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A Long-Acting Glucagon-like Peptide 2 Protracted by Coomassie Brilliant Blue to Enhance Intestinal Growth in Mice

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Cite This: *Bioconjugate Chem.* 2026, 37, 973−980

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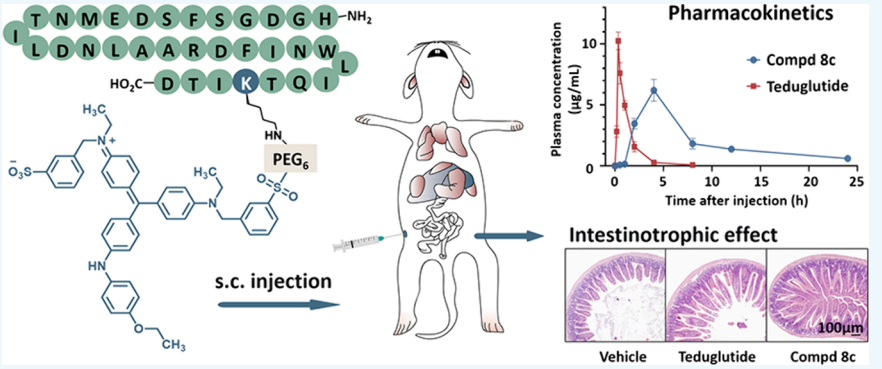

ABSTRACT: Glucagon-like peptide 2 (GLP-2) has therapeutic potential in the treatment of intestinal diseases. Its analogue, teduglutide, is clinically used for the treatment of short bowel syndrome (SBS). Despite the benefit of reduced parenteral support and a consistent safety profile in patients with SBS, teduglutide requires subcutaneous injection once daily. Long-acting GLP-2 analogues and the associated lower injection-frequency compliance are an unmet medical need in drug development. Here, we conjugated the pan-protein binder Coomassie brilliant blue (CBB) to the Lys^{30} residue of teduglutide to obtain a long-acting analogue **8c** that retains potency and selectivity comparable to teduglutide. In the rat subcutaneous pharmacokinetic study, the CBB protracted peptide **8c** displays a greatly extended half-life ($T_{1/2}$: 7.97 vs 0.56 h) in the circulation and a measured release (T_{max}: 4 vs 0.33 h) from the subcutaneous depot, which is in line with its enhanced affinity to albumin and special protracting mechanism. The unique pharmacokinetic profile can translate into a significantly enhanced intestinal growth in mice, measured as the 1.25-fold greater weight of the small intestine, 1.75-fold higher villus of jejunum, and 1.1-fold deeper crypt compared to the vehicle control. Its prolonged kinetics and intestinotrophic action would be beneficial for a reduced dosing frequency and better treatment outcome in SBS.

INTRODUCTION

Short bowel syndrome (SBS) is a common cause of intestinal failure in which the small intestine cannot absorb enough nutrients from food and drinks.[1] It often happens to patients who have had a large part of their small intestine surgically removed. The malabsorption associated with SBS can cause severe diarrhea and even require long-term parenteral nutrition. SBS conditions could be adapted by treatment with Glucagon-like peptide 2 (GLP-2) and its analogue to promote intestinal adaptation.[2] GLP-2 is a gastrointestinal hormone produced by intestinal L-cells. Its biological action on the gastrointestinal tract includes the modulation of motility, stimulation of crypt cell proliferation, enlargement of the mucosal surface area, and inhibition of cell death.[3] Notably, the trophic effect of GLP-2 is specific for the intestinal mucosa, and the growth of other tissues has not been reported, albeit the coexpression of the GLP-2 receptor (GLP2R) in some other mouse organs.[4] Native GLP-2 is of limited use as a therapeutic agent because of the enzymatic cleavage and very short half-life (~7 min in humans).[5,6] Its analogue teduglutide, which has an Ala-to-Gly substitution at amino acid (aa) position 2 to resist dipeptidyl peptidase-4 (DPP-4) cleavage, displays a terminal half-life of 3.0−5.5 h when injected subcutaneously in humans.[7] It has been approved by the FDA for once daily treatment of patients suffering from SBS.[8]

Despite being efficacious in SBS therapy, teduglutide requires daily subcutaneous injections, which can lead to decreased compliance and suboptimal treatment outcomes.[9] Various additional analogues with reduced clearance are under development for SBS, including apraglutide (VectivBio AG) and glepaglutide (Zealand Pharma). These two analogues rely on

Received: January 25, 2026
Revised: March 30, 2026
Accepted: March 31, 2026
Published: April 10, 2026



ACS Publications

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A

Peptide compds	Amino acid sequence																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
hGLP-1	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	G		
hGLP-2	H	A	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	W	L	I	Q	T	K	I	T	D
rGLP-2	H	A	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	T	R	D	F	I	N	W	L	I	Q	T	K	I	T	D
mGLP-2	H	A	D	G	S	F	S	D	E	M	S	T	I	L	D	N	L	A	T	R	D	F	I	N	W	L	I	Q	T	K	I	T	D
Teduglutide	H	G	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	W	L	I	Q	T	K	I	T	D

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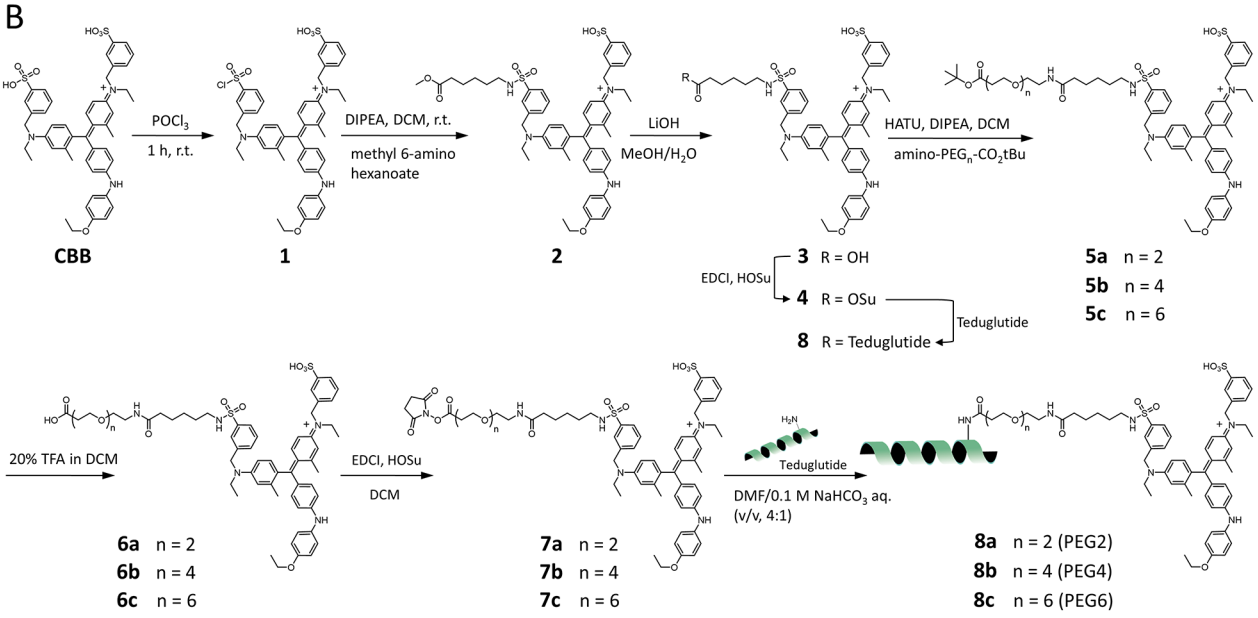

Figure 1. Generation of teduglutide analogues by site-specific CBB conjugation. (A) Amino acid sequences of human, mouse, and rat GLP-2, along with teduglutide and human GLP-1. The colored segments denote amino acid variations among peptides, while the Lys^{30} for CBB conjugation is marked as green in the teduglutide sequence. (B) Synthetic route to illuminate the CBB modification and teduglutide conjugation.

either the lipophilic amino acid substitution (apraglutide) or sequence modification (glepaglutide) to enhance the plasma protein binding, which eventually prolongs their retention and half-lives in the circulation.[10−12] Other strategies have been used to extend the half-life of GLP-2, including the PEGylation, XTEN fusion, and lipidation at the stapled Cys residues.[13−15]

Recently, the small-molecule protractor has become more popular for the half-life extension of peptide drugs.[16−18] For example, the long-chain fatty diacids (C18 and C20 diacids) have been used in several FDA-approved drugs to protract GLP-1, insulin, and other peptides for once weekly administration.[19] These fatty diacid protractors have largely converged on leveraging endogenous plasma proteins, primarily serum albumin, for long-circulation times.[20−22] Once conjugated with protractors, peptides could attach piggyback on albumin and remain in the blood circulation for a very long time, given the long half-life of albumin in humans ($t_{1/2}$ ~29 days).[23] In this regard, Gadgaard et al. have conjugated a C16 diacid protractor with human GLP-2 (hGLP-2) to generate a series of long-acting peptides (half-life of ~9.5 h in rats).[24] However, the introduction of fatty diacids to hGLP-2 residues leads to an attenuated potency, especially at the native Lys^{30}, which shows the 45-fold lowered activity.[24] Moreover, the impact on arrestin recruitment and receptor internalization were impaired, suggesting a desensitization protection after protractor conjugation.[25] The mechanism is unclear, but it demonstrates that C16 fatty diacid can cause malfunctions in the pathway, and it is not an ideal protractor for GLP-2 peptides.

In this context, we set out to evaluate Coomassie brilliant blue (CBB) as a new protractor to potentiate GLP-2 for long-term SBS therapy. CBB is a water-soluble dye for protein staining in biochemistry laboratories. It is also an FDA-approved drug and used in clinics under the trade name Brilliant Peel for retinal surgery.[26] Our previous work serendipitously found that CBB could protract peptide GLP-1 in both the blood circulation and subcutaneous depot, achieving a long-term glucose control in db/db mouse models.[27] Its analogue, Evan blue, could also prolong the circulation half-life of Exendin-4 in mice, as independently reported by the Chen group.[16] As a pan-protein binder, CBB has a special protracting mechanism that allows it to minimally affect the peptide potency. Herein, we use teduglutide as the model GLP-2 peptide and hypothesize that the covalent attachment of CBB to the native Lys^{30} might produce a minimal impact on its potency but render a long half-life in vivo. We report a highly selective condition to conjugate the CBB protractor with teduglutide, yielding a site-specific Lys^{30} modification with high efficiency. Our study systematically demonstrates that PEG spacer length critically modulates a triad of key properties: in vitro GLP2R potency, albumin-binding affinity, and the in vivo pharmacokinetic−pharmacodynamic profile. The optimized lead conjugate, compound **8c**, achieves a 15-fold extension in plasma half-life ($T_{1/2}$), a shift in elimination pathways, and a superior intestinotrophic effect in vivo compared to native teduglutide. This work not only presents compound **8c** as a promising long-acting GLP-2 for SBS therapy

https://doi.org/10.1021/acs.bioconjchem.6c00041
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but also demonstrates the versatility of CBB as a small-molecule protractor in peptide development.

RESULTS AND DISCUSSION

Site-Specific Conjugation of CBB to Lys^{30} to Generate Long-Acting Teduglutides

GLP-2 is an evolutionarily conserved 33-aa polypeptide that plays a crucial role in intestinal growth and healing.[28] Its sequence is highly conserved across species, with only two amino acid variations (positions 11 and 19) observed among human, mouse, and rat orthologues (Figure 1A). GLP-2 also shares a sequence similarity to GLP-1 because the two hormones are encoded by one gene (preproglucagon), albeit their distinctive biological activity.[29] Teduglutide, the sole FDA-approved GLP-2 analogue for clinical use, incorporates a single alanine-to-glycine substitution at the second N-terminal residue and enhances its resistance to DPP-4 mediated degradation.[7] Due to its favorable stability and commercial availability, we selected teduglutide as the GLP-2 peptide model for CBB conjugation. The lysine residue at position 30 (Lys^{30}) of teduglutide is spatially accessible and reactive, which we chose to conjugate with CBB.

CBB was functionalized by a PEG spacer and conjugated to teduglutide Lys^{30} via an *N*-hydroxysuccinimide (NHS) ester-mediated amide coupling, as depicted in Figure 1B. One sulfonic acid group of the CBB molecule was selectively converted to sulfonyl chloride in phosphorus oxychloride ($POCl_3$) at a low temperature (0 °C). The reactive group was then coupled with methyl aminohexanoate to afford a stable intermediate (compound **2**), which allows the chromatographic separation to remove the byproduct with disulfonylation. The methyl ester was hydrolyzed to release the carboxylic group, which can be functionalized under much milder conditions as compared to the sulfonic acid group. The hexanoate end was extended with a PEG spacer or directly activated by the NHS ester for peptide conjugation in the aqueous phase. Accordingly, a series of teduglutide-CBB conjugates (**8** and **8a**−**c**), differentiating by the length of the PEG spacer (0, 2, 4, or 6 repeating ethylene glycol units), was generated to screen for the best in biological activity.

The most challenging step in the synthesis is the site-specific conjugation of CBB on the Lys^{30} residue of teduglutide. Structurally, teduglutide possesses two primary amine groups: N-terminal α-amine and Lys^{30} ε-amine, both of which have chemical reactivity with NHS ester. However, the Lys^{30} ε-amine has a much higher pK_a value (around 10.0−10.5) than the terminal α-amine (around 7.5−8.0). By carefully tuning the alkaline state in the reaction, we may enable the deprotonation of Lys^{30} ε-amine selectively and achieve a site-specific conjugation on it. With this hypothesis, we screened the reaction conditions by gradually increasing the proportion of 0.1 M $NaHCO_3$ aqueous solution (v/v%) in the reaction. As shown in Figure 2, HPLC tracing of reaction products confirmed that $NaHCO_3$ had a significant impact on the conjugation selectivity. Within the DMF cosolvent containing a 20% 0.1 M $NaHCO_3$ aqueous solution, it resulted in near-exclusive acylation at the Lys^{30} ε-amine with a complete conversion (approximately 100%). In contrast, when the base concentration was increased beyond 40%, we observed the presence of acylation both at the Lys^{30} ε-amine and N-terminal α-amine, showing as a peak dimer (two CBBs per peptide) in the HPLC chromatographs. This observation underscores the profound and nuanced influence of the cosolvent on the nucleophilic reactivity of distinct amine

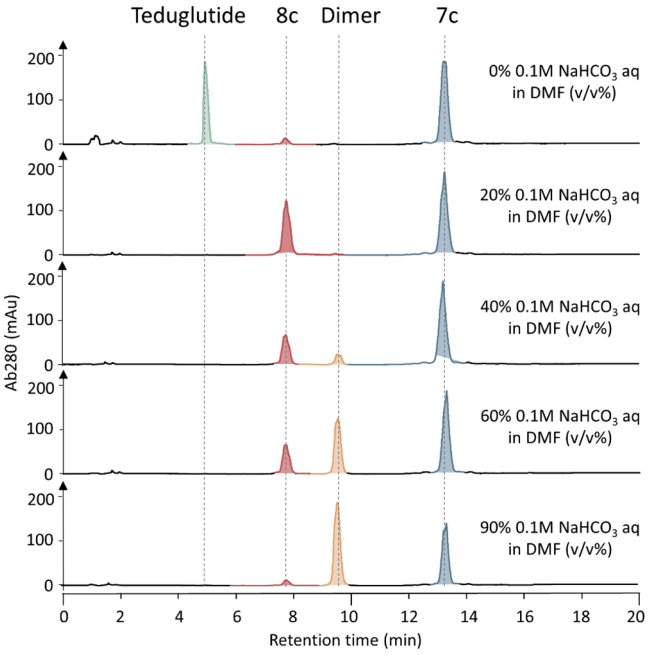

Figure 2. HPLC analysis of reaction mixtures to identify the best alkaline condition for Lys^{30}-specific conjugation. Teduglutide and **7c** were coupled at a fixed molar ratio of 1:4, and all were incubated for 0.5 h at room temperature. The alkaline state of reaction was controlled by the gradual increase of 0.1 M $NaHCO_3$ aqueous solution proportion (v/v%).

groups within a structured peptide. The fine-tuning of conjugation specificity, as exemplified here through the simple modulation of solvent alkali, provides a feasible methodology for the precise modification of therapeutic peptides.

Effect of Linker Length on Peptide Potency and Selectivity

PEG is a widely adopted linker in peptide development due to its good solubility and low immunogenicity. An appropriate PEG length would enable the peptide to be spaced from the CBB protractor and its associated albumin, which may maximize the peptide potency. To systematically investigate the impact of linker length, we evaluated the bioactivity of teduglutide analogues with PEG spacers of varying lengths. The activity assay employed HEK-293 cells integrating with the constitutive cAMP response elements (CRE) and firefly luciferase reporter gene cassette under control of the human GLP-2 receptor (GLP2R). Activation of GLP2R by teduglutide could trigger the intracellular cAMP production, which in turn drives the expression of luciferase and yields a luminescent signal for quantitative readout of receptor activation (Figure S1). The sequential parallel comparison in Figure 3 reveals that the PEG spacer has a determinative effect on the peptide potency, with over 10 times reduction by the shortest spacer (no linker) and almost retained potency by the longest PEG6 linker. The trend maintains consistency in the assay of the GLP2R crossing species (human, mouse, and rat). Importantly, teduglutide analogues exhibit a similar potency on the GLP2R activation of several species we tested. The cross-species efficacy may accelerate the preclinical development of peptide drugs in animal models with disease characteristics resembling those in humans, such as rodents and nonhuman primates.

The broad action across species raises a concern about the selectivity of teduglutide analogues at GLP2R over other G protein-coupled receptor (GPCR) pathways, for example,

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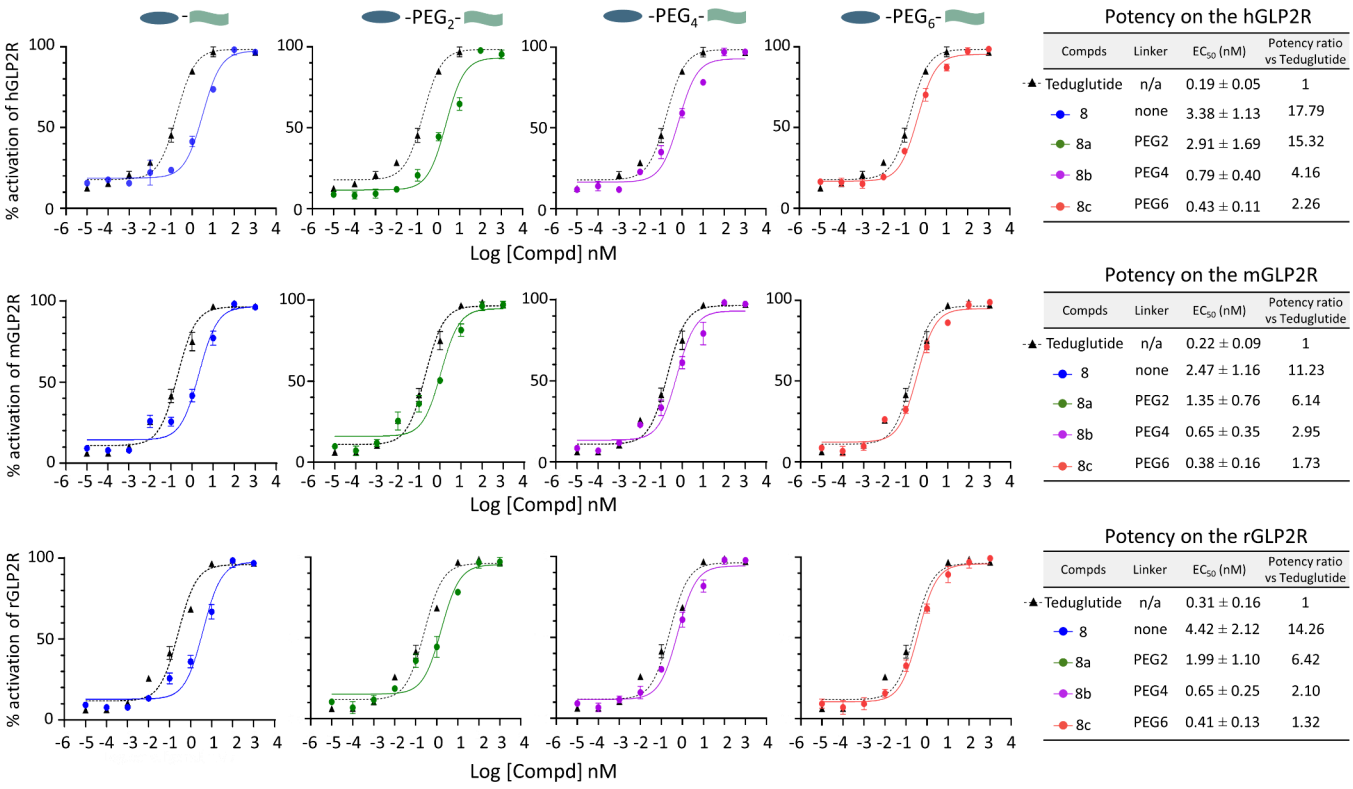


Potency on the hGLP2R

Compds	Linker	EC_{50} (nM)	Potency ratio vs Teduglutide
Teduglutide	n/a	0.19 ± 0.05	1
8	none	3.38 ± 1.13	17.79
8a	PEG2	2.91 ± 1.69	15.32
8b	PEG4	0.79 ± 0.40	4.16
8c	PEG6	0.43 ± 0.11	2.26

Potency on the mGLP2R

Compds	Linker	EC_{50} (nM)	Potency ratio vs Teduglutide
Teduglutide	n/a	0.22 ± 0.09	1
8	none	2.47 ± 1.16	11.23
8a	PEG2	1.35 ± 0.76	6.14
8b	PEG4	0.65 ± 0.35	2.95
8c	PEG6	0.38 ± 0.16	1.73

Potency on the rGLP2R

Compds	Linker	EC_{50} (nM)	Potency ratio vs Teduglutide
Teduglutide	n/a	0.31 ± 0.16	1
8	none	4.42 ± 2.12	14.26
8a	PEG2	1.99 ± 1.10	6.42
8b	PEG4	0.65 ± 0.25	2.10
8c	PEG6	0.41 ± 0.13	1.32

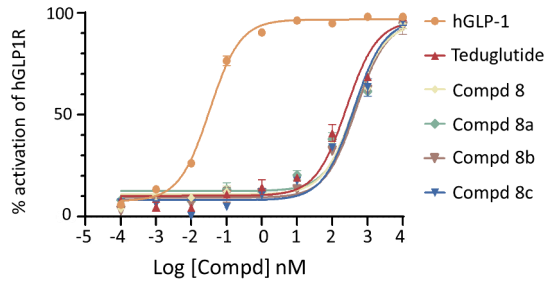
Figure 3. Agonistic effects of teduglutide analogues on human, mouse, and rat GLP2R. Signaling pathway analysis via the CRE-luciferase reporter assay in GLP1R-HEK-293 cells following transient transfection. The dashed lines represent teduglutide, while the solid lines represent each of the conjugates. Data are representative of three independent experiments, and values are expressed in mean ± SD (n = 3).

GLP1R where a low activity is preserved owing to the sequence homogeneity.[30] To determine the selectivity, we tested the hGLP1R activation by comparing the analogues and native hGLP-1. Here, those with Lys^{30} acylation all show similar potency on hGLP1R, despite the difference in spacer length (Figure 4A). A slightly higher response was observed for teduglutide but not that prominently. To get a clear overview of the selectivity, we calculated the selectivity index as a ratio of AUCs (area under the curves) that reflects the ability to activate the GLP2R relative to GLP1R. As shown in Figure 4B, all the analogues exhibit a similar selectivity to the parent teduglutide, suggestive of the retaining selectivity after conjugation.

CBB Interacts with Albumin and Protracts Teduglutide In Vivo

In search of a long-acting efficacy, CBB was conjugated as a protractor to enhance the peptide's albumin-binding ability. The interaction of the protractor with albumin is reversible, which allows peptide shuttle from the albumin carrier to the target receptor at its destination.[19] However, a higher affinity to albumin is still favored for strong protraction in the circulation. To profile the affinity, we employed biolayer interferometry (BLI) to determine the binding kinetics between the teduglutide analogues and human serum albumin (HSA). The step-binding sensorgrams in Figure 5A revealed a clear trend that the peptide affinity to HSA increased with the length of the PEG spacer. Compound **8c**, the analogue with the longest PEG spacer, has a K_D value of 16 μM, which is almost 3 times lower than compound **8** (with no spacer). This structure−activity relationship (SAR) is in line with our observation on GLP2R stimulation, suggesting that the spacer length has a determinative effect on both peptide potency and protractor activity. It

A



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Compds	Linker	AUC		Selectivity index
		hGLP2R[a]	hGLP1R[b]	
Teduglutide	n/a	4.47 ± 0.10	2.17 ± 0.093	2.06
8	none	3.49 ± 0.13	1.88 ± 0.081	1.85
8a	PEG2	3.59 ± 0.10	1.93 ± 0.082	1.86
8b	PEG4	3.73 ± 0.094	2.08 ± 0.094	1.80
8c	PEG6	3.98 ± 0.093	2.14 ± 0.083	1.87

[a] The values were obtained from AUCs in Figure 3.
[b] The AUC values were calculated from curves in Figure 4A.

Figure 4. Selectivity test of teduglutide analogues on GLP2R over GLP1R. (A) HEK-293 cells were transiently transfected with the hGLP1R reporter plasmid and assessed for hGLP1R activity upon stimulation by the hGLP-1 peptide, teduglutide, and its analogues. (B) Comparison of the AUC change of each analogue after activation of hGLP2R and hGLP1R. The selectivity index is calculated as $AUC_{hGLP2R}/AUC_{hGLP1R}$. Data are representative of three independent experiments, and values are expressed in mean ± SD (n = 3).

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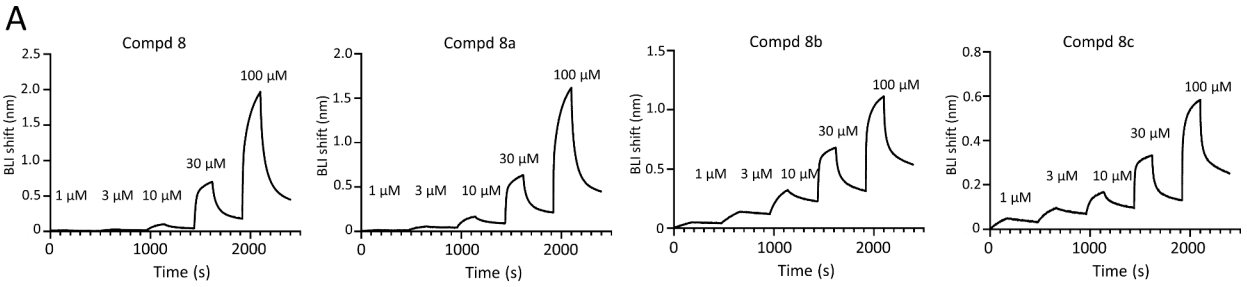

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Compds	Human Serum Albumin (HSA)			EC_{50} (nM) on hGLP2R		Ratio 2%/0% HSA
	K_{on} (1/Ms)	K_{off} (1/s)	K_D (μm)*	0% HSA	2% HSA	
Teduglutide	-	-	-	0.41 ± 0.32	0.48 ± 0.40	1.17
8	1.86×10^{3}	7.69×10^{-2}	41.34	4.20 ± 2.26	23.59 ± 8.44	5.62
8a	1.94×10^{3}	7.40×10^{-2}	38.13	2.00 ± 0.73	17.11 ± 6.82	8.50
8b	2.62×10^{3}	5.88×10^{-2}	22.45	0.65 ± 0.37	13.00 ± 4.12	20.00
8c	2.82×10^{3}	4.53×10^{-2}	16.06	0.59 ± 0.26	15.76 ± 6.37	26.71

* Kinetic K_D values are defined by $K_D = K_{off}/K_{on}$.
- Response is uninterpretable using standard Langmuir binding kinetics.

C

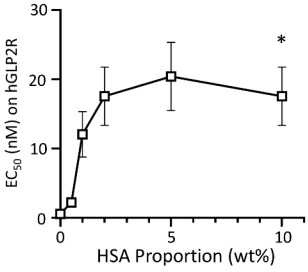
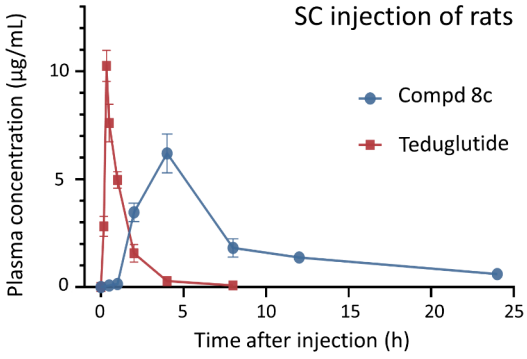


Figure 5. Assessment of albumin interaction and its impact on peptide potency. (A) BLI traces to show the binding kinetics of HSA to teduglutide analogues. On the trace of each cycle was marked by the analogue concentration, which ranges from 1 to 100 μM. The data represents as the typical results from three independent experiments. (B) Summary of binding affinity to HSA and EC_{50} on GLP2R activation in the presence or absence of 2% HSA. (C) HSA proportion titration to explore the impact of albumin interaction on compound **8c** potency. Data are expressed as mean ± SD (n = 3). *P < 0.05.

is worth noting that the candidate compound **8c** also has the highest K_{on} and lowest K_{off} values among all analogues, indicating its fast association and dissociation with albumin, a sign of good protracting capability.

To further validate the tracing effect, we tested the peptide potency on hGLP2R activation in cells with or without 2% HSA presence (Figure 5B, and the EC_{50} curves are available in Figure S2). A negative impact on the peptide potency was observed when HSA was supplemented, especially in the group treated by **8c**, which has a difference ratio of 26.7 (2/0% HSA). It is understandable, considering the circumstance that a peptide associated with albumin is usually inaccessible by GLP2R. The high ratio value indicates that compound **8c** has the highest albumin binding and thereby a longer duration of action in vivo than others. Since the HSA proportion (wt %) in the plasma is much higher than 2%, we set out to titrate the potency (EC_{50}) over HSA concentrations. As shown in Figure 5C and Figure S3, the activity of compound **8c** reaches its plateau at HSA proportions over 2%. It indicates that peptide interaction with albumin is saturable at a relatively low concentration of HSA, and most peptides would have been fully protracted in the blood circulation where the albumin concentration is ∼40 mg/mL (4%).[31]

The in vivo characterization of compound **8c** started by profiling the pharmacokinetics in Sprague−Dawley (SD) rats. Teduglutide and compound **8c** were administrated via the subcutaneous route independently, and the peptides remaining in plasma over time were quantified by LC-MS/MS. As shown in Figure 6, we observed a greatly extended half-life of compound **8c** compared to teduglutide ($T_{1/2}$: 7.97 vs 0.56 h). The T_{max} and MRT_{last} were also dramatically delayed in rats treated with compound **8c**, which suggested a slow and measured release of peptides from the subcutaneous depot. In accordance with the slow release, the peak concentration (C_{max}) of compound **8c** was attenuated to almost half that of teduglutide, reflecting a reduced risk of potential acute toxicity. Other pharmacokinetic



SD rats	T_{max} (h)	C_{max} (μg/mL)	$T_{1/2}$ (h)	MRT_{last} (h)	AUC_{last} (h·μg/mL)
Teduglutide	0.33	10.26	0.56	1.27	11.79
Compd 8c	4	6.20	7.97	7.80	45.81

Figure 6. Plasma concentration−time curves to show the kinetics of teduglutide and compound **8c** in SD rats following a single subcutaneous (SC) administration (2000 μg/kg; n = 3). Peptide concentrations in blood were quantified by LC-MS/MS. The key pharmacokinetic parameters were summarized in a table below. MRT, mean residence time. AUC, area under the curve.

parameters were improved by the CBB protraction as well. For example, the AUC of peptides increases 3.8 times after CBB conjugation, suggestive of better bioavailability. The notable improvement in the bioavailability might be attributed to the suppression of renal elimination of peptides after CBB-mediated albumin association. To better illuminate the metabolic divergence, we set out to track and compare the deposition of sulfo-Cy7-labeled peptides in mouse organs. Near infrared imaging in Figure S4 showed that teduglutide was predominantly deposited in the kidney, whereas the fluorescence signal

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of compound **8c** was found primarily in the liver. The metabolic shift from kidney to liver is in agreement with the change of apparent molecular size of teduglutide after albumin hitchhiking, which reduces the kidney clearance.

Long-Term Activity Promotes Small Intestinal Hypertrophy in Mice

The long-term action is an unmet medical need for many therapeutic peptides. CBB takes advantage of its pan-protein interaction ability to protract peptides in both the subcutaneous depot and bloodstream.[27] It is hypothesized that CBB could bind with matrix proteins, such as collagens, in the skin and slow down the peptide absorption by the vasculature. The drug bolus, supported by the collagen skeleton under the skin after subcutaneous injection, can serve as a natural "depot formulation" like the FluidCrystal technology,[32] enabling peptide release in a sustained and controlled manner (Figure 7A). In the bloodstream, CBB-conjugated peptides hitchhike serum albumin to obtain a long residence time thanks to the high abundance of albumin in plasma and the large size of the peptide−albumin complex that surpasses the renal threshold.[33] As aforementioned, albumin interaction may attenuate the peptide activity, but the impact is generally temporal and reversible because the CBB protracted peptide could disassociate itself from albumin and shuttle to GLP2R on the intestinal cells, which induces a biological function on the digestive tract.

To assess the long-acting effect in vivo, peptides were tested in a mouse model for intestinal growth over 15 days. BALB/C mice received daily subcutaneous injections of either teduglutide (2 mg/kg), compound **8c** (2 mg/kg), or 100 μL of phosphate-buffered saline (PBS) as a vehicle control. The specificity of the actions was demonstrated by the lack of changes in body weight and colon weight but an increased weight of the small intestine in the treated mice (Figure 7B). Strikingly, in the long-acting **8c** group, this tissue-specific effect underscores the enhanced and sustained GLP2R activation in the proximal intestine conferred by CBB protraction. It is worth noting that GLP-2 treatment promotes the nutrient handling efficiency, but this action is generally not paralleled by an increase in food intake and body weight gain in healthy animals, likely owing to the regulation of glucose homeostasis under normal conditions.[34,35] In contrast, GLP-2 therapy is associated with weight gain in human patients with the SBS syndrome because of its counteraction on malnutrition and dehydration.[36]

Previous studies have shown that the hypertrophic change is temporal and site-specific, with growth of the intestinal weight and crypt−villus height.[10,37] To confirm the effect in our study, we investigated the histomorphometry of small intestine tissues. Images of hematoxylin and eosin (H&E) staining reveal that the peptide treatment increases the villus height of the jejunum and crypt depth in both the jejunum and ileum compared to the control (Figure 7C and Figure S5). Meanwhile, the architectural alteration is indicative of an enhanced mucosal growth, expanded absorptive surface area, and potentially improved nutrient uptake capacity—key benefits of GLP-2 therapy in conditions of intestinal insufficiency. Meanwhile, it is worth noting that the extraintestinal effects of GLP-2 stimulation have been observed more recently, particularly on the gallbladder where the GLP2R is also expressed.[38] The striking side effect after long-term receptor activation is the inhibition of gallbladder motility and increased gallbladder refilling, which may contribute to an increased risk of gallbladder diseases.[39] Nevertheless, the benefit of the long-acting GLP-2 therapy could outweigh the risk. Altogether, our animal results confirmed that the long-acting teduglutide analogue **8c** has an enhanced and sustained pharmacological activity, positioning it as a promising candidate for further therapeutic development.

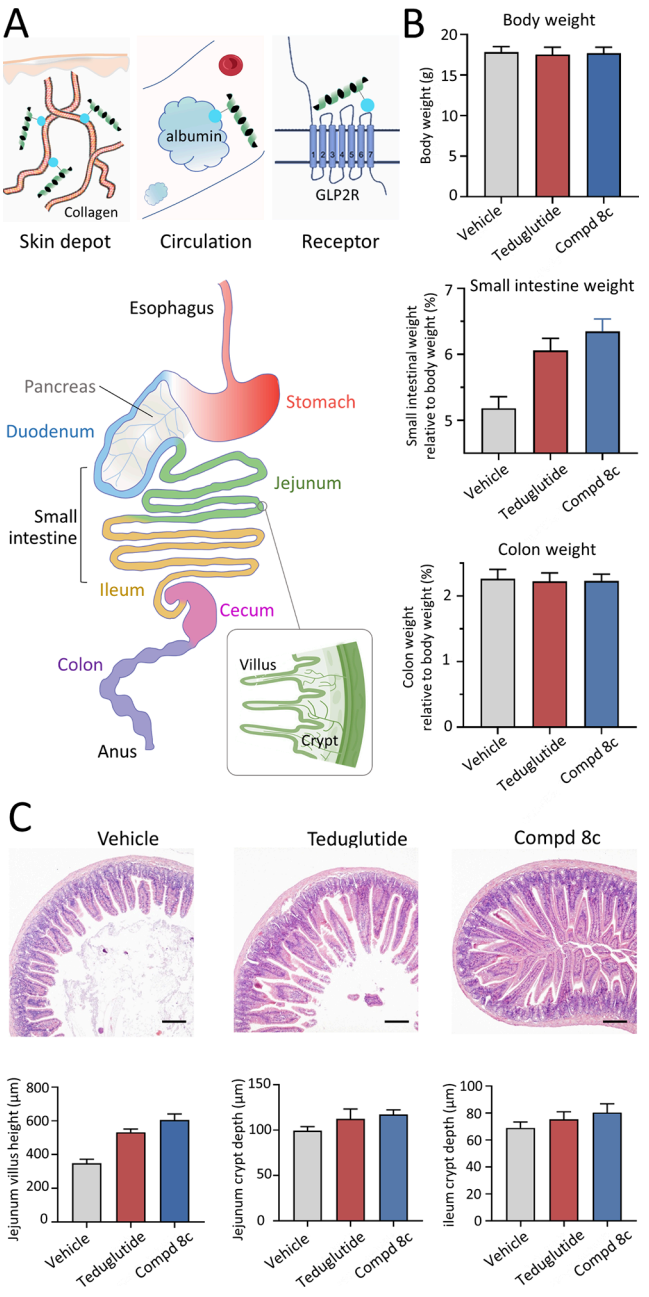

Figure 7. Effect of teduglutide and its analogue **8c** on the intestinal growth in mice. (A) Scheme showing the CBB protraction and its hypertrophic effect on digestive tracts, including the growth of villus and crypt. (B) Body weight, small intestinal weight, and colon weight in mice treated by the PBS vehicle (100 μL), compound **8c** (2 mg/kg, once a day), and teduglutide (2 mg/kg, once a day) for 15 days (n = 8 mice per group). (C) Representative H&E-stained histological sections of the jejunum tissue. Villus height and crypt depth were measured and plotted as histograms for comparison. All data are shown as mean ± SD. Scale bars, 100 μm.

CONCLUSIONS

In the development of GLP-2 therapeutics, this work established CBB as a structurally novel and synthetically tractable small-

https://doi.org/10.1021/acs.bioconjchem.6c00041
Bioconjugate Chem. 2026, 37, 973−980

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molecule protractor for GLP-2 half-life extension. We have rationally designed and synthesized a series of novel conjugates based on the FDA-approved GLP-2 analogue teduglutide, in which the well-characterized protein-binding dye CBB was site-specifically attached via variable length PEG linkers. This design leverages CBB's interaction with HSA while employing PEG as a flexible, biocompatible spacer to maintain the intrinsic receptor-binding activity of the peptide. A pivotal finding of this work is the profound pharmacokinetic enhancement conferred by CBB conjugation. Affinity studies using biolayer interferometry confirmed the weak, reversible, spacer-dependent binding to HSA. In vivo, this feature translated to a remarkable extension of systemic peptide exposure following subcutaneous administration in rodents. The improved pharmacokinetics correlated to an enhanced intestinotropic effect in the mouse model.

In summary, this study introduced a solution to address a key challenge of the GLP-2 peptide—the short in vivo half-life. The profound pharmacodynamic effects position our lead compound as a promising long-acting peptide for conditions such as short bowel syndrome, where long-term therapy is required to reduce the dosing frequency, improve patient adherence, and enhance clinical outcomes. Future studies would focus on a comprehensive preclinical safety assessment, dose optimization in relevant disease models, and further investigation into the molecular determinants of peptide−albumin−receptor interactions. This work not only expands the therapeutic potential of GLP-2 but also contributes to the growing toolkit of small-molecule protractors, which may broaden the translational impact of many other peptide drugs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.6c00041.

EC_{50} curves, HSA proportion titration, NIR fluorescence imaging, H&E-stained ileum tissue images, experimental methods, and HPLC spectra of compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the National Key R&D Program of China (grant no. 2021YFA0909900) and the National Natural Science Foundation of China (grant no. 82273860).

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