al., 2016; Soergel et al., 2014). Within class B1 GPCRs, the recently FDA-approved co-agonist tirzepatide, targeting the GLP-1R and the receptors for glucose-dependent insulinotropic polypeptide (also called gastric inhibitory polypeptide, GIP), acts as biased agonist on the GLP-1R with strong G protein-mediated signalling, but impaired receptor internalization as part of its apparently superior mechanism of actions (Holst et al., 2021; Willard et al., 2020). Recently, we were able to show that an N-terminal modification of the DPP-4 site in GLP-1 (GLP-1[ABV]) confers G protein bias, relative to native GLP-1(7-36) NH2 (van der Velden et al., 2021).

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**Conclusion and Implications:** G protein-biased GLP-2R agonists with diminished receptor desensitization have superior intestinotrophic effects and may represent improved treatment of intestinal insufficiency including SBS.

**KEYWORDS**

bias, GLP-2 receptor (GLP-2R), glucagon-like peptide-2 (GLP-2), internalization, intestinal growth

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**What is already known?**

- GLP-2 has clinically relevant intestinotrophic actions.
- Biased agonism designates selective activation of intracellular pathway(s) relative to reference agonist.

**What does this study add?**

- Alterations of the GLP-2 N-terminus may result in selective intracellular pathway activation.
- Biased GLP-2 receptor agonists, tailored towards Gs-coupling with diminished receptor desensitization, have improved intestinotrophic actions.

**What is the clinical significance?**

- Biased GLP-2 receptor agonists are potentially superior in treatment of intestinal insufficiency.
- Other therapeutic class B1 receptor agonists may be improved by similar desensitization protection.
Here we hypothesized that G protein-biased GLP-2R agonists would have improved intestinotrophic actions due to lesser receptor desensitization, and that N-terminal amino acid substitutions in the GLP-2 sequence would result in such biased profiles. The latter assumption is based on current knowledge of the role of the N-terminus in class B1 receptor activation, where recent observations suggest that receptor activation induces a very complex network of conformational changes (Liang et al., 2018; van der Velden et al., 2021; Venneti & Hewage, 2011; Wu et al., 2020; Y. Zhang et al., 2017). Thus, crystal and cryo-EM structures of the class B1 GPCRs, including the GLP-1R, the glucagon receptor (GCGR), the GIP receptor (GIPR), and recently also the GLP-2R, have revealed a number of amino acid residues of particular importance for ligand binding and receptor activation within this receptor family (Parthier et al., 2007; Parthier et al., 2009; Song et al., 2017; Sun et al., 2020; H. Zhang et al., 2018; Y. Zhang et al., 2017). Attempting to create bias, we carried out substitutions of the first seven N-terminal residues of the GLP-2 peptide with either alanine (Ala, A) or tryptophan (Trp, W) and examined how these substitutions affected the GLP-2R activation with respect to both cAMP accumulation and β-arrestin 1 and 2 recruitment. For the most potent variants with unselective (GLP-2 wild-type like) and selective (biased) activation profiles, we furthermore probed their ability to induce GLP-2R internalization. Finally, we selected one biased variant, namely the most potent (in terms of cAMP accumulation) with a low ability to recruit β-arrestins and induce receptor internalization, and examined its intestinotrophic effect relative to native GLP-2 in mice. Our results suggest that, within the GLP-2 system, it is possible to develop biased agonists that are less liable to cause desensitization and tachyphylaxis with potential superiority to the existing agents for the therapy of intestinal disorders such as SBS based on GLP-2R targeting.

2 | METHODS

2.1 | Materials

Human GLP-2 and GLP-1 were purchased from Bachem, Bubendorf, Switzerland (4039611 and 4030663, respectively). N-terminally amino acid substituted GLP-2 variants (GLP-2 Ala and Trp scan peptides) based on the sequence of human GLP-2, were synthesized by Schafer-n, Copenhagen, Denmark. All peptides had a purity of at least 95% by HPLC analysis and correct mass spectrometry controlled molecular weight. cDNAs of human GLP-2R, human GLP-1R and mouse GLP-2R were purchased from Origene, Rockville, Maryland, USA (SC111108, SC124060 and MC217338, respectively). SNAP-tagged GLP-2R was custom produced by Cisbio, Codolet, France. GLP-2(1-33)[M10V] was 125I-labelled using the standard stoichiometric chloramine T method, as previously described (Gadgaard et al., 2022). HEK293A and HEK293A Δβ-Arr1/2 KO cells were kindly provided by Asuka Inoue, Tohoku University, Japan (O’Hayre et al., 2017).

2.2 | Transfection and tissue cultures

COS-7 cells (RRID:CVCL_0224) were cultured at 10% CO₂ and 37°C in Dulbecco’s Modified Eagle Medium (DMEM) 1885 supplemented with 10% fetal bovine serum (FBS), 2-mM glutamine, 180 units per milliliter penicillin and 45-g·mL⁻¹ streptomycin. Transient transfection of COS-7 cells was performed using the calcium phosphate precipitation method as previously described (van der Velden et al., 2021). HEK293 (RRID:CVCL_0045) cells were cultured at 10% CO₂ and 37°C in DMEM-GlutaMAX™-I supplemented with 10% FBS, 180 units per milliliter penicillin and 45-g·mL⁻¹ streptomycin. These cells were transfected using polyethylenimine (PEI) for the arrestin recruitment assay as described in detail in (van der Velden et al., 2021). HEK293A (RRID:CVCL_6910) and HEK293A β-arrestin 1 and 2 knockout (Δβ-Arr1/2 KO) cells were cultured at 5% CO₂ and 37°C in DMEM-GlutaMAX™-I supplemented with 10% FBS, 180 units per milliliter penicillin and 45-g·mL⁻¹ streptomycin. These cells were transfected using Lipofectamine 2000 from Thermo Fischer Scientific, Massachusetts, USA, according to the manufacturer’s instructions for the real-time internalization assay.

2.3 | cAMP assay

Transiently transfected COS-7 cells were seeded in white 96-well plates the day after transfection with a density of 35,000 cells per well. The next day the assay was initiated by washing with HEPES buffered saline (HBS) followed by an incubation step with assay buffer (HBS with 1 mM 3-isobutyl-1-methylxanthine [IBMX]) for 30 min at 37°C. Assay buffer was pH adjusted to 8.3 using 4 M NaOH before added. The ligands were then added and incubated for an additional 30 min at 37°C. After ligand incubation, the HitHunter® cAMP assay (Eurofins DiscoverX, Fremont, USA) was carried out according to the manufacturer’s instructions. Luminescence was measured by PerkinElmer™ EnVision 2014 Multilabel Reader (PerkinElmer, Waltham, MA).

2.4 | β-Arrestin 1 and 2 recruitment assay

HEK293 cells were transiently transfected with either human or mouse GLP-2R, the donor RLuc8-Arrestin-2-Sp1 (arrestin 2 recruitment) or RLuc8-Arrestin-3-Sp1 (arrestin 3 recruitment), the acceptor Δβ-Arr1/2 KO cells were cultured at 5% CO₂ and 37°C in DMEM-GlutaMAX™-I supplemented with 10% FBS, 180 units per milliliter penicillin and 45-g·mL⁻¹ streptomycin. Following 10-min incubation, increasing concentrations of the ligands were added and incubated for an additional 30 min. Luminescence was measured with the Berthold Technologies Mithras Multilabel Reader (RLuc8 at 485 ± 40 nm and YFP at 530 ± 25 nm).
2.5 | Competition binding

COS-7 cells transiently expressing human GLP-2R were seeded in clear 24-well plates the day after transfection at a density of 100,000 cells per well. The number of cells added per well was adjusted to ensure 5%-10% specific binding of the radioligand, \(^{125}\text{I}\)-GLP-2(1-33) [M10Y] (Gadgaard et al., 2022). The following day, the cells were assayed by competition binding for 3 h at 4°C using 15–40 pM of \(^{125}\text{I}\)-GLP-2(1-33)[M10Y] and increasing amounts of unlabelled ligand in a total volume of 210 \(\mu\)L per well in 50-mM HEPES buffer (pH 7.2) supplemented with 0.5% bovine serum albumin (BSA) (binding buffer). After incubation, the cells were washed twice in 400 \(\mu\)L per well ice-cold binding buffer and lysed using 500 \(\mu\)L per well of 200-mM NaOH with 1% SDS for 30 min. The samples were counted using the Wizard Gamma Counter (PerkinElmer, Waltham, MA).

2.6 | Real-time receptor internalization

HEK293A and HEK293A \(\Delta\beta\)-Arr1/2 KO cells transiently expressing human SNAP-GLP-2R were seeded at a density of 15,000 cells per well in white 384-well plates on the day of transfection. The following day media were removed and new, fresh culture media were added. On day three, the assay was run. First, the SNAP-GLP-2R expressing cells were labelled with 100-nM Tag-Lite SNAP-Lumi4-Tb (donor) in OptiMEM for 60 min at 37°C. Next, the cells were washed four times with internalization buffer Hank’s Balanced Salt Solution (HBSS) supplemented with 1-mM CaCl\(_2\), 1-mM MgCl\(_2\) and 20-mM (HEPES) followed by addition of 100 \(\mu\)M preheated fluorescein-O’-acetic acid (acceptor). Only internalization buffer was added for donor recording. The plate was incubated at 37°C for 5 min prior to ligand stimulation for temperature adjustment before reading the plate. The cells were then stimulated with 37°C preheated ligand solution and internalization was measured every third minute for 60 min at 37°C in PerkinElmer™ EnVision 2014 Multilabel Reader (PerkinElmer, Waltham, MA). The same donor signal was achieved in the HEK293A and HEK293A \(\Delta\beta\)-Arr1/2 KO cells by adjustment of SNAP-GLP-2R DNA concentrations.

2.7 | Intestinal growth in mice

Animal experiments were performed with permission from the Danish Animal Experiments Inspectorate (licence 2018-15-0201-01397) and the local ethical committee in accordance with the guidelines of Danish legislation governing animal experimentation (1987) and the National Institutes of Health (publication number 85-23). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). Female C57Bl/6Jj mice (12 weeks old) obtained from Janvier Labs (Saint Berthevin Cedex, France) were housed in groups of eight in individually ventilated cages under a 12-h light cycle. Mice had ad libitum access to standard chow and water and were left to acclimatize for at least 1 week prior to the study. The mice were given either 2.5 \(\mu\)g per mouse GLP-2, 2.5 \(\mu\)g per mouse GLP-2[57W] or vehicle subcutaneously morning and evening for 10 days. On day 10, the mice were euthanized by cervical dislocation and the small intestine and colon were removed surgically, gently flushed with 0.9% NaCl to remove luminal content, and weighted.

2.8 | Histology

Fresh tissue from the duodenum, jejunum, ileum and colon was fixed in a 4% formaldehyde solution before being dehydrated and paraffin-embedded. A microtome was used to cut the embedded tissue (4 \(\mu\)m) and the sections were stained with haematoxylin and eosin (H&E). The length of 20 well-oriented villi and crypts were measured per section using a light microscope connected to a camera (Zeiss Axio Lab.A1, Brock & Michelsen, Birkeroed, Denmark) and Zeiss Zen lite software (Carl Zeiss Microscopy GmbH, Göttingen, Germany). The observer was blinded to the origin of the sections.

2.9 | Data and statistical analysis

For the in vitro studies, \(IC_{50}\), \(EC_{50}\) and \(K_i\) values were determined by nonlinear regression using GraphPad Prism 9 (San Diego, California, USA) (GraphPad Prism, RRID:SCR_002798). Sigmoid curves were fitted logistically with a Hill slope of 1.0 for activation curves and –1.0 for binding curves. For the in vivo studies, the experiment number is \(n = 3\) due to very concise replicability. To uniform the assay outputs (cAMP and \(\beta\)-arrestin assays), each experiment, performed in duplicate, was normalized to the absence of ligand (i.e., only buffer addition) as baseline (0%) and the highest tested concentration of the endogenous hormone (100%). For the in vivo study, group size calculation was performed based on small intestinal weight related to bodyweight (BW) as a primary endpoint. Based on previous experiments, we expected a mean of 6.6% of BW in the group treated with GLP-2 (Hunt et al., 2020). We aimed to detect a difference of 5% (±0.3% of BW) between treatment groups with a probability rate of 0.05. When using a typical standard deviation on ±0.35% of BW, we needed at least 15 mice per group to reach a power of 80%. We selected 16 mice per group to account for unexpected mortality and technical issues. Differences between groups were evaluated by one-way ANOVA followed by Tukey’s multiple comparison test. The post hoc tests were conducted only if significance was obtained in ANOVA analyses. Statistical analysis was undertaken only for the in vivo experiment, where each group size was \(n > 5\). The declared group size is the number of independent values and the statistical analysis was done using these independent values. Statistical significance was accepted at \(P < .05\). Outliers were in general included in the data analysis and presentation. The manuscript complies with BJPs recommendations and requirements on experimental design and BJPs recommendations on experimental design and presentation (Curtis et al., 2022).
2.10 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 RESULTS

3.1 Binding profiles of N-terminally substituted GLP-2 variants

Ala and Trp substitutions were made in the first seven N-terminal amino acids of GLP-2 (Figure 1a) to study how this would affect receptor binding and activation (cAMP accumulation and β-arrestin recruitment). With an Ala scan, the side chain of the original amino acid is deleted, whereas the Trp substitution introduces potential steric hindrance. All peptides were able to compete with $^{125}$I-GLP-2 [M10Y] for GLP-2R binding; however, the affinity was affected in most cases (Table 1). Substituting Ala at positions 4, 5 and 7 resulted in less than 5-fold decrease in affinity (Figure 1b and Table 1). Bearing in mind that native GLP-2 has Ala in position 2, the largest effects were observed when Ala was introduced at positions 1, 3 and 6 where 16- to 25-fold shifts in $K_i$ were observed (Figure 1b and Table 1). Trp substitutions at positions 1 and 2 had a moderate effect on the affinity (4- to 6.3-fold decreases) (Figure 1c and Table 1), whereas Trp in positions 3 to 7 resulted in 13- to 32-fold decreases with the largest shift observed for GLP-2[D3W] and GLP-2[F6W] (Figure 1c and Table 1). Altogether, the largest affinity impairments were observed with substitution at positions 3 and 6 for both the Ala and Trp scan.

3.2 Bias can arise upon introduction of Ala or Trp in the N-terminus of GLP-2

We went on to study how the GLP-2 variants activated the human GLP-2R based on cAMP accumulation. Teduglutide, a human GLP-2 with an Ala to Gly substitution at position 2, was included to allow comparisons of our GLP-2 amino acid substituted variants with an established drug. Ala substitution of the N-terminal amino acid of GLP-2, [H1A], had the largest effect on both the potency and efficacy, with a 162-fold decreased potency and ~50% reduced efficacy compared to GLP-2 (Figure 2a1 and Table 1), despite a mere 16-fold decrease in affinity (Table 1). Consistent with the loss of affinity, a decrease in potency and efficacy was observed with Ala substitutions at position 3 ([D3A]) and 6 ([F6A]) with 55-fold and 21-fold decreases in potency and reductions in efficacy to 84% and 87% of GLP-2, respectively (Figure 2a3 and 2a6 and Table 1). Looking at the Trp scan, the largest effect on efficacy was observed when residues at positions 2 ([A2W]), 3 ([D3W]) and 4 ([G4W]) were substituted. Here the efficacy decreases to 46%, 59% and 52% of GLP-2, respectively (Figure 2a2-a4 and Table 1). For comparison, teduglutide resulted in a
TABLE 1  Affinity, potency and efficacy values of the Ala and Trp scan of GLP-2 on the human GLP-2R. The table displays a summary of the affinity, potency and efficacy values of the Ala and Trp scan of the first seven N-terminal amino acids of GLP-2 on the human GLP-2R tested in binding, cAMP accumulation and β-arrestin 1 and 2 recruitment experiments. The data shown are from n = 3 independent experiments carried out in duplicate.

<table>
<thead>
<tr>
<th>Binding</th>
<th>cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log IC₅₀ ± SEM</td>
<td>Kᵢ (nM)</td>
</tr>
<tr>
<td>GLP-2</td>
<td>−8.9 ± 0.20</td>
</tr>
<tr>
<td>GLP-2[H1A]</td>
<td>−7.7 ± 0.22</td>
</tr>
<tr>
<td>GLP-2[D3A]</td>
<td>−7.5 ± 0.20</td>
</tr>
<tr>
<td>GLP-2[G4A]</td>
<td>−8.7 ± 0.44</td>
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<tr>
<td>GLP-2[S5A]</td>
<td>−8.3 ± 0.21</td>
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<tr>
<td>GLP-2[F6A]</td>
<td>−7.6 ± 0.20</td>
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<tr>
<td>GLP-2[S7A]</td>
<td>−8.3 ± 0.32</td>
</tr>
<tr>
<td>GLP-2[H1W]</td>
<td>−8.3 ± 0.12</td>
</tr>
<tr>
<td>GLP-2[A2W]</td>
<td>−8.1 ± 0.19</td>
</tr>
<tr>
<td>GLP-2[D3W]</td>
<td>−7.4 ± 0.21</td>
</tr>
<tr>
<td>GLP-2[G4W]</td>
<td>−7.6 ± 0.23</td>
</tr>
<tr>
<td>GLP-2[S5W]</td>
<td>−7.8 ± 0.26</td>
</tr>
<tr>
<td>GLP-2[F6W]</td>
<td>−7.5 ± 0.37</td>
</tr>
<tr>
<td>GLP-2[S7W]</td>
<td>−7.7 ± 0.14</td>
</tr>
</tbody>
</table>

10-fold higher potency compared to that of GLP-2 (Figure 2a2 and Table 1).

Regarding β-arrestin 1 and 2 recruitments to the human GLP-2R, most variants completely lost this property and are therefore biased towards G protein signalling. Only [G4A], [S5A], [S7A] and [S7W] recruited β-arrestin 1 or 2 with similar potencies as GLP-2, however, with decreased efficacy for [S5A] and [S7W] (Figures 2b2/c2 and Table 1). Teduglutide recruited arrestins as efficiently as GLP-2 (Figure 2b2/c2 and Table 1). To get a combined view of the bias introduced, we calculated the area under the curve (AUC) for each peptide in each readout (Figure 2d1-d7). This illustrated that [H1W] (Figure 2d1), [S5W] (Figure 2d5), [F6A] and [F6W] (Figure 2d6) and [S7W] (Figure 2d7) resulted in the largest bias towards G protein signalling compared to GLP-2 whereas no bias was observed for teduglutide (Figure 2d2), [G4A] (Figure 2d4) or [S7A] (Figure 2d7).

3.3 Complex patterns of receptor internalization induced by the GLP-2 variants

We tested the most potent GLP-2 variants (as estimated from the cAMP accumulation experiments): the unbiased [G4A], [S5A] and [S7A] and the biased [H1W], [S5W], [F6A], [F6W] and [S7W] for their ability to induce GLP-2R internalization using the N-terminally SNAP-tagged version of the human GLP-2R (Figure 3). GLP-2 induced GLP-2R internalization with an EC₅₀ of 42 nM (Figure 3a/b). The unbiased [G4A] (Figure 3e/f) and [S7A] (Figure 3k/l) induced internalization equal to GLP-2, but likewise did the biased [S5A] (Figure 3g/h). In contrast, the biased [F6A] resulted in a 71% decrease in efficacy compared to GLP-2 at 1-μM stimulation (Figure 3i/j). For the biased Trp substitutions, [H1W] (Figure 3m/n) and [S7W] (Figure 3o/p), the internalization profiles mimicked that of GLP-2, whereas the other biased [F6W] (Figure 3q/r) and [S7W] (Figure 3s/t) forms had a decrease in efficacy by 39% and 37%, respectively, compared to GLP-2 following 1-μM stimulation.

3.4 Human GLP-2R internalizes in the absence of β-arrestins

Because some of the G protein biased GLP-2 variants induced GLP-2R internalization as efficiently as native GLP-2 despite their inability to recruit β-arrestins ([H1W] and [S5W]), we probed the ability of the human GLP-2R to internalize in the absence of β-arrestin 1 or 2 by employing HEK293A Δβ-arrestin 1/2 KO cells (Figure 4). In the absence of β-arrestin 1 or 2, the human GLP-2R still internalized following GLP-2 stimulation, however not as efficiently as in the presence of arrestins (Figure 4a) despite similar receptor expression levels (Figure 4b). Furthermore, the receptor seemed to internalize faster when the arrestins were present with an internalization rate of 0.16 ± 0.014 min−1 compared to 0.12 ± 0.017 min−1 in the absence of arrestins following 1 μM GLP-2 stimulation (Figure 4a). Thus, the GLP-2R can internalize in the absence of arrestins, but with lower efficiency and speed compared to when arrestins are present.
Table 1 (Continued)

<table>
<thead>
<tr>
<th>GLP-2</th>
<th>β-Arrestin 1</th>
<th></th>
<th></th>
<th>GLP-2</th>
<th>β-Arrestin 2</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log EC50 ± SEM</td>
<td>EC50 (nM)</td>
<td>EC50 fold change from GLP-2</td>
<td>E_max ± SEM (%)</td>
<td>Log EC50 ± SEM</td>
<td>EC50 (nM)</td>
<td>EC50 fold change from GLP-2</td>
</tr>
<tr>
<td>GLP-2</td>
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<td>-</td>
<td>100 ± 2.1</td>
<td>−8.8 ± 0.06</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>GLP-2[H1A]</td>
<td>No recruitment</td>
<td></td>
<td></td>
<td>No recruitment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLP-2[D3A]</td>
<td>No recruitment</td>
<td></td>
<td></td>
<td>No recruitment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLP-2[G4A]</td>
<td>−9.2 ± 0.20</td>
<td>0.63</td>
<td>0.4</td>
<td>74 ± 4.6</td>
<td>−9.3 ± 0.17</td>
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<td>48 ± 5.5</td>
<td>−8.7 ± 0.25</td>
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<tr>
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<td>43 ± 5.5</td>
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Figure 2  
cAMP accumulation and β-arrestin 1 and 2 recruitment of Ala and Trp substituted GLP-2 variants on human GLP-2R. Human GLP-2R was transiently expressed in COS-7 cells and assessed for (a1–a7) cAMP accumulation or HEK293 cells and assessed for (b1–b7) β-arrestin 1 recruitment and (c1–c7) β-arrestin 2 recruitment upon stimulation with the GLP-2 modified variants. The bars at the top of the figure indicate which GLP-2 modified variant was tested. The dashed line in each graph represents native GLP-2. (d1–d7) Area under the curve (AUC) is calculated for each graph and shown as an activity index. Data are shown as mean ± SEM, n = 3 independent experiments carried out in duplicate.
FIGURE 3  Real-time internalization of human GLP-2R following Ala and Trp GLP-2 modified variant stimulation. HEK293A cells were transiently transfected with the N-terminally SNAP-tagged GLP-2R and assayed for internalization following stimulation of the most potent Ala and Trp substituted GLP-2 variants from the cAMP accumulation experiments. Both the real-time internalization data and corresponding dose–response curves are shown for (a/b) GLP-2, (c/d) teduglutide, (e/f) [G4A], (g/h) [S5A], (i/j) [F6A], (k/l) [S7A], (m/n) [H1W], (o/p) [S5W] and (q/r) [F6W] and (s/t) [S7W]. The dashed line in each dose–response graph represents native GLP-2. Data are shown as mean ± SEM, n = 3 independent experiments carried out in triplicate.
3.5 | GLP-1R activation reduced by the GLP-2 variants

Because GLP-2 is a low potency agonist of the GLP-1R (Gadgaard et al., 2022), and N-terminal truncations of GLP-2 result in increased binding to the GLP-1R (Gabe et al., 2022), we wanted to test how the Ala and Trp substituted GLP-2 variants affected the selectivity of the peptides and therefore included experiments with the human GLP-1R. Several variants did not activate the GLP-1R, but [G4A], [G4W], [S5A], [S5W], [F6A] and [F6W] and [S7A] and [S7W]. In all graphs the modified GLP-2 variants are compared to native GLP-2. The dashed line in each graph represents native GLP-1. (h–n) A comparison of AUC change of each GLP-2 variant compared to native GLP-2 on human GLP-2R and GLP-1R. Data are shown as mean ± SEM, n = 3 independent experiments carried out in duplicate.

3.6 | The potent and biased GLP-2 variant [S7W] causes enhanced gut trophic actions in mice

Before testing the potential impact of the introduced bias on intestinal growth in mice, we first screened the interaction of the peptides with the mouse GLP-2R (Figure S1 and Table S1). The activation profiles were roughly the same on the human and mouse GLP-2R. Based on the functional profiles, we chose the biased variants with the strongest cAMP activation and weakest receptor internalization...
for the in vivo studies (Figure 6a/b). We calculated the ratio between the cAMP and internalization capabilities (AUC for both) (Figure 6c) for the biased GLP-2 variants with strong $G_s$ protein activation (defined as a cAMP AUC of at least 90% of GLP-2). The [S7W] variant had the highest ratio, indicating that it had the most severely impaired GLP-2R internalization response relative to strong $G_s$ coupling and was therefore chosen for studies of intestinotrophic actions. We subcutaneously injected C57Bl/6Jrj mice with 2.5-μg GLP-2, [S7W] or vehicle twice daily for 10 days. No difference in the overall body weight between groups was observed (Figure 7a), but there was a significant increase in the weight of the small intestine after [S7W] treatment compared to GLP-2 (Figure 7b). The colon weight decreased slightly after both GLP-2 and [S7W] (Figure 7c).

Morphological investigations were carried out in the small intestinal tissue and revealed that [S7W] increased villus height in both the duodenum and jejunum and crypt depth in the duodenum comparable to GLP-2 (Figure 7d/e/g). Taken together, the biased [S7W] displayed stronger intestinotrophic actions compared to GLP-2.

4 | DISCUSSION

Biased agonists are pharmacologically important due to their potential to improve treatments in the sense of better efficacy and/or possibility of reduced side-effects (Wootten et al., 2018). Here we successfully developed biased GLP-2R agonists by modifications of the GLP-2 N-terminus and show that G protein-biased GLP-2 agonism over arrestin recruitment and receptor internalization improves the intestinotrophic actions in vivo in mice compared with native GLP-2.

Within the class B1 GPCR family, the N-terminus is essential for receptor activation. This has been confirmed by NMR, cryo-EM, in silico modelling, as well as in vitro and in vivo experiments for the GIPR, GLP-1R, GCCR and GLP-2R (Parthier et al., 2007; Sun et al., 2020; Venneti & Hewage, 2011; H. Zhang et al., 2018; Y. Zhang et al., 2017). The GLP-2 system has, however, not been studied as extensively as other members of the class B1 system, for example the GIP and GLP-1 systems, possibly due to lesser focus from a drug development point of view, although GLP-2R agonists have considerable potential in the treatment of various intestinal diseases and potentially for bone diseases also (Brubaker, 2018; Schiellerup et al., 2019). A previous Ala scan of GLP-2[A2G] showed that Ala substitutions in the N-terminus resulted in less GLP-2R activation (DaCambra et al., 2000). Furthermore, the truncated metabolite GLP-2(3-33) has been shown to be a partial agonist of the GLP-2R (Gabe et al., 2022; Thulesen et al., 2002). These studies emphasize the importance of the N-terminus for proper GLP-2R activation, in line with what has previously been shown for other members of the class B1 family (Gabe et al., 2018; Hansen et al., 2016; Smit et al., 2021; Sparre-Ulrich et al., 2016; van der Velden et al., 2021). In addition, we recently described that removal of the GLP-2 N-terminus resulted in lack of GLP-2R selectivity (Gabe et al., 2022). In contrast, we here find that N-terminal substitutions of GLP-2 do not result in impaired GLP-2R selectivity (Figure 5). Our Ala and Trp scan confirmed the importance of the N-terminus, with the largest effect on the cAMP accumulation profile being observed after substitutions within the first four residues (Figure 2a1-a4). Specifically, the [H1A] substitution had a major impact on both the cAMP potency and efficacy, but lesser effect on affinity (Figure 1b, 2a1 and Table 1). Having in mind that the $\alpha$-helix of GLP-2 spans from amino acid no. 4 to 29 of the peptide (Sun et al., 2020) (Figure 1a), this correlates well with the notion regarding the class B1 peptides that the $\alpha$-helix is important for the affinity whereas the N-terminus ensures the efficacy (Hoare, 2005; Schwartz & Frimurer, 2017). The major effect of the[H1A] substitution on the activation profile correlates well with the recently published cryo-EM structure of GLP-2R, where the N-terminal His forms a hydrogen bond with H268 and hydrophobic contacts with V271, W340 and R344 of the GLP-2R (Sun et al., 2020). We furthermore found that position 3 was important for both receptor binding and activation as both Ala and Trp substitution at this position dramatically affected both the affinity and receptor activation profiles.

![FIGURE 6](image-url) Selection of biased GLP-2 variant for their in vivo study assessing intestinal growth. Spider web of activation profiles (AUC) normalized to GLP-2 of (a) Ala and (b) Trp substituted GLP-2 variants tested in cAMP accumulation, $\beta$-arrestin 1 and 2 recruitment and receptor internalization. (c) The Ala and Trp variants with a cAMP AUC of at least 90% of GLP-2 were selected and the ratio between their cAMP AUC and internalization AUC was calculated (the black dotted line represents native GLP-2).
FIGURE 7  Effect of GLP-2 and biased GLP-2 variant [S7W] on intestinal growth in mice. Female mice were treated with either 2.5 μg per mouse GLP-2 or [S7W], morning and evening for 10 days, and on day 10 the mice were sacrificed and their (a) body weight, (b) small intestinal weight relative to body weight (%) and (c) colon weight relative to body weight (%) were measured. Additionally, the villus height of the (d) duodenum, (e) jejunum and (f) ileum and crypt depth of the (g) duodenum, (h) jejunum and (i) ileum were measured. (j) Representative HE sections of the duodenum of each group of mice treated with either vehicle, GLP-2 or [S7W]. Data are shown as mean ± SEM with n = 16 mice in each group. Significant differences between groups were analysed by one-way ANOVA followed by Tukey’s multiple comparison test where * = P < 0.05.
Our study confirms the pharmacological importance of biased agonists because they can result in treatments with improved efficacy, as observed for the systems of the chemokines (Jørgensen et al., 2021), the opioids (Manglik et al., 2016; Soergel et al., 2014) and the incretins (Willard et al., 2020), where biased profiles of compounds have been linked to therapeutic advantages.

Teduglutide is currently the only GLP-2 analogue on the market. It is approved for the treatment of patients suffering from SBS. This variant has been optimized to be protease (DPP-4) resistant by introduction of Gly instead of Ala at position 2 thereby prolonging its half-life in the circulation to 3.0–5.5 hours when injected subcutaneously (Marier et al., 2008). No other modifications were made and teduglutide, therefore, requires daily injections. To make it more convenient for the patients, a treatment modality that can be taken less frequently is warranted. Currently, two long-acting GLP-2 analogues, apraglutide and glepaglutide, are under development and in clinical trial phase 3 for the treatment of SBS (Hargrove et al., 2020; Hvistendahl et al., 2020; Naimi et al., 2019). Of importance, apraglutide and teduglutide retain potency and selectivity at the GLP-2R comparable with native GLP-2 regarding Gs signalling, whereas glepaglutide is both less potent and less selective since it also activates the GLP-1R to a larger degree than the two others (Hargrove et al., 2020).

Our in vitro screening confirmed the reported cAMP activation for teduglutide, but also that it is unbiased compared to native GLP-2 (Figures 2a/b/c/d and 3d). Both glepaglutide and apraglutide have been shown to have greater intestinotrophic activity than teduglutide in rats (Hargrove et al., 2020). These effects are probably mainly related to their prolonged half-lives compared to teduglutide, and nothing has been reported regarding biased action.

In conclusion, we show that it is possible by N-terminal modifications of GLP-2 to create biased agonism with respect to cAMP generation versus arrestin recruitment and internalization and that this translates into enhanced small intestinal growth in mice compared to native GLP-2. We expect the decreased receptor internalization to cause less tachyphylaxis, which would be valuable for a drug that has to be given chronically. These changes may translate into greater clinical efficacy (Figure 7).

**AUTHOR CONTRIBUTIONS**

**Maria Buur Nordskov Gabe:** Conceptualization (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); visualization; writing - original draft (lead); writing - review and editing (lead). 

**Liv von Voss:** Conceptualization (supporting); investigation (supporting); visualization (supporting); writing - review and editing (supporting). 

**Jenna Elizabeth Hunt:** Formal analysis (supporting); investigation (supporting); methodology (supporting); visualization (supporting); writing - review and editing (supporting). 

**Darina Gadgaard:** Investigation (supporting); methodology (supporting); writing - review and editing (supporting). 

**Lærke SmidtGasbjerg:** Methodology (supporting); visualization (supporting); writing - review and editing (supporting). 

**Jens Juul Holst:** Conceptualization (supporting); funding acquisition (lead); investigation (supporting); methodology (supporting); writing - original draft (supporting); writing - review and editing (supporting).
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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. M. B. N. G., L. S. G., J. J. H. and M. M. R. are co-founders of Antag Therapeutics ApS. B. H., J. J. H. and M. M. R. are co-founders of Bainan Biotech ApS.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the _BJP_ guidelines for _Design and Analysis and Animal Experimentation_, as recommended by the funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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