Synthesis and Pharmacological Characterization of Novel, Potent and Low Clearance GLP-2 Analogues

FERRING PHARMACEUTICALS

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Introduction

GLP-2, **1**, is a 33 amino acid peptide released from intestinal L-cells following food ingestion and acts at G protein coupled GLP-2 receptors in the small intestine and colon to promote intestinal growth and increase nutrient absorption. Native hGLP-2 has a high systemic clearance (CL) due in part to proteolytic cleavage of its N-terminus by dipeptidyl peptidase IV (DPP4), limiting its potential clinical use. A DPP4 resistant analogue, teduglutide, [Gly[2]]hGLP-2 (**2**), displays similar intestinotrophic properties with an improved pharmacokinetic profile.[1] **2** has been shown to be effective in patients with short bowel syndrome[2] and Crohn's disease.[3] Two other analogues with C-terminal hexalysine extensions, ZP1846 and ZP1848 are also in clinical trials for the treatment of chemotherapy-induced diarrhea and for the treatment of Crohn's disease, respectively.[4]

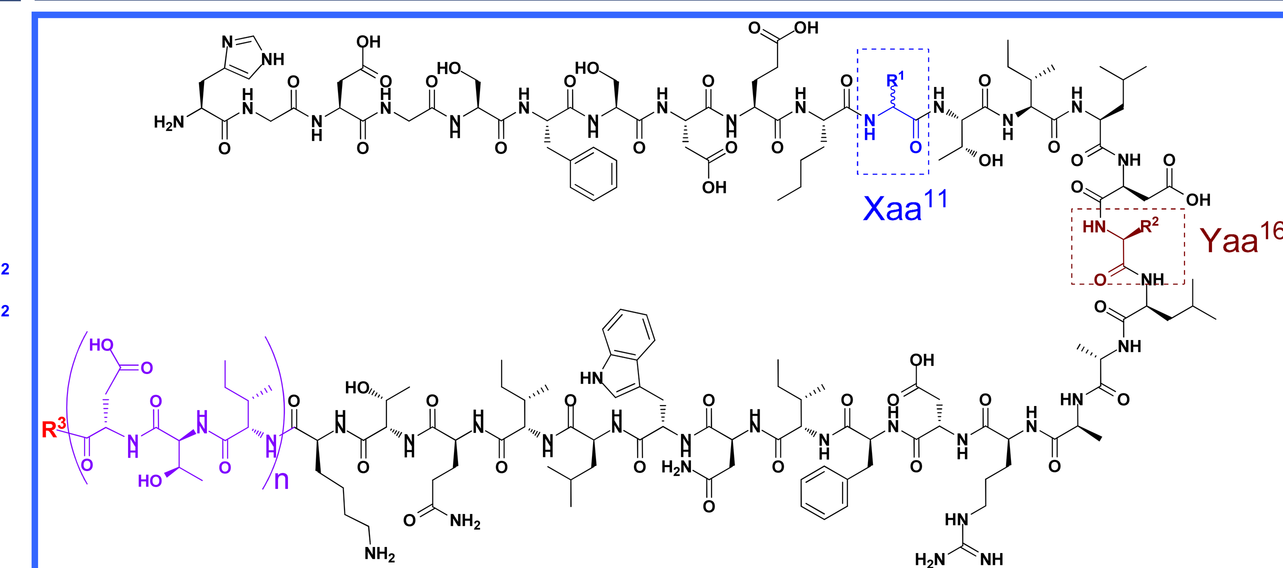
In search of GLP-2 agonists pharmacologically superior to compounds currently in clinical development, we synthesized and biologically evaluated (*in vitro* receptor potency and selectivity, *in vivo* rat pharmacokinetics), a series of analogues based on [Gly[2]]hGLP-2 (1-30) peptide amides where the Met[10] residue was replaced by the more stable isosteric norleucine. Based on our internal data and literature,[5] positions 11 and 16 were selected for modifications. The most promising modifications were then incorporated in full length 1-33 peptides. Here we report on the discovery of potent, low-clearance and clinically relevant GLP-2 analogues.

Sequences of hGLP-2 analogues

	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 34 35 36 37 38 39
hGLP-2, **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
teduglutide, **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
ZP1846	H **G** **E** G **S** F S **S** E **L** **S** T I L D **A** L A A R D F I **A** W L I **A** T K I T D **K K K K K K**-NH_2
ZP1848	H **G** **E** G **T** F S **S** E **L** **A** T I L D **A** L A A R D F I **A** W L I **A** T K I T D **K K K K K K**-NH_2
Compounds **3-29**	H **G** D G S F S D E **Nle** **Xaa** T I L D **Yaa** L A A R D F I N W L I Q T K-NH_2
Compounds **30-35**	H **G** D G S F S D E **Nle** **Xaa** T I L D **Yaa** L A A R D F I N W L I Q T K I T D **R³**

Differences with native hGLP-2 highlighted in blue

Structure



Structure and pharmacological profile of GLP-2 analogues

Analogue	Structure[a]				Analytical data			In vitro potency (EC50, nM) at receptor		Potency ratio at hGLP-2R vs. compound 1	Selectivity[b] hGLP-1/hGLP-2	Rat CL (ml/kg/min)
	Xaa11	Yaa16	n	R3	HPLC purity	M+H calculated	M+H observed	hGLP-2	hGLP-1			
1	Asn	Asn	1	-OH	96.2	3764.8	3764.9	0.07	520	1.0	7400	25
2	Asn	Asn	1	$-NH_2$	94.0	3750.8	3750.8	0.09	>1000[c]	0.78	>11000	9.9
3	Phe	Asn	0	$-NH_2$	100.0	3435.7	3435.6	0.15	16	0.47	100	NT[d]
4	Cpa	Asn	0	$-NH_2$	99.7	3469.7	3469.8	0.16	8.9	0.44	55	NT[d]
5	His	Asn	0	$-NH_2$	98.3	3425.7	3426.0	0.44	300[e]	0.16	680	NT[d]
6	Cha	Asn	0	$-NH_2$	99.7	3441.8	3442.0	0.51	8.9	0.14	17	NT[d]
7	Leu	Asn	0	$-NH_2$	100.0	3401.7	3401.8	0.15	180[e]	0.47	1200	2.8
8	D-Leu	Asn	0	$-NH_2$	100.0	3401.7	3401.8	0.31	>1000[c]	0.23	>3200	3.3
9	D-Phe	Asn	0	$-NH_2$	100.0	3435.7	3435.8	0.09	120[e]	0.78	1300	1.2
10	D-Cpa	Asn	0	$-NH_2$	98.4	3469.7	3469.8	0.09	60	0.78	660	0.51
11	D-3-Cpa	Asn	0	$-NH_2$	96.0	3469.7	3470.2	0.11	45	0.64	400	0.32
12	D-Thi	Asn	0	$-NH_2$	99.1	3441.7	3442.0	0.10	80[e]	0.7	800	1.1
13	D-Tyr	Asn	0	$-NH_2$	99.1	3451.7	3452.2	0.18	32[e]	0.39	170	4.9
14	D-His	Asn	0	$-NH_2$	97.8	3425.7	3426.0	0.20	>1000[c]	0.35	>5000	NT[d]
15	Asn	Leu	0	$-NH_2$	98.8	3401.7	3401.8	0.10	>1000[c]	0.7	>10000	0.84
16	Asn	Cha	0	$-NH_2$	99.9	3441.8	3442.0	0.10	>1000[c]	0.7	>10000	0.41
17	Asn	Tyr	0	$-NH_2$	98.1	3451.7	3452.0	0.11	>1000[c]	0.64	>9000	1.2
18	Asn	Aph	0	$-NH_2$	98.0	3450.7	3451.0	0.13	>1000[c]	0.54	>7600	1.9
19	Asn	Phe	0	$-NH_2$	98.7	3435.7	3436.2	0.14	>1000[c]	0.5	>7100	NT[d]
20	Asn	Trp	0	$-NH_2$	98.6	3474.7	3475.0	0.15	>1000[c]	0.47	>6600	0.50
21	Asn	Thi	0	$-NH_2$	98.4	3441.7	3442.0	0.20	>1000[c]	0.35	>5000	NT[d]
22	Asn	Cpa	0	$-NH_2$	100.0	3469.7	3469.8	0.22	>1000[c]	0.32	>4500	NT[d]
23	Asn	His	0	$-NH_2$	97.4	3425.7	3426.0	0.42	>1000[c]	0.17	>2300	NT[d]
24	D-Phe	Tyr	0	$-NH_2$	99.5	3484.8	3485.2	0.07	90[e]	1.0	1200	0.48
25	D-Phe	Leu	0	$-NH_2$	98.8	3434.8	3435.2	0.08	>1000[c]	0.88	>12000	0.37
26	D-Phe	Phe	0	$-NH_2$	100.0	3468.8	3469.2	0.09	>1000[c]	0.78	>11000	0.30
27	D-Thi	Tyr	0	$-NH_2$	99.8	3490.7	3491.0	0.07	68[e]	1.0	970	0.52
28	D-Thi	Leu	0	$-NH_2$	99.8	3440.7	3441.2	0.08	>1000[c]	0.88	>12000	0.33
29	D-Thi	Phe	0	$-NH_2$	99.3	3474.7	3475.2	0.08	>1000[c]	0.88	>12000	0.26
30	D-Phe	Leu	1	-OH	95.6	3764.9	3764.4	0.03	>1000[c]	2.3	>33000	0.22
31	D-Phe	Leu	1	$-NH_2$	96.1	3763.9	3764.2	0.03	>1000[c]	2.3	>33000	0.27
32	D-Phe	Phe	1	-OH	96.3	3798.9	3798.6	0.06	>1000[c]	1.2	>16000	0.15
33	D-Phe	Phe	1	$-NH_2$	90.2	3797.9	3797.4	0.06	>1000[c]	1.2	>16000	0.24
34	D-Thi	Phe	1	$-NH_2$	87.5	3803.9	3803.9	0.07	>1000[c]	1.0	>14000	0.15
35	D-3-Cpa	Phe	1	$-NH_2$	92.8	3831.9	3831.3	0.13	>1000[c]	0.54	>7600	0.12

[a] *Compound **1** (hGLP-2) has Ala in position 2 and Met in position 10. Compound **2** has Met in position 2.* [b] *Ratio EC_{50}(hGLP-1R)/EC_{50}(hGLP-2R);* [c] *No significant agonism at the highest concentration tested – 1000 nM;* [d] *NT – not tested;* [e] *partial agonist, efficacy <70%*

Results and discussion

- Based on our preliminary C-terminal truncation study (results not shown here) the 1-30 peptide amide was selected for initial SAR studies.
- To prevent side reactions associated with aspartimide formation[6] due to the presence of the Asp[3]-Gly[4] motif, peptides were synthesized by assembling sequences up to position 5 and coupling the 1-4 fragment prepared separately on trityl resin.
- The introduction of single hydrophobic residues in positions 11 or 16 resulted in analogues nearly as potent *in vitro* as the natural hormone, **1**. Compounds with D-aromatic amino acids in position 11 (**9**, D-Phe[11]; **10**, D-Cpa[11]; **12**, D-Thi[11]) or aromatic/aliphatic L-amino acids in position 16 (**15**, Leu[16]; **16**, Cha[16]; **17**, Tyr[16]; **19**, Phe[16]) were the most potent in the series.
- When combined, these modifications resulted in compounds equipotent *in vitro* with **1** (i.e. **24**, **27**).
- Some analogues modified in position 11 (e.g. **3**, **4**, **6**) showed decreased selectivity vs hGLP-1 receptor. The selectivity was considerably improved when the L-amino acid residues in this position were replaced with their D-enantiomers.
- The introduction of aromatic D-amino acid residues in position 11 yielded compounds with greatly improved pharmacokinetic profiles in rat as illustrated by their low systemic clearance (CL) values after iv administration (i.e. the 3-Cpa[11] compound **11**).
- Combination of hydrophobic modifications in positions 11 and 16 led to compounds **24**-**29** with low CL values in rat.
- The full length peptides **30-35** were equipotent or more potent *in vitro* than the parent hormone (i.e. analogues **30**, **31** were 2-fold more potent than **1**)
- CL values trended lower in peptides **30-35** as compared to shortened analogues **24-29**.
- The C-terminal acid peptides **30** and **32** had similar pharmacological profiles as their corresponding primary amide compounds **31** and **33**.

Conclusions

- A series of potent and selective GLP-2 analogues modified in position 11 and/or 16 with pharmacokinetic characteristics superior to that of native hormone and/or teduglutide have been discovered.
- A member of this series, compound **31** (FE 203799), is a potent, selective and low CL analogue that has been selected for clinical development as a potential treatment of gastrointestinal diseases and disorders.
- More comprehensive accounts on pharmacological[7] and pharmacokinetic[8] profiles of FE 203799 have been presented elsewhere.

References

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Experimental

General

Amino acid derivatives and resins were purchased from the following suppliers: EMD Biosciences (Novabiochem), Bachem and Peptide International. Other chemicals and solvents were purchased from Sigma-Aldrich, Fisher Scientific and VWR.

Peptide synthesis.

The C-terminal fragments up to position 5 were assembled by Fmoc chemistry using ABI 433A automatic peptide synthesizer on a 0.25 mmol or 0.5 mmol scale. The following side chain protecting groups were used: Trt for Gln and Asn, tBu for Glu and Asp, Pbf for Arg, Boc for Lys and Trp. Single HATU-mediated couplings with 5-fold excess of reagents were employed. Rink amide resin (for primary peptide amides, **2-29**, **31**, **33-35**) or Fmoc-Asp(OtBu)-O-NovaSyn TGT resin, cat# 856126 (for peptide acids, **1**, **30**, **32**) were used. The 1-4 fragment was subsequently added by a manual, DIC/HOBt-mediated coupling of tetrapeptide Boc-His(Trt)-Gly-Asp(OtBu)-Gly-OH to the resin-bound peptides. To prepare hGLP-2 (**1**), tetrapeptide Boc-His(Trt)-Ala-Asp(OtBu)-Gly-OH was used to introduce the N-terminal fragment.

Cleavage, cyclization and purification

All peptides were cleaved with concomitant side chain protecting groups removal using the TFA/H2O/TIS 90/6/4 (v/v/v) cocktail. For methionine-containing reference peptides **1** and **2**, reagent K (TFA/thioanisole/EDT/H2O 88/5/5/2 (v/v/v/v)) was used instead. The peptides were purified by preparative HPLC in triethylammonium phosphate buffer at pH = 2.3 and desalted in a TFA buffer. When necessary, extra purification steps in triethylammonium phosphate buffer at pH = 7.0 and/or in triethylammonium perchlorate buffer at pH = 2.3 were added prior to the desalting. The fractions with purity exceeding 97% were pooled and lyophilized.

In vitro receptor assays

In vitro receptor assays were performed in human embryonic kidney 293 (HEK-293) cells expressing the human GLP-2 or GLP-1 receptors and a luciferase reporter gene under the control of transcriptional regulatory elements responsive to receptor activation. hGLP-2 assays were conducted with transiently transfected cells, hGLP-1 assays were conducted using a stable cell line. To monitor the agonist-induced activity, expression of the luciferase gene was determined after 5-hour incubation with various concentrations of test compounds. Compound potency was expressed as the EC50, the concentration that produced a half maximal response relative to the respective endogenous ligand, calculated by four parameter non-linear regression analysis of concentration-response curves using ActivityBase™ software. The efficacy was expressed in relative terms as % maximal possible effect (MPE) calculated based on the maximal response of the reference agonist included in each assay.

Rat Pharmacokinetics and Bioanalysis

Catheterized male Sprague Dawley rats (~0.3 kg) were used for the PK studies. The jugular vein was used for compound administration and the carotid artery was used for blood sampling. Dosing solutions of compounds **2-35** were prepared in 25 mM phosphate buffer, pH 7.4 with no NaCl and dosing solution of **1** was prepared in 25 mM phosphate buffer, pH 7.4 isotonic with NaCl. Blood was collected at multiple time points up to 5 h post-injection. PK parameters were determined by non-compartmental analysis using PK solutions and WinNonLin software.

The blood samples collected were processed to plasma by centrifugation, then flash frozen and stored at -20ºC. Bioanalysis was conducted using protein precipitation followed by electrospray ionization LC/MS/MS methods (AB Sciex API4000 MS, Shimadzu Prominence HPLC (CBM-20A) and SIL-20ACHT Autosampler). The gradient HPLC method involved reverse-phase column (Phenomenex Jupiter 00B-4053-B0, 50x2.0 mm, 5um, 300Å, C18) and mobile phase (A: 0.01% TFA and 1.0% formic acid in water, B: 0.01% TFA and 1.0% formic acid in 70% CH_3CN) at a flow rate of 0.5 ml/min.

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