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Synthesis and Pharmacological Characterization of Novel Glucagon-like Peptide-2 (GLP-2) Analogues with Low Systemic Clearance

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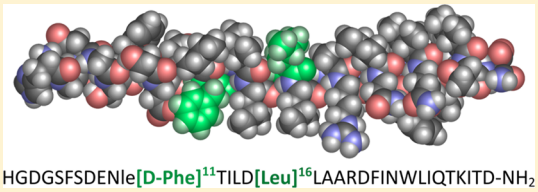
Supporting Information

ABSTRACT: Glucagon-like peptide-2 receptor agonists have therapeutic potential for the treatment of intestinal diseases. The native hGLP-2, a 33 amino acid gastrointestinal peptide, is not a suitable clinical candidate, due to its very short half-life in humans. In search of GLP-2 receptor agonists with better pharmacokinetic characteristics, a series of GLP-2 analogues containing Gly substitution at position 2, norleucine in position 10, and hydrophobic substitutions in positions 11 and/or 16 was designed and synthesized. *In vitro* receptor potency at the human GLP-2, selectivity vs the human GLP-1 and GCG receptors, and PK profile in rats were determined for the new analogues. A number of compounds more potent at the hGLP-2R than the native hormone, showing excellent receptor selectivity and very low systemic clearance (CL) were discovered. Analogues **69** ([Gly^2,Nle^{10},D-Thi^{11},Phe^{16}]hGLP-2-(1−30)-NH$_2$), **72** ([Gly^2,Nle^{10},D-Phe^{11},Leu^{16}]hGLP-2-(1−33)-OH), **73** ([Gly^2,Nle^{10},D-Phe^{11},Leu^{16}]hGLP-2-(1−33)-NH$_2$), **81** ([Gly^2,Nle^{10},D-Phe^{11},Leu^{16}]hGLP-2-(1−33)-NHEt), and **85** ([Gly^2,Nle^{10},D-Phe^{11},Leu^{16}]-hGLP-2-(1−33)-NH-((CH$_2$)$_2$O)$_4$-(CH$_2$)$_2$-CONH$_2$) displayed the desired profiles (EC$_{50}$ (hGLP-2R) < 100 pM, CL in rat <0.3 mL/min/kg, selective vs hGLP-1R and hGCGR). Compound **73** (FE 203799) was selected as a candidate for clinical development.



INTRODUCTION

Glucagon-like peptide 2 (GLP-2) is a 33 amino acid gastrointestinal (GI) hormone that is produced by enteroendocrine cells in the small and large intestine and released into circulation following food intake. GLP-2 is synthesized by posttranslational processing of proglucagon by prohormone convertase 1/3.[1] The sequences of the human (hGLP-2) **1**,[2] and the rat (rGLP-2) form of the hormone as well as the sequence of an analogue in the clinical development are shown in Figure 1.

Compound **1** exerts its numerous biological responses through a specific GLP-2 G-protein coupled receptor that is abundantly expressed in the small intestine, colon, and stomach.[3,4] The biological actions of **1** have been extensively reviewed in the scientific literature.[5−10] In mice, exogenous **1** was shown to stimulate intestinal growth through increased proliferation and decreased apoptosis of mucosal epithelial cells. The trophic effect was specific for the intestinal mucosa, as **1** had no effect on the growth of other tissues.[11,12] Other biological actions of **1** in the gastrointestinal tract include enhancement of nutrient absorption,[13,14] stimulation of mesenteric[15] and intestinal blood flow,[16] and modulation of motility.[17] Compound **1** exhibits anti-inflammatory activity in the small[18,19] and large intestine and improves barrier function.[7] Despite being efficacious in these models, the potential use of **1** as a therapeutic agent is hampered by its unfavorable pharmacokinetic profile with elimination half-life in humans of about 7 min.[20] This short circulating half-life stems from the hormone sensitivity to cleavage of its N-terminal dipeptide by the ubiquitous serine protease enzyme dipeptidyl-peptidase IV (DPP IV) and formation of the cleavage product GLP-2 (3−33),[21] a weak partial agonist/antagonist at the GLP-2 receptor.[22]

Although a substantial effort has been undertaken to identify GLP-2 agonists suitable for clinical development, the literature on SAR studies of GLP-2 analogues is sparse and in most cases limited to patents and patent applications.[23,24] A single replacement of the Ala^2 residue with Gly led to the DPP IV resistant analogue **2** (teduglutide, [Gly^2]hGLP-2, Figure 1), with an improved PK profile and superior intestinotrophic properties as compared to the native hormone in mice.[25]

Compound **2** has been demonstrated to be efficacious in a variety of animal models of disease, including inflammatory bowel disease,[26] short bowel syndrome and TPN-induced intestinal atrophy,[27,28] and chemotherapy[29,30] and radiation-induced gastrointestinal mucositis.[31]

The Drucker group conducted a comprehensive Ala-scan of **2** and an SAR study on position 2 substitutions.[32] Only

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	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	
1	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	OH
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	OH
2	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	OH

Figure 1. Sequences of human and rat GLP-2 and an analogue in the clinical development. Differences from the hGLP-2 sequence are marked in bold.

Table 1. In Vitro Evaluation of Truncated Analogues of the [Gly^2]hGLP-2 (1−*n*)-NH$_2$ General Structure

		hGLP-2R		hGLP-1R		Selectivity[a]
Compound	n	EC_{50} (nM)[b]	Potency ratio[c] vs **2**	EC_{50} (nM)	Efficacy (%)	hGLP-1R/hGLP-2R
1[d]	33	0.07	0.78	520	71	7400
2[d]	33	0.09	1.0	>1000[e]		>11000
4	32	0.29	3.2	>1000[e]		>3400
5	31	0.48	5.3	>1000[e]		>2000
6	30	0.22	2.4	>1000[e]		>4500
7	29	0.63	7.0	>1000[e]		>1500
8	28	1.1	12	>1000[e]		> 900
9	27	1.1	12	>1000[e]		>900
10	25	>30	>330	>1000[e]		N/A

[a] EC_{50} (hGLP-1R)/EC_{50} (hGLP-2R). [b] All compounds are fully efficacious at the hGLP-2R except for inactive compound **10**. [c] EC_{50} (analogue)/EC_{50} (**2**). [d] Compound **1** is the native hormone and an Ala^2 peptide (see Figure 1 for the sequence). Compounds **1** and **2** are C-terminal acids. [e] No agonism up to the highest concentration tested (1000 nM).

conservative replacements (Gly, Ile, Pro, Aib, or D-Ala) of the Ala^2 residue were well tolerated by the rGLP-2 receptor and resulted in analogues more potent than the native hormone in a functional cAMP-based assay. Receptor binding data for the Ala-scan showed that many residues in **2** (i.e., 17, 20, 22, 23, 25, 26, and 30) were crucial for rGLP-2R recognition. This was recently confirmed in an NMR and molecular modeling-based study.[33] Most of the Ala substituted compounds exhibited markedly reduced production of cAMP, with notable exceptions of analogues modified in positions 7, 8, 11, 16, and 24.[32]

Analogue **2** has shown a half-life of 3.0−5.5 h in humans following subcutaneous administration.[34] In patients with short bowel syndrome, once daily treatment with **2** significantly reduced the parental nutrition requirements and improved intestinal function.[35−39] In patients with moderate to severe Crohn's disease, treatment with **2** resulted in a trend for improvement in the Crohn's Disease Activity Index score.[40] Compound **2** has been recently approved by the US FDA and the EMA for use in patients with short bowel syndrome requiring parenteral support. Other GLP-2 analogues in clinical trials are [Gly^2,Glu^3,Ser^{8,11},Leu^{10},Ala^{16,24,28}]hGLP-2-(1−33)-Lys$_6$-NH$_2$ (ZP1846)[41] for the treatment of chemotherapy-induced diarrhea and [Gly^2,Glu^3,Thr^5,Ser^8,Leu^{10},Ala^{11,16,24,28}]hGLP-2-(1−33)-Lys$_6$-NH$_2$ (ZP1848)[41] for the treatment of Crohn's disease (www.zealandpharma.com).

In search of GLP-2R agonists with superior pharmacodynamics (PD) and PK profiles relative to **2** and the compounds in clinical development, we designed and synthesized a new series of 83 GLP-2 analogues modified in positions 11 and/or 16. To render DPP IV stability, position 2 was fixed with Gly and the Met^{10} residue was replaced with the more chemically stable isosteric norleucine. The compounds were screened in *in vitro* functional assays at the hGLP-1, hGLP-2, and hGCG receptors and their pharmacokinetic properties determined in rats. We describe here the discovery of potent and selective GLP-2 analogues with unprecedented exceptionally low systemic clearance (CL).

RESULTS AND DISCUSSION

The native GLP-2 contains five Asp residues including one (Asp^3) immediately followed by a Gly residue. This particular sequence is very prone to side-reactions due to the formation of aspartimide and subsequent ring opening. Indeed, our initial efforts to synthesize the analogues by Fmoc strategy in a single run resulted in rather complex mixtures containing multiple byproducts (*β*-shifted peptides, piperidides and truncated peptides). The presence of desHis[1] peptides indicated that the peptide chain termination via nucleophilic opening of aspartimide by the *α*-amino group of the Gly^2 residue was forming the corresponding 1,4-piperazine-2,5-diones[42] or 1,4-diazepine-2,5-diones.[43] Aspartimide formation has been extensively studied in the literature.[44−46] Strategies to suppress this unwanted side reaction include the addition of HOBt to the piperidine solution,[47] and the use of piperazine instead of piperidine for the Fmoc group removal.[48] Various aspartic acid side chain protecting groups, more sterically hindered than the commonly used t-butyl group, have been proposed.[49,50] TFA-labile backbone protecting groups offering total suppression of aspartimide formation such us Hmb,[51] Dmb[52] or Dcpm,[53] have also been introduced. In our laboratory, we have elaborated yet another strategy to avoid the aspartimide formation. Since all analogues would have a fixed N-terminal tetrapeptide ending with the Gly^4 residue, the C-terminal parts of the peptides were assembled up to position 5 and the remaining four amino acids were coupled racemization-free as tetrapeptide Boc-His(Trt)-Gly-Asp(tBu)-Gly-OH, **3**, prepared separately on 2-chlorotrityl resin.[54] Because of the bulkiness of the 2-chlorotrityl linker, side reactions such as diketopiperazine and aspartimide formation are greatly suppressed on chlorotrityl resin. Indeed, the crude **3** could be used in the fragment condensation step without purification as it contained the des-trityl compound as the sole impurity.

Our initial SAR effort was focused on reducing the size of the GLP-2 molecule, which could result in analogues with potentially lower cost of manufacturing. Since hGLP-2 (3−33) is an antagonist/weak agonist of the hGLP-2R[22] and hGLP-2 (11−33) is a very weak agonist with poor efficacy *in vitro*,[55] C-terminal

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truncations seemed to be the only viable strategy to generate shortened and still potent GLP-2R agonists. Position 2 in the truncated peptides was fixed with Gly for DPP IV stability.[21] The truncated analogues were evaluated *in vitro* at the hGLP-1R, hGLP-2R and hGCGR. The shortest analogue synthesized in this series, [Gly^2]hGLP-2 (1−25)-NH_2, **10**, was found to be inactive as a hGLP-2R agonist *in vitro*. Somewhat surprisingly, the 30-mer peptide **6** turned out to be the most potent truncated agonist *in vitro* with a modest 2.4-fold loss of potency at the hGLP-2R *in vitro* relative to **2** (Table 1). It is interesting to note that this corresponds to shortening the peptide chain by one full helical turn, but investigating the structural consequences of this change was beyond the scope of our work. Peptides **4**−**10** showed excellent selectivity vs both hGLP-1R (Table 1) and hGCGR (data not shown). Based on the results of this preliminary evaluation, the SAR program was continued with 30-mer peptide amides. Also, based on the SAR exploration of position 2 in rGLP-2 reported by DaCambra et al.,[32] and the results of an expanded internal exploratory study of position 2 (data not shown) we elected to prepare all further analogues with the Gly^2 modification and with replacement of the oxidation and alkylation-prone Met residue in position 10 by the isosteric Nle (analogues **11**−**86**, Tables 2−6).

Table 2. Results of a D-Scan of the [Gly^2,Nle^{10}]hGLP-2 (1−30)-NH_2 (6) Molecule

		hGLP-2R		hGLP-1R	Selectivity[a]
Compound	Position (D-Aaa)	EC_{50} (nM)[b]	Potency ratio vs **6**[c]	EC_{50} (nM)	hGLP-1R/ hGLP-2R
6[d]	N/A	0.22	1	>1000[e]	>4500
11	4 (D-Ala)	0.20	0.91	>1000[e]	>5000
12	5 (D-Ser)	8.1	37	>1000[e]	>120
13	7 (D-Ser)	0.19	0.86	>1000[e]	>5200
14	8 (D-Asp)	16[f]	73	>1000[e]	>62
15	9 (D-Glu)	7.1	32	>1000[e]	>140
16	10 (D-Nle)	0.23	1.0	>1000[e]	>4300
17	11 (D-Asn)	0.16	0.73	>1000[e]	>6200
18	12 (D-Thr)	1.5	6.8	>1000[e]	>660
19	13 (D-Ile)	0.35	1.6	>1000[e]	>2800
20	14 (D-Leu)	8.9	40	>1000[e]	>110
21	16 (D-Asn)	1.5	6.8	>1000[e]	>660
22	17 (D-Leu)	2.6	12	>1000[e]	>380
23	18 (D-Ala)	4.5	20	>1000[e]	>220
24	19 (D-Ala)	14	64	>1000[e]	>71
25	20 (D-Arg)	2.2	10	>1000[e]	>450
26	21 (D-Asp)	0.47	2.1	>1000[e]	>2100
27	24 (D-Asn)	0.43	2.0	>1000[e]	>2300
28	25 (D-Trp)	> 30	> 130	>1000[e]	N/A
29	29 (D-Thr)	4.5	20	>1000[e]	>220
30	30 (D-Lys)	0.38	1.7	>1000[e]	>2600

[a]EC_{50} (hGLP-1R)/EC_{50} (hGLP-2R). [b]Compounds are fully efficacious at the hGLP-2R unless indicated otherwise. [c]EC_{50} (analogue)/EC_{50} (**6**). [d]Compound **6** is a Met^{10} analogue. [e]No agonism up to the highest concentration tested (1000 nM). [f]Partial agonist at the hGLP-2R.

To better understand the SAR of the GLP-2 molecule, a D-scan of the [Gly^2,Nle^{10}]hGLP-2 (1−30)-NH_2 peptide (**6**, Table 2) was performed. Replacing each residue with its D-enantiomer could provide valuable information on the structural requirements for the GLP-2 receptor recognition/activation. Moreover, this strategy could render analogues with improved hGLP-2R specificity, potentially more resistant against proteolytic degradation in plasma, and with better PK properties. A D-scan of the closely related gastrointestinal hormone GLP-1 (GLP-1-(7−36)-NH_2) identified several positions (8, 17, 18, 30, 34, 36) where the inversion of configuration was well tolerated by the GLP-1R in both binding and functional assays.[56]

The modifications of compound **6** with D-enantiomers of the native amino acids were well tolerated in positions 4, 7, 10, 11, 13, 21, 24, and 30, with the D-Asn^{11} analogue **17** being slightly more potent *in vitro* than the parent compound **6**. In the N-terminal portion of the peptide, loss of potency was observed in positions 5, 8, 9, 12, and 14. In the C-terminal portion, losses by epimerization appeared in positions 17, 18, 19, 20, 25, and 29, all of which were found to be oriented toward a hydrophobic binding cavity in a model of the extracellular domain of the hGLP-2R.[33] The most severe loss of *in vitro* potency was observed for the Trp^{25} analogue **28**. These results do not completely mirror the losses of binding potency or cAMP production at the rGLP-2R (at 1 nM peptide concentration) in the DaCambra Ala-scan.[32] For example, Ala replacement in positions 4, 10, 13, or 30 of **1** resulted in significant loss of functional activity at the rGLP-2R while we observed only minimal effect on EC_{50} at the hGLP-2R in our D-scan for these positions. Conversely, we observed a significant decrease in potency and partial agonism for the D-Asp^8 analogue **14** while its Ala replacement had little effect on cAMP production at the rGLP-2R.[32] All D-scan analogues **11**−**30** were inactive at both the hGLP-1R (Table 2) and hGCGR (data not shown).

Based on the outcome of our D-scan and the results of the previously reported Ala-scan,[32] positions 11 and 16 were selected for further modifications. Amino acids used in position 11 were of both L- and D-configurations. For position 16, only L-amino acids were employed, as the D-Asn^{16} analogue **21** was considerably (6.8-fold) less potent than **6**. In addition to the *in vitro* screening for hGLP-2 receptor potency and selectivity, we also determined CL after intravenous administration to rats.

First, the asparagine residue in position 11 was replaced with a variety of natural and unnatural amino acids of both L- and D-configuration (Table 3).

Compound **31**, where the Asn^{11} residue was substituted with a positively charged D-Dab showed a 3-fold loss of potency as compared to **6**. In most cases, the hydrophobic replacements led to compounds equipotent or slightly more potent than **6.** However, introduction of residues with bulky hydrophobic side chains resulted in less potent analogues (i.e., Cha, **38,** D-Bip, **51**). These modifications also increased the potency of analogues at the hGLP-1R, reducing hGLP-1/hGLP-2 receptor selectivity (e.g., compounds **37**−**39**, **41**). Although we do not know if our GLP-2 analogues bind to the hGLP-1R in exactly the same orientation and register as hGLP-1, it is interesting to note that hGLP-1 has a hydrophilic serine in the equivalent position. The replacement of the hydrophobic residue of L-configuration with its D-counterpart considerably improved selectivity vs the hGLP-1 receptor and, in the aromatic residues series, also increased the potency at the hGLP-2R by 2-fold (as exemplified by the EC_{50} values for compounds **40**, **42**−**44**, **46**, and **47**−**49**, which were in the 0.09−0.11 nM range). Compounds **31**−**51** were inactive at the hGCGR (data not shown). One unexpected finding was that all analogues with a lipophilic amino acid (L or D) in position 11 in this series had significantly reduced CL values in rat as compared to those of reference compounds **1**, **2**, and **6**. Within the series, the analogues modified with aromatic residues (**33**, **39**−**45**) and cyclic Cha displayed lower CL (0.32−4.9 mL/min/

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Table 3. Pharmacological Profile of [Gly^2,Nle^{10},X^{11}]hGLP-2 (1−30)-NH$_2$

	Structure[a]	hGLP-2R	hGLP-1R		Selectivity[b]	Rat iv PK
Compound	X	EC_{50} (nM)[c]	EC_{50} (nM)	Efficacy (%)	hGLP-1R/hGLP-2R	CL (mL/min/kg)
1	Asn	0.07	520	71	7400	25
2	Asn	0.09	>1000[d]		>11000	9.9
6	Asn	0.22	>1000[d]		>4500	20
31	D-Dab	0.66	>1000[d]		>1500	9.7
32	His	0.44	300	35	680	NT[e]
33	D-His	0.20	>1000[d]		>5000	0.73
34	Leu	0.15	180	44	1200	2.8
35	D-Leu	0.31	>1000[d]		>3200	3.3
36	Ile	0.13	110	92	840	5.6
37	Hol	0.29	10	87	34	NT[e]
38	Cha	0.51	8.9	83	17	0.69
39	Phe	0.15	16	94	100	1.8
40	D-Phe	0.09	120	64	1300	1.2
41	Cpa	0.16	8.8	89	55	0.55
42	D-Cpa	0.09	60	86	660	0.51
43	D-2Cpa	0.11	100	36	900	0.73
44	D-3Cpa	0.11	45	73	400	0.32
45	D-Tyr	0.18	32	68	170	4.9
46	D-Thi	0.10	80	49	800	1.1
47	D-3Thi	0.09	83	39	920	1.3
48	Trp	0.21	56	35	260	0.71
49	D-Trp	0.10	40	80	400	1.2
50	Bip	0.18	21	83	110	NT[e]
51	D-Bip	1.9	39	108	20	NT[e]

[a]Compounds **1** and **2** are the C-terminal acid 33-mer peptides (Figure 1). Analogue **6** has Met in position 10. [b]EC_{50} (hGLP-1R)/EC_{50} (hGLP-2R). [c]Compounds fully efficacious at the hGLP-2R. [d]No agonism up to the highest concentration tested (1000 nM). [e]Not tested.

Table 4. Pharmacological Profile of [Gly^2,Nle^{10},Y^{16}]hGLP-2 (1−30)-NH$_2$

	Structure[a]	hGLP-2R	hGLP-1R	Selectivity[b]	Rat iv PK
Compound	Y	EC_{50} (nM)[c]	EC_{50} (nM)	hGLP-1R/hGLP-2R	CL (mL/min/kg)
1	Asn	0.07	520	7400	25
2	Asn	0.09	>1000[d]	>11000	9.9
6	Asn	0.22	>1000[d]	>4500	20
52	Leu	0.10	>1000[d]	>10000	0.84
53	Cha	0.10	>1000[d]	>10000	0.41
54	Phe	0.14	>1000[d]	>7100	5.9
55	Tyr	0.11	>1000[d]	>9000	1.2
56	Cpa	0.22	>1000[d]	>4500	0.67
57	Thi	0.20	>1000[d]	>5000	6.1
58	Aph	0.13	>1000[d]	>7600	1.9
59	His	0.42	>1000[d]	>2300	0.13
60	hPhe	0.11	>1000[d]	>9000	0.44
61	1Nal	0.19	>1000[d]	>5200	0.43
62	2Nal	0.13	>1000[d]	>7600	0.28
63	Trp	0.15	>1000[d]	>6600	0.50
64	Bip	0.35	>1000[d]	>2800	NT[e]

[a]Analogues **1** and **2** are the C-terminal acid 33-mer peptides (Figure 1), and compounds **1**, **2**, and **6** have Met in position 10. [b]EC_{50} (hGLP-1R)/EC_{50} (hGLP-2R). [c]Compounds fully efficacious at the hGLP-2R. [d]No agonism up to the highest concentration tested (1000 nM). [e]Not tested.

kg) than analogues with aliphatic residues (**34**−**36**) (2.8−5.6 mL/min/kg). Moreover, the size/type of the aromatic ring and the type and the location of substituents in this aromatic ring had a huge impact on CL values in this series. For example, the 4-hydroxy compound **45** showed relatively high (albeit still lower than **2**) CL value of 4.9 mL/min/kg. On the other hand, the D-Cpa compounds **42**−**44** displayed CL values in the range of 0.32−0.73 mL/min/kg. The lowest value in this series (0.32 mL/min/kg), about 30 times lower than that of **2**, was found for the 3-chloro isomer **44**.

To explore the SAR of position 16, compound **6** was modified with hydrophobic aliphatic and aromatic residues to yield analogues **52**−**64** (Table 4). Most of the modifications were well tolerated by the hGLP-2R and resulted in compounds equipotent or up to 2-fold more potent *in vitro* than **6**. The aliphatic Leu^{16} and Cha^{16} analogues were the most potent compounds in this series (EC_{50} = 0.10 nM) whereas analogues

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Table 5. Pharmacological Profile of [Gly^2,Nle^{10},X^{11},Y^{16}]hGLP-2 (1−30)-NH$_2$

	Structure[a]		hGLP-2R	hGLP-1R		Selectivity[b]	Rat iv PK
Compound	X	Y	EC_{50} (nM)[c]	EC_{50} (nM)	Efficacy (%)	hGLP-1R/hGLP-2R	CL (mL/min/kg)
1	Asn	Asn	0.07	520	71	7400	25
2	Asn	Asn	0.09	>1000[d]		>11000	9.9
6	Asn	Asn	0.22	>1000[d]		>4500	20
65	D-Phe	Leu	0.08	>1000[d]		>12000	0.37
66	D-Phe	Phe	0.09	>1000[d]		>11000	0.30
67	D-Phe	Tyr	0.07	90	58	1200	0.48
68	D-Thi	Leu	0.08	>1000[d]		>12000	0.33
69	D-Thi	Phe	0.08	>1000[d]		>12000	0.26
70	D-Thi	Tyr	0.07	70	51	7000	0.52
71[a]	D-Phe	Leu	0.04	130	41	3200	0.49

[a]Analogues **1** and **2** are the C-terminal acid 33-mer peptides (Figure 1). Compound **6** has Met in position 10, compound **71** is a rGLP-2 analogue with Thr in position 19. [b]EC_{50} (hGLP-1R)/EC_{50} (hGLP-2R). [c]Compounds fully efficacious at the hGLP-2R. [d]No agonism up to the highest concentration tested (1000 nM).

Table 6. Pharmacological Profile of [Gly^2,Nle^{10},X^{11},Y^{16}]hGLP-2 (1−33)-R

	Structure[a]			hGLP-2R	hGLP-1R	Selectivity[b]	Rat iv PK
Compound	X	Y	R	EC_{50} (nM)[c]	EC_{50} (nM)	hGLP-1R/hGLP-2R	CL (mL/min/kg)
1	Asn	Asn	OH	0.07	520	7400	25
2	Asn	Asn	OH	0.09	>1000[d]	>11000	9.9
72	D-Phe	Leu	OH	0.03	>1000[d]	>33000	0.28
73	D-Phe	Leu	NH_2	0.03	>1000[d]	>33000	0.27
74	D-Phe	Phe	OH	0.06	>1000[d]	>16000	0.15
75	D-Phe	Phe	NH_2	0.06	>1000[d]	>16000	0.24
76	D-Thi	Phe	NH_2	0.07	>1000[d]	>14000	0.15
77	D-3Cpa	Leu	NH_2	0.09	>1000[d]	>11000	0.20
78	D-3Cpa	Phe	NH_2	0.13	>1000[d]	>7600	0.12
79	D-3Cpa	2-Nal	NH_2	0.51	>1000[d]	>1900	0.11
80[a]	D-Thi	Leu	NH_2	0.05	>1000[d]	>20000	0.28
81	D-Phe	Leu	NHEt	0.05	>1000[d]	>20000	0.19
82	D-Phe	Leu	NH-iBu	0.06	>1000[d]	>16000	0.17
83	D-Phe	Leu	NHBn	0.10	>1000[d]	>10000	0.10
84	D-Phe	Leu	NH-4Pic	0.04	>1000[d]	>25000	0.27
85	D-Phe	Leu	NH-R^1 [e]	0.03	>1000[d]	>33000	0.23
86	D-Phe	Leu	NH-R^2 [f]	0.03	>1000[d]	>33000	0.28

[a]Compounds **1** and **2** have Met in position 10. Compound **80** is a rat (Thr[19]) analogue. [b]EC_{50} (hGLP-1R)/EC_{50} (hGLP-2R). [c]Compounds fully efficacious at the hGLP-2R. [d]No agonism up to the highest concentration tested (1000 nM). [e]R^1 is -$((CH_2)_2O)_4$-$(CH_2)_2$-$CONH_2$. [f]R^2 is −CH_2-$((CH_2)_2O)_3$-$(CH_2)_3$-NHCO-CH_2-O-CH_2-$CONH_2$.

with heterocyclic His (**59**) and bulky Bip (**64**) showed a minor, 2-fold loss in potency as compared to **6**. Unlike modifications in position 11, changes in position 16 did not have any deteriorating effect on selectivity vs the hGLP-1R as none of the compounds **52**−**64** showed any activity at this receptor *in vitro* up to the maximum concentration tested (1000 nM). The peptides in this series showed no activity at the hGCGR up to 1000 nM (data not shown). A somewhat different PK SAR was seen in this series compared to compounds modified in position 11. Remarkably low CL values were observed for analogues with bulky, hydrophobic side chains, both aliphatic and aromatic (e.g., **53**, **60**−**63**). However, for compounds with an unsubstituted phenyl (**54**) and 2-thienyl (**57**) rings, higher CL values were determined. The introduction of various substituents in position 4 of the Phe[16] phenyl ring resulted in compounds with lower CL than the parent analogue **54**. Rather surprisingly, the His[16] compound **59** showed the lowest CL (0.13 mL/min/kg) in the series. As it was observed for position 11, replacement of a single amino acid (Asn[16]) by a lipophilic one reduced the clearance of a 30 amino acid long peptide by a large factor.

Modifications in positions 11 (D-Thi and D-Phe) and 16 (Leu, Phe, Tyr) that resulted in the most potent and selective analogues *in vitro* were combined in analogues **65**−**71** (Table 5). All compounds turned out to be very potent hGLP-2R agonists *in vitro* (EC_{50} < 0.1 nM). The 2.4-fold loss of potency associated with truncating the GLP-2 molecule from 33 to 30 residues (compound **6**) was fully compensated by the replacements of the Asn[11,16] residues with the lipophilic amino acids. Compounds **65**−**71** displayed a receptor selectivity profile similar to the analogues modified only in position 11 with the notable exemption of those with Tyr[16] and the rat GLP-2 analogue **71**. The modifications in positions 11 and/or 16 appeared to have a somewhat additive effect on CL as extremely low values in the 0.26−0.52 mL/min/kg range were consistently observed for analogues **65**−**71**. The combination of the D-Phe[11] residue with Phe[16] or Leu[16] residues resulted in compounds with the most desirable pharmacological profile (equipotent *in vitro* with **1**, selective and extremely low-CL in rat).

Shortening the sequence of **2** by just three residues (compounds **31**−**71**) would have only a minor impact on

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manufacturing costs if one of these analogues was selected for development. Moreover, CL of the shortened analogue **6** was more than 2-fold higher than that of **2** so we explored whether adding back the native C-terminal tripeptide to the best analogues from the 1−30-NH_2 series would result in compounds with even better pharmacological profile than those of peptides **65**−**71**.

To address that question the full length analogues **72**−**86** were designed with the best hydrophobic modifications identified in the 1−30 series. In addition, compounds **81**−**86** were synthesized as analogues of **73** modified with various substituents at the C-terminal amide (Table 6). A majority of the peptides in this series were very potent as hGLP-2R agonists *in vitro* with the exception of analogue **79** containing the bulky 2Nal[16] substitution and hydrophobic 3Cpa[11] which was 5 times less potent *in vitro* than **2**. Analogues in this subset displayed much improved selectivity vs the hGLP-1R as compared to their 1−30 counterparts. Compounds **72**−**86** were also very selective vs, hGCGR (data not shown). The combination of D-Phe[11]/Leu[16] resulted in the most potent analogues *in vitro* and C-terminal amides were as potent as their corresponding C-terminal acids (see **72** vs **73** and **74** vs **75**). The CL values of the full length analogues was similar or lower than those of the 30-mer peptides. The *N*-alkyl amides **81**−**83** had CL values lower than 0.2 mL/min/kg and the N-benzyl amide **83** had the lowest CL value in the series (0.10 mL/min/kg). The more polar analogues with short PEG chains at the C-terminus (**85** and **86**) had CL values similar to the parent compound **73**.

In general, modifications with hydrophobic residues in just one or two positions (11 and/or 16) in these 33 amino acid long peptides produced remarkable reductions in metabolic clearance in rats, from 9.9 mL/min/kg for the DPP IV stable **2** to less than 0.3 mL/min/kg for compounds **72**−**80**. A variety of strategies to reduce clearance and lengthen half-life of peptides have been described, either by increasing molecular weight to decrease the rate of renal filtration or by facilitating their binding to plasma proteins to reduce their free fraction, which may be subject to proteolysis and renal filtration.[57] These strategies require modification of the structure of the peptides; examples include lipidization (i.e., palmitoylation), PEG-ylation and fusion to long half-life polypeptides (e.g., XTEN) and proteins (e.g., albumin, Fc moieties). The singly palmitoylated GLP-1 analogue liraglutide is marketed for the treatment of diabetes mellitus and is suitable for once a day subcutaneous administration.[58] A number of compounds described in this work display a PK profile that, if it translates to humans, may allow more convenient dosing than with **2** or other GLP-2 analogues in clinical development. Remarkably, the decrease in rat clearance in our analogues has been achieved without employing any of the strategies to prolong plasma half-life mentioned above, i.e., without drastically increasing molecular weight and/or adding synthetic complexity.

As noted above, the introduction of modifications in just one or two positions in 30- or 33-mer GLP-2 analogues resulted in remarkable reductions in their clearance. We thought it was unlikely that these substitutions would render the peptides highly resistant to proteolysis as point modifications in positions 11 and/or 16 would not be able to prevent enzymatic attack at multiple and very distant potential proteolysis sites, if proteolysis is actually the reason for the high clearance of **1** and **2** (but see the discussion on helicity later). The value of the residual clearance in rats for analogues **72**−**86**, < 0.3 mL/min/kg, is much lower than the glomerular filtration rate in these animals, about 9 mL/min/kg.[59] Since the MW of these peptides (<4100) is not sufficiently high to slow down glomerular filtration *per se*,[60] the low total clearance pointed at a reduced free fraction of the peptides in plasma and to plasma protein binding (PPB) as the cause of their low clearance. We therefore tested selected analogues with low CL for PPB in rat plasma using an ultracentrifugation method (Table 7).

Table 7. Rat Plasma Protein Binding and Helicity Values for Selected Compounds

Compound	Major Modifications[a]	PPB (%)	Helicity (%)
1	None	66.2	21
2		58.1	22
39	Phe[11], C-term NH_2	N.D.[b]	47
40	D-Phe[11], C-term NH_2	96.1	29
52	Leu[16], C-term NH_2	98.7	39
65	D-Phe[11], Leu[16], C-term NH_2	97.6	42
72	D-Phe[11], Leu[16]	99.5	29
73	D-Phe[11], Leu[16], C-term NH_2	99.2	27
81	D-Phe[11], Leu[16], C-term NHEt	N.D.[b]	55
82	D-Phe[11], Leu[16], C-term NHiBu	98.4	73
83	D-Phe[11], Leu[16], C-term NHBn	N.D.[b]	75
84	D-Phe[11], Leu[16], C-term NH-4Pic	N.D.[b]	49

[a]All compounds are the Gly[2] analogues except for **1**. **1** and **2** are the Met[10] analogues, and all other compounds are the Nle[10] analogues. **1**, **2**, **72**, **73**, and **81**−**84** are 33-mer peptides, and **39**, **40**, **52**, and **65** are 30-mer peptides; [b]N.D. = not determined.

Surprisingly, whereas **1** and **2** exhibited very moderate protein binding, all the compounds with low CL showed extensive PPB (>95%). It appears that the single replacement of Asn either in position 11 (compound **40**) or 16 (compound **52**) with a hydrophobic residue is sufficient to cause a significant increase in PPB. Since hormones of the glucagon family have been shown to have considerable helical character in solution[33] and some albumin-binding motifs reported in the literature contain multiple helical domains,[61,62] we investigated whether the high PPB in our analogues might be attributable to an enhanced helical character as compared to **1** or **2**. Selected compounds were evaluated by circular dichroism for their secondary structure in solution at physiological pH and ionic strength. All analogues tested showed higher helical content than **1** or **2** in PBS buffer. It can be speculated that the introduction of a lipophilic amino acid in positions 11, 16, or both, might contribute to stabilizing a helical conformation in this segment of the peptide via new hydrophobic interactions. In an α-helix, the side chain of a hydrophobic amino acid in position 11 would be located close to that of Leu[14] and one in position 16 would be in close proximity to the side chain of Ile.[13] Interestingly, peptides **81**−**84** which are C-terminally alkylated analogues of compound **73** showed particularly high helical content (>50%) in PBS buffer (Figure 2, numeric values in Table 7). It is tempting to speculate that a similar set of hydrophobic interactions between the side chain of Ile[31] and the C-terminal amide groups might be responsible for this peculiar "capping effect". However, as there was no clear correlation between helicity and PPB or CL we cannot conclude that the increase in helicity in our analogues is directly responsible for their strong protein binding.

Based on the data presented here and other considerations, compound **73** was selected for further evaluation. To investigate the effect of its lower clearance and higher plasma protein binding on its *in vivo* activity, the intestinotrophic activity of analogue **73** was tested in normal male Sprague−Dawley rats.

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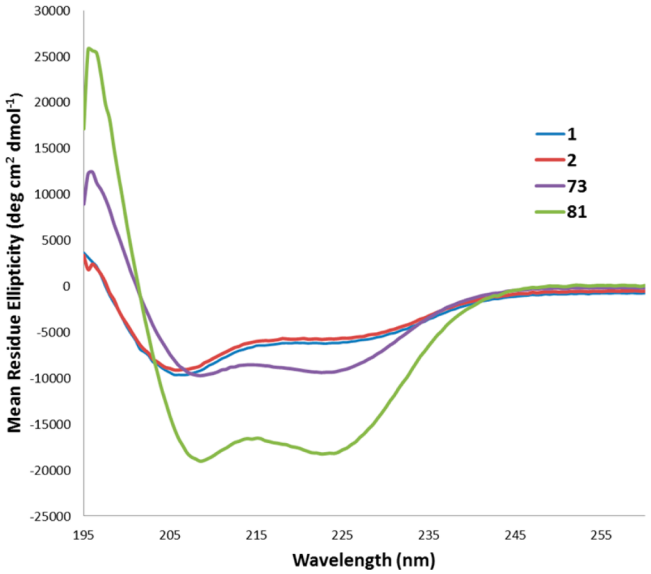

Figure 2. CD spectra of reference peptides **1**, and **2**, and compounds **73** and **81**.

Compound **73,** reference **2**, or vehicle control were injected once daily at doses ranging from 3 to 1000 nmol/kg for 5 days by the subcutaneous route and the wet weight of the small intestine was determined after the final dose. Compounds **2** and **73** had similar potency at the rat GLP-2R *in vitro*, but **73** achieved a larger increase in small intestinal growth at all doses tested (Table 8 and Figure 3). Thus, the decrease in plasma free fraction of **73** was more than compensated by the decrease in its clearance and consequent increase in half-life relative to **2** (details will be reported elsewhere), and **73** appears to be a promising compound for the treatment of conditions requiring intestinotrophic activity.

Table 8. Comparison of 73 and 2 Potency *in Vitro* in the rGLP-2R Assay and Effect *in Vivo* in the Small Intestine Growth Stimulation Assay at the Highest Dose Tested

	In vitro rGLP-2R[a]	*In vivo* Rat intestinal growth at dose of 1000 nmol/kg[b]		Rat iv PK
Compound	EC_{50} (nM)	Increase wet weight over vehicle (%)	N[c]	CL (mL/min/kg)
2	0.08	39.0	18	9.9
73	0.04	61.2	17	0.27

[a]In vitro potency at the rat GLP-2 receptor. [b]Compounds were administered once daily for 5 days; see Figure 3. [c]N -= number of rats per compound.

CONCLUSION

A new series of GLP-2 analogues modified in positions 2, 10, 11, and 16 and at the C-terminus has been designed, synthesized, and evaluated in pharmacological models. The new compounds are potent hGLP-2R agonists and selective vs the related hGLP-1 and hGCG receptors *in vitro*. Hydrophobic modifications in positions 11 and/or 16 resulted in compounds of extremely low CL in rats, mostly by increasing plasma protein binding. These low CL values were achieved without employing the conjugation strategies typically used to improve the half-life of peptides, such as lipidation, pegylation, or fusion with proteins. Compound **73** (FE 203799), which showed a promising pharmacological profile including *in vivo* potency in a rat model, has been selected for clinical development and is being considered as a treatment for gastrointestinal disorders. A comprehensive pharmacological evaluation of the compound will be reported elsewhere.

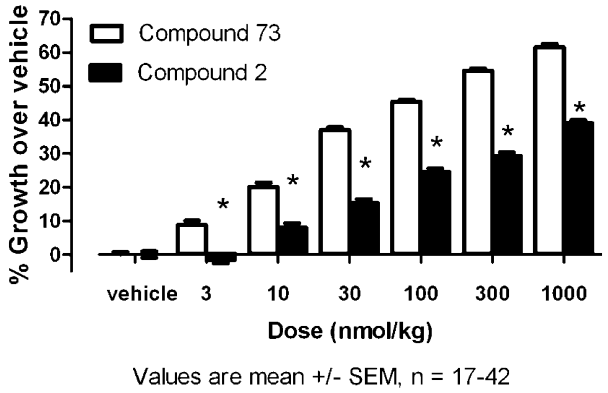

Figure 3. Stimulation of intestinal growth by compounds **2** (closed bars) and **73** (open bars) after daily s.c. administrations for 5 days. Small intestine wet weight for each rat was normalized to body weight and expressed as % increase over the mean of a vehicle control group run in the same experiment.

EXPERIMENTAL SECTION

Chemistry. *General.* Amino acid derivatives were purchased from Novabiochem, Bachem, and Peptide International and resins were obtained from Novabiochem and Matrix Innovation. Other chemicals and solvents were purchased from Sigma-Aldrich, VWR and Fisher Scientific.

Analytical HPLC was performed on a Waters 600 Liquid Chromatograph using a Vydac C_{18}, 5 μm, 4.6 × 250 mm column at a flow rate of 2 mL/min. Preparative HPLC was performed on a Waters 2000 Liquid Chromatograph using a C18, 300 Å, 15 μm PrePak 47 × 300 mm cartridge at a flow rate of 100 mL/min. Final purity of analogues was assessed on a 1100 Agilent Liquid Chromatograph as described in Supporting Information. Mass spectra were recorded on a Finnigan MAT LCQ spectrometer.

Boc-His(Trt)-Gly-Asp(tBu)-Gly-OH (**3**). The fully protected tetrapeptide was assembled manually by Fmoc chemistry starting from H-Gly-O-2Cl-Trityl resin (Novabiochem, cat # 856053). The couplings were performed with DIC/HOBt using 3-fold excess of amino acid derivatives. The Fmoc protecting group was removed with 20% PIP/DMF. The fully protected tetrapeptide was cleaved from the resin with 30% HIPF/DCM (v/v).[63] The compound was lyophilized twice from t-butanol and the crude product (about 90% HPLC purity) was used in the subsequent step without purification.

*Synthesis of 30-mer (**4**–**71**) or 33-mer (**73**, **75**–**80**) peptide primary amides.* The 5–30 or 5–33 fragments were assembled on Rink amide resin by Fmoc chemistry using ABI 433A (Applied Biosystems) or 9050 Plus (Perceptive Biosystems) automatic peptide synthesizers on a 0.25 mmol or a 0.5 mmol scale. The following side chain protecting groups were used for all peptides discussed in this paper: Trt for Gln and Asn, tBu for Glu and Asp, Pbf for Arg, Boc for Lys and Trp. Single HATU-mediated couplings with a 5-fold excess of reagents were used. The 1–4 fragment was subsequently added by a manual, DIC/HOBt-mediated coupling of tetrapeptide **3** using a 2-fold excess of reagents. The peptides were cleaved with the TFA/H_2O/TIS 90/6/4 cocktail (2 h). For analogues containing methionine (**4**–**10**), 2-mercaptopyridine was added to the cleavage mixture.[64]

*Synthesis of 33-mer C-terminal acids (**1**, **2**, **72**, **74**).* The 5–33 fragments were assembled on Fmoc-Asp(OtBu)-O-NovaSyn TGT resin (EMD, cat# 856126) using ABI 433A synthesizer. The 1–4 fragment was added manually as described above. To synthesize **1**, the N-terminal tetrapetide Boc-His(Trt)-Ala-Asp(tBu)-Gly-OH was used instead of **3**.

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For methionine-containing peptides (**1**, **2**) 2-mercaptopyridine was added to the cleavage mixture.[64]

*Synthesis of 33-mer C-terminal secondary amides (**81**–**84**).* The 1–33 peptide acids were assembled using Fmoc-Asp(OtBu)-O-TGT-NovaSyn resin as described in the previous paragraph. The fully protected analogues were cleaved from the resin with the HIPF/DCM 1/4 (v/v) cocktail,[63] the solvents were evaporated and the peptides were precipitated with ethyl ether and separated by filtration. The crude protected analogues were coupled with an appropriate amine using DIC/HOAt in the chloroform/2,2,2-trifluoroethanol 3/1 (v/v) solvent system.[65] The side-chain protecting groups were removed with the TFA/H_2O/TIS 90/6/4 (v/v/v) cocktail.

*Synthesis of 33-mer C-terminal mini-PEG secondary amides (**85**, **86**).* The compounds were prepared on Rink ChemMatrix resin. The mini-PEG fragments (Fmoc-15-amino-4,7,10,13-tetraoxapentadecanoic acid, EMD, product number 851035, for compound **85** and O-(N-Fmoc-3-aminopropyl)-O′-(N-diglycolyl-3-aminopropyl)-diethylene glycol, EMD, product number 851031, for compound **86**, respectively) were coupled using PyBOP/DIPEA in NMP. The resins were subsequently capped with 5% acetic anhydride, 6% 2,4-lutidine in DMF. Fmoc-Asp(OtBu)-OH was then manually added and the resulting resins were transferred to automatic synthesis vessels. The 5–32 fragments were assembled on the ABI 433A synthesizer and the 1–4 fragment was added manually by coupling peptide **3** using PyBOP/DIEA in NMP. The peptides were cleaved from the resin as described above.

Purification. The peptides were purified by preparative HPLC in triethylammonium phosphate buffers at pH 2.3 or 7.0. For more complex crude products an additional run in perchlorate buffer at pH 2.3 was performed. The compounds were eluted with a gradient of acetonitrile. The pooled fractions were diluted with at least one volume of water, reloaded on the prep column and eluted with a fast gradient of acetonitrile in 0.1% TFA. The fractions with HPLC purity exceeding 95% were pooled and lyophilized.

Biological methods. *In vitro receptor assays.* The agonist activity and potency of compounds at the rat and human Glucagon-like peptide 2 receptors (rGLP-2R and hGLP-2R) were determined in a transcriptional reporter gene assay (RGA) by transiently transfecting the human or rat GLP-2 receptor expression DNA construct into a human embryonic kidney (HEK-293) cell line in concert with a reporter DNA construct containing intracellular cAMP responsive promoter elements regulating expression of firefly luciferase.[66] Two days following transfection, cells were exposed to serial dilutions of compounds diluted half-log per dose (concentration range of 0.0003–30 nM), incubated at 37 °C for 5 h, followed by lysis of cells in the presence of luciferin and the total luminescence was measured. Compound **1** (30 nM) was used as an internal control in each experiment. The data displayed normal variation in individual assays performed.

To determine hGLP-1 receptor selectivity, compounds were tested in a luciferase-based transcriptional reporter assay (RGA) in a HEK-293 cell line stably expressing both the human Glucagon-like peptide 1 (hGLP-1) receptor and a reporter DNA construct containing intracellular cAMP responsive promoter elements. hGLP-1 was used as an internal control for the hGLP-1 receptor assay. To determine hGCG receptor selectivity, compounds were tested in luciferase-based transcriptional reporter gene assays by transiently transfecting the human Glucagon receptor (hGCGR) expression DNA construct into a HEK-293 cell line in concert with a reporter DNA construct containing intracellular cAMP responsive promoter elements. hGlucagon was used as an internal control for the hGCG receptor assay. In both assays compounds were tested in at least three independent experiments. Dose–response curves were analyzed using a one-site, four parameter model from Xlfit (IDBS) and used to estimate EC_{50} and efficacy values. Agonist potency is presented as geometric means in nanomol/L (nM) and efficacy is presented as the arithmetic means in percentage of internal control efficacy. Selectivity values are given as ratios of the EC_{50} values at the receptor of interest to the corresponding EC_{50} values at the hGLP-2R.

PK studies. Animals. All animals procedures were approved by the Ferring Research Institute Institutional Animal Care and Use Committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council. The single-dose pharmacokinetic profiles of GLP-2 analogues were investigated following *i.v.* administration in male Sprague–Dawley rats (230–300 g). Animals with chronic jugular vein and carotid artery catheters inserted surgically were obtained from Harlan Laboratories Inc. (Indianapolis USA). The rats were given free access to food (18% protein Rodent Diet, Harlan Teklad, Madison USA) and water. They were housed in a conventional animal facility in individually ventilated caging (LabProducts Inc., Seaford USA) with appropriate air flow under controlled environmental conditions (20–22 °C, 12 h light/dark cycle).

Dosing and sampling. The GLP-2 analogues were dissolved in 25 mM phosphate buffer in water, pH 7.4 (no tonicity adjustment with NaCl). Each rat was given a single dose of 0.2 mg/kg of each test compound in cassette mode through the jugular vein.[67] Cassettes were constituted of 3 to 6 GLP-2 analogues with each compound at a concentration of 0.2 mg/mL. Blood samples (250 μL) were collected from the carotid artery catheter at nominal times of either 2, 4, 6, 10, 20, 30, 60, 90, and 120 min or 2, 5, 8, 13, 20, 35, 55, 80, 120, 210, and 300 min after administration into prechilled tubes containing K_2EDTA as anticoagulant. Blood that was drawn from the animal was replaced with an equal volume of saline. The samples were centrifuged at 4 °C and plasma was separated. All samples were immediately frozen on dry ice and stored at −50 °C until further analysis.

Bioanalysis. The concentrations of GLP-2 analogues in rat plasma were determined using a liquid chromatography tandem mass spectrometry (LC/MS/MS) method. The dynamic range of the assays was 2–4000 ng/mL. Standard and internal standard solutions were prepared in 50% DMSO in acetonitrile. Typically, 40 μL of rat plasma sample was mixed with 20 μL of the internal standard and 100 μL of the crashing solution (50% DMSO in acetonitrile). After shaking for 2 min and centrifugation at 5700 g for 30 min, supernatant was injected into a Jupiter 5 μm 300 Å C18 50 × 2.0 mm HPLC column (Phenomenex, Torrance USA) coupled to a Shimadzu LC-20AD series LC (Shimadzu, Kyoto Japan) system. The analytes were eluted using a gradient of solvent B (solvent A - 0.01% TFA and 1% formic acid in water; solvent B - 0.01% TFA, 1% formic acid and 70% acetonitrile in water) at a flow rate of 0.5 mL/min and detected using an API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Ontario Canada) in the positive electrospray ionization mode. Analyte concentrations were calculated by linear regression analysis using the peak area ratio of analyte to the internal standard on the Applied Biosystems Analyst software version 1.4.2.

PK data analysis. PK parameters were calculated using a non-compartmental curve stripping method (PK Solutions 2.0, Summit Research Services, Ashland, USA). The area under the plasma concentration–time curve to infinity (AUC_∞) was calculated by combining the area under the curve to the last time point ($AUC_{0\text{-}t}$) with the extrapolated AUC value of the terminal phase. Body weight normalized CL values (mL/min/kg) were calculated as dose divided by $AUC_{(0\text{-}\infty)}$ and rat body weight.

Determination of rat plasma protein binding. Rat plasma protein binding (PPB) analysis was performed by an ultracentrifugation (UC) method. Briefly, 5 μL of a 20–100 mg/mL solution of the GLP-2 analogue in DMSO was added to a 1 mL Sorvall polycarbonate thick-walled UC tube (Fisher Scientific, product number 45237) containing 495 μL of rat plasma to a final peptide concentration of 200–1000 ng/mL. The peptide plasma mixture was incubated at 37 °C for 15 min in a Sorvall S140-AT 35° fixed angle rotor, and then spun at 120,000 rpm (773,000 xg) for 30 min at 37 °C in a Sorvall MX150 micro-ultracentrifuge (Asheville NC, USA). After centrifugation, a 50 μL sample in the middle layer of the UC tube was collected at the 300 μL volume meniscus level with a 250 μL pipettor tip. The compound concentrations in the collected sample was quantified immediately by the LC/MS/MS method described in the Experimental Section. Each compound was assayed in quadruplicate. The percentage of binding was calculated as

$$\text{PPB}\ (\%) = \frac{Ci - Cm}{Ci} * 100$$

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Where Ci is the initial concentration of peptide and *Cm* is the concentration of peptide remaining in the middle layer after ultracentrifugation.

Rat intestinal growth assay. Male Sprague–Dawley rats (Harlan, Indianapolis) (body weight 235–275 g) were housed in a controlled environment with free access to food and water. On the first day of experimentation animals were randomly assigned to treatment groups (typically n = 6 per treatment) with doses standardized by body weight. Compounds or vehicle (1 mL/kg) were administered by s.c. bolus injection, once daily at approximately the same time of day for five consecutive days. Animals were weighed at the beginning of each day of dosing to determine dose volumes and to track body weight. Animals were euthanized after dosing on the final day (96 h after the first dose), the gastrointestinal tract was excised and the small intestine was carefully dissected. Intestines were flushed with saline to expel contents. Excess saline was removed and the wet weight of intestines was recorded.

Dosing solutions. Peptides were dissolved in 25 mM phosphate buffer, pH 7.4. On the first day of experimentation, compounds were formulated at concentrations allowing administration of the desired dose in a volume of 1 mL per kg of animal body weight (3–1000 nmol/mL), and stored at 4 °C over the course of the experiment.

Data calculation and statistical analysis. For each animal, wet weight of the intestine was normalized to body weight and percent increase over the mean of the vehicle group run in the same experiment was calculated. Data from multiple experiments were combined and data for each treatment are expressed as mean with SEM. For statistical comparisons, data were analyzed by ANOVA followed by Student–Newman–Keuls method for *post hoc* comparisons. Differences between groups were considered to be statistically significant at $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01909.

In vitro, PK, PPB, and helicity data with statistical parameters, protocol for helicity determinations, and physiochemical properties of analogues **1**, **2**, and **4–86** (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Aph, β-(4-aminophenyl)alanine; Bip, β-(4,4′-biphenyl)alanine; Cha, β-cyclohexylalanine; Cpa, β-(4-chlorophenyl)alanine; 2Cpa, β-(2-chlorophenyl)alanine; 3Cpa, β-(3-chlorophenyl)-alanine; Dab, 2,4-diaminobutyric acid; Dcpm, dicyclopropylmethyl; Dmb, 2,4-dimethoxybenzyl; DPP IV, dipeptidyl peptidase IV; GLP-1R, GLP-1 receptor; GLP-2R, GLP-2 receptor; hGCGR, human glucagon receptor; Hmb, 2-hydroxy-4-methoxybenzyl; Hol, homoleucine; 1Nal, β-(1-naphtyl)-alanine; 2Nal, β-(2-naphtyl)alanine; 4Pic, 4-picolyl (4-pyridinylmethyl); RGA, reporter gene assay; rGLP-2R, rat GLP-2 receptor; SAR, structure–activity relationship; Thi, β-(2-thienyl)alanine; 3Thi, β-(3-thienyl)alanine

■ REFERENCES

(1) Wideman, R. D.; Covey, S. D.; Webb, G. C.; Drucker, D. J.; Kieffer, T. J. A switch from prohormone convertase (PC)-2 to PC1/3 expression in transplanted alpha-cells is accompanied by differential processing of proglucagon and improved glucose homeostasis in mice. *Diabetes* **2007**, *56*, 2744–2752.

(2) Buhl, T.; Thim, L.; Kofod, H.; Orskov, C.; Harling, H.; Holst, J. J. Naturally occurring products of proglucagon 111–160 in the porcine and human small intestine. *J. Biol. Chem.* **1988**, *263*, 8621–8624.

(3) Munroe, D. G.; Gupta, A. K.; Kooshesh, F.; Vyas, T. B.; Rizkalla, G.; Wang, H.; Demchyshyn, L.; Yang, Z. J.; Kamboj, R. K.; Chen, H.; McCallum, K.; Sumner-Smith, M.; Drucker, D. J.; Crivici, A. Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 1569–1573.

(4) Pedersen, J.; Pedersen, N. B.; Brix, S. W.; Grunddal, K. V.; Rosenkilde, M. M.; Hartmann, B.; Orskov, C.; Poulsen, S. S.; Holst, J. J. The glucagon-like peptide 2 receptor is expressed in enteric neurons and not in the epithelium of the intestine. *Peptides* **2015**, *67*, 20–28.

(5) Drucker, D. J. Glucagon-like peptide 2. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 1759–1764.

(6) Holst, J. J. Gut hormones as pharmaceuticals. From enteroglucagon to GLP-1 and GLP-2. *Regul. Pept.* **2000**, *93*, 45–51.

(7) Estall, J. L.; Drucker, D. J. Glucagon-like peptide-2. *Annu. Rev. Nutr.* **2006**, *26*, 391–411.

(8) Wallis, K.; Walters, J. R.; Forbes, A. Review article: Glucagon-like peptide 2–current applications and future directions. *Aliment. Pharmacol. Ther.* **2007**, *25*, 365–372.

(9) Thulesen, J. Glucagon-like peptide 2 (GLP-2), an intestinotrophic mediator. *Curr. Protein Pept. Sci.* **2004**, *5*, 51–65.

(10) Drucker, D. J.; Yusta, B. Physiology and pharmacology of the enteroendocrine hormone glucagon-like peptide-2. *Annu. Rev. Physiol.* **2014**, *76*, 561–583.

(11) Drucker, D. J.; Erlich, P.; Asa, S. L.; Brubaker, P. L. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 7911–7916.

(12) Tsai, C. H.; Hill, M.; Asa, S. L.; Brubaker, P. L.; Drucker, D. J. Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. *Am. J. Physiol.* **1997**, *273*, E77–84.

(13) Meier, J. J.; Nauck, M. A.; Pott, A.; Heinze, K.; Goetze, O.; Bulut, K.; Schmidt, W. E.; Gallwitz, B.; Holst, J. J. Glucagon-like peptide 2 stimulates glucagon secretion, enhances lipid absorption, and inhibits gastric acid secretion in humans. *Gastroenterology* **2006**, *130*, 44–54.

(14) Hsieh, J.; Longuet, C.; Maida, A.; Bahrami, J.; Xu, E.; Baker, C. L.; Brubaker, P. L.; Drucker, D. J.; Adeli, K. Glucagon-like peptide-2 increases intestinal lipid absorption and chylomicron production via CD36. *Gastroenterology* **2009**, *137*, 997–1005.

(15) Bremholm, L.; Hornum, M.; Henriksen, B. M.; Larsen, S.; Holst, J. J. Glucagon-like peptide-2 increases mesenteric blood flow in humans. *Scand. J. Gastroenterol.* **2009**, *44*, 314–319.

(16) Stephens, J.; Stoll, B.; Cottrell, J.; Chang, X.; Helmrath, M.; Burrin, D. G. Glucagon-like peptide-2 acutely increases proximal small intestinal blood flow in tpn-fed neonatal piglets. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2006**, *290*, R283–289.

(17) Nagell, C. F.; Wettergren, A.; Pedersen, J. F.; Mortensen, D.; Holst, J. J. Glucagon-like peptide-2 inhibits antral emptying in man, but is not as potent as glucagon-like peptide-1. *Scand. J. Gastroenterol.* **2004**, *39*, 353–358.

(18) Sigalet, D. L.; Wallace, L. E.; Holst, J. J.; Martin, G. R.; Kaji, T.; Tanaka, H.; Sharkey, K. A. Enteric neural pathways mediate the anti-inflammatory actions of glucagon-like peptide 2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *293*, G211–221.

(19) Alavi, K.; Schwartz, M. Z.; Palazzo, J. P.; Prasad, R. Treatment of inflammatory bowel disease in a rodent model with the intestinal growth factor glucagon-like peptide-2. *J. Pediatr. Surg.* **2000**, *35*, 847–851.

DOI: 10.1021/acs.jmedchem.5b01909
J. Med. Chem. 2016, 59, 3129–3139

(20) Hartmann, B.; Harr, M. B.; Jeppesen, P. B.; Wojdemann, M.; Deacon, C. F.; Mortensen, P. B.; Holst, J. J. In vivo and in vitro degradation of glucagon-like peptide-2 in humans. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 2884−2888.
(21) Drucker, D. J.; Shi, Q.; Crivici, A.; Sumner-Smith, M.; Tavares, W.; Hill, M.; DeForest, L.; Cooper, S.; Brubaker, P. L. Regulation of the biological activity of glucagon-like peptide 2 in vivo by dipeptidyl peptidase IV. *Nat. Biotechnol.* **1997**, *15*, 673−677.
(22) Thulesen, J.; Knudsen, L. B.; Hartmann, B.; Hastrup, S.; Kissow, H.; Jeppesen, P. B.; Orskov, C.; Holst, J. J.; Poulsen, S. S. The truncated metabolite GLP-2 (3−33) interacts with the GLP-2 receptor as a partial agonist. *Regul. Pept.* **2002**, *103*, 9−15.
(23) Larsen, B. D.; Petersen, Y. M. Selective glucagon-like-peptide-2 (GLP-2) analogues. WO2008056155, 2008.
(24) Just, R.; Bovbjerg, K. L.; Riber, D.; Russel, W. S. Glucagon-like-peptide-2 (GLP-2) analogues. WO2013164484, 2013.
(25) Drucker, D. J.; DeForest, L.; Brubaker, P. L. Intestinal response to growth factors administered alone or in combination with human [Gly2]glucagon-like peptide 2. *Am. J. Physiol.* **1997**, *273*, G1252−1262.
(26) Boushey, R. P.; Yusta, B.; Drucker, D. J. Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. *Am. J. Physiol.* **1999**, *277*, E937−947.
(27) Scott, R. B.; Kirk, D.; MacNaughton, W. K.; Meddings, J. B. GLP-2 augments the adaptive response to massive intestinal resection in rat. *Am. J. Physiol.* **1998**, *275*, G911−921.
(28) Washizawa, N.; Gu, L. H.; Gu, L.; Openo, K. P.; Jones, D. P.; Ziegler, T. R. Comparative effects of glucagon-like peptide-2 (GLP-2), growth hormone (GH), and keratinocyte growth factor (KGF) on markers of gut adaptation after massive small bowel resection in rats. *JPEN, J. Parenter. Enteral Nutr.* **2004**, *28*, 399−409.
(29) Tavakkolizadeh, A.; Shen, R.; Abraham, P.; Kormi, N.; Seifert, P.; Edelman, E. R.; Jacobs, D. O.; Zinner, M. J.; Ashley, S. W.; Whang, E. E. Glucagon-like peptide 2: A new treatment for chemotherapy-induced enteritis. *J. Surg. Res.* **2000**, *91*, 77−82.
(30) Boushey, R. P.; Yusta, B.; Drucker, D. J. Glucagon-like peptide (GLP)-2 reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor. *Cancer Res.* **2001**, *61*, 687−693.
(31) Torres, S.; Thim, L.; Milliat, F.; Vozenin-Brotons, M. C.; Olsen, U. B.; Ahnfelt-Ronne, I.; Bourhis, J.; Benderitter, M.; Francois, A. Glucagon-like peptide-2 improves both acute and late experimental radiation enteritis in the rat. *Int. J. Radiat. Oncol., Biol., Phys.* **2007**, *69*, 1563−1571.
(32) DaCambra, M. P.; Yusta, B.; Sumner-Smith, M.; Crivici, A.; Drucker, D. J.; Brubaker, P. L. Structural determinants for activity of glucagon-like peptide-2. *Biochemistry* **2000**, *39*, 8888−8894.
(33) Venneti, K. C.; Hewage, C. M. Conformational and molecular interaction studies of glucagon-like peptide-2 with its N-terminal extracellular receptor domain. *FEBS Lett.* **2011**, *585*, 346−352.
(34) Marier, J. F.; Beliveau, M.; Mouksassi, M. S.; Shaw, P.; Cyran, J.; Kesavan, J.; Wallens, J.; Zahir, H.; Wells, D.; Caminis, J. Pharmacokinetics, safety, and tolerability of teduglutide, a glucagon-like peptide-2 (GLP-2) analog, following multiple ascending subcutaneous administrations in healthy subjects. *J. Clin. Pharmacol.* **2008**, *48*, 1289−1299.
(35) Jeppesen, P. B.; Gilroy, R.; Pertkiewicz, M.; Allard, J. P.; Messing, B.; O'Keefe, S. J. Randomised placebo-controlled trial of teduglutide in reducing parenteral nutrition and/or intravenous fluid requirements in patients with short bowel syndrome. *Gut* **2011**, *60*, 902−914.
(36) Vipperla, K.; O'Keefe, S. J. Targeted therapy of short-bowel syndrome with teduglutide: The new kid on the block. *Clin. Exp. Gastroenterol.* **2014**, *7*, 489−495.
(37) Jeppesen, P. B. Teduglutide for the treatment of short bowel syndrome. *Drugs Today (Barc)* **2013**, *49*, 599−614.
(38) Naberhuis, J. K.; Tappenden, K. A. Teduglutide for safe reduction of parenteral nutrient and/or fluid requirements in adults: A systematic review. *JPEN, J. Parenter. Enteral Nutr.* **2015**. [Online early access]. DOI: 10.1177/0148607115582063. Published Online: April 16, 2015.
(39) Seidner, D. L.; Joly, F.; Youssef, N. N. Effect of teduglutide, a glucagon-like peptide 2 analog, on citrulline levels in patients with short bowel syndrome in two phase III randomized trials. *Clin. Transl. Gastroenterol.* **2015**, *6*, e93.
(40) Buchman, A. L.; Katz, S.; Fang, J. C.; Bernstein, C. N.; Abou-Assi, S. G. Teduglutide, a novel mucosally active analog of glucagon-like peptide-2 (GLP-2) for the treatment of moderate to severe Crohn's disease. *Inflamm. Bowel Dis.* **2010**, *16*, 962−973.
(41) Larsen, B. D.; Petersen, Y. M.; Ebbehoej, K. Glucagon-like-peptide-2 (GLP-2) analogues. WO2006117565, 2006.
(42) Schon, I.; Kisfaludy, L. Formation of aminosuccinyl peptides during acidolytic deprotection followed by their transformation to piperazine-2,5-dione derivatives in neutral media. *Int. J. Pept. Protein Res.* **1979**, *14*, 485−494.
(43) Suli-Vargha, H.; Schlosser, G.; Ilas, J. 1,4-diazepine-2,5-dione ring formation during solid phase synthesis of peptides containing aspartic acid beta -benzyl ester. *J. Pept. Sci.* **2007**, *13*, 742−748.
(44) Mergler, M.; Dick, F. The aspartimide problem in Fmoc-based SPPS. Part III. *J. Pept. Sci.* **2005**, *11*, 650−657.
(45) Mergler, M.; Dick, F.; Sax, B.; Stahelin, C.; Vorherr, T. The aspartimide problem in Fmoc-based SPPS. Part II. *J. Pept. Sci.* **2003**, *9*, 518−526.
(46) Mergler, M.; Dick, F.; Sax, B.; Weiler, P.; Vorherr, T. The aspartimide problem in Fmoc-based SPPS. Part I. *J. Pept. Sci.* **2003**, *9*, 36−46.
(47) Lauer, J. L.; Fields, C. G.; Fields, G. B. Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. *Lett. Pept. Sci.* **1995**, *1*, 197−205.
(48) Wade, J. D.; Mathieu, M. N.; Macris, M.; Tregear, G. W. Base-induced side reactions in fmoc-solid phase peptide synthesis: Minimization by use of piperazine as N-alpha deprotection reagent. *Lett. Pept. Sci.* **2000**, *7*, 107−112.
(49) Okada, Y.; Iguchi, S.; Kawasaki, K. Synthesis of beta −1- and beta −2-adamantyl aspartates and their evaluation for peptide synthesis. *J. Chem. Soc., Chem. Commun.* **1987**, 1532−1534.
(50) Karlstrom, A.; Unden, A. Design of protecting groups for the beta-carboxylic group of aspartic acid that minimize base-catalyzed aspartimide formation. *Int. J. Pept. Protein Res.* **1996**, *48*, 305−311.
(51) Quibell, M.; Owen, D.; Peckman, L. C.; Johnson, T. Suppression of piperidine-mediated side product formation for Asp(OBut)-containing peptides by the use of N-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide protection. *J. Chem. Soc., Chem. Commun.* **1994**, 2343−2344.
(52) Cardona, V.; Eberle, I.; Barthelemy, S.; Beythien, J.; Doerner, B.; Schneeberger, P.; Keyte, J.; White, P. D. Application of Dmb-dipeptides in the Fmoc SPPS of difficult and aspartimide-prone sequences. *Int. J. Pept. Res. Ther.* **2008**, *14*, 285−292.
(53) Röder, R.; Henklein, P.; Weisshoff, H.; Mugge, C.; Patzel, M.; Schubert, U.; Carpino Louis, A.; Henklein, P. On the use of N-dicyclopropylmethyl aspartyl-glycine synthone for backbone amide protection. *J. Pept. Sci.* **2010**, *16*, 65−70.
(54) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. 2-chlorotrityl chloride resin. Studies on anchoring of Fmoc-amino acids and peptide cleavage. *Int. J. Pept. Protein Res.* **1991**, *37*, 513−520.
(55) Yamazaki, K.; Kagaya, T.; Watanabe, M.; Terauchi, H.; Iida, D.; Fukumoto, H.; Suzuki, S.; Arai, T.; Aoki, M.; Takase, K.; Seiki, T.; Tsukahara, K.; Nagakawa, J. A novel truncated glucagon-like peptide 2 (GLP-2) as a tool for analyzing GLP-2 receptor agonists. *Biomed. Res.* **2013**, *34*, 129−136.
(56) Goke, R.; Hareter, A.; Vahl, T.; Bode, H.-P.; Hoffmann, E. Glucagon-like peptide-1 receptor in relation to its ligand. In *The insulinotropic gut hormone glucagon-like peptide-1*; Fehmann, H. C., Goke, B., Eds.; Karger: Basel, 1997; pp 85−104.
(57) Pollaro, L.; Heinis, C. Strategies to prolong the plasma residence time of peptide drugs. *MedChemComm* **2010**, *1*, 319−324.
(58) Neumiller, J. J.; Campbell, R. K. Liraglutide: A once-daily incretin mimetic for the treatment of type 2 diabetes mellitus. *Ann. Pharmacother.* **2009**, *43*, 1433−1444.

DOI: 10.1021/acs.jmedchem.5b01909
J. Med. Chem. 2016, 59, 3129−3139

(59) Ahmed, M. H.; Balment, R. J.; Ashton, N. Renal action of acute chloroquine and paracetamol administration in the anesthetized, fluid-balanced rat. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 478−483.
(60) Bailon, P.; Won, C. Y. PEG-modified biopharmaceuticals. *Expert Opin. Drug Delivery* **2009**, *6*, 1−16.
(61) Jonsson, A.; Dogan, J.; Herne, N.; Abrahmsen, L.; Nygren, P. A. Engineering of a femtomolar affinity binding protein to human serum albumin. *Protein Eng., Des. Sel.* **2008**, *21*, 515−527.
(62) Nilvebrant, J.; Hober, S. The albumin-binding domain as a scaffold for protein engineering. *Comput. Struct. Biotechnol. J.* **2013**, *6*, e201303009.
(63) Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E. A new reagent for the cleavage of fully protected peptides synthesized on 2-chlorotrityl chloride resin. *J. Chem. Soc., Chem. Commun.* **1994**, 2559−2560.
(64) Taichi, M.; Kimura, T.; Nishiuchi, Y. Suppression of side reactions during final deprotection employing a strong acid in boc chemistry: Regeneration of methionyl residues from their sulfonium salts. *Int. J. Pept. Res. Ther.* **2009**, *15*, 247−253.
(65) Inui, T.; Bodi, J.; Kubo, S.; Nishio, H.; Kimura, T.; Kojima, S.; Maruta, H.; Muramatsu, T.; Sakakibara, S. Solution synthesis of human midkine, a novel heparin-binding neurotrophic factor consisting of 121 amino acid residues with five disulphide bonds. *J. Pept. Sci.* **1996**, *2*, 28−39.
(66) Himmler, A.; Stratowa, C.; Czernilofsky, A. P. Functional testing of human dopamine d1 and d5 receptors expressed in stable camp-responsive luciferase reporter cell lines. *J. Recept. Res.* **1993**, *13*, 79−94.
(67) Frick, L. W.; Adkison, K. K.; Wells-Knecht, K. J.; Woollard, P.; Higton, D. M. Cassette dosing: Rapid in vivo assessment of pharmacokinetics. *Pharm. Sci. Technol. Today* **1998**, *1*, 12−18.

DOI: 10.1021/acs.jmedchem.5b01909
J. Med. Chem. 2016, 59, 3129−3139