Supporting Information for

Design of potent and proteolytically stable oxyntomodulin analogs

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Materials and General Procedures

All peptides were purchased from Cellmano Biotech Co., Ltd., Hefei, China and InnoPep, San Diego, CA. 4,4'-Bis-bromomethyl-biphenyl (Bph) and 3,3'-Bis-bromomethyl-biphenyl (mBph) were purchased from TCI America and used directly in cross-linking reaction. 4,4'-Bis-bromomethyl-bipyridyl (Bpy), *p*-Phenylene-3,3'bis-allylbromide (Alk), Bis-bromomethyl-phenazine (Phe), were synthesized according to published procedure.[12] Cross-linked peptides were purified using a Gilson or Shimadzu semi-preparative reverse-phase HPLC system equipped with a Phenomenex C18 column with a flow rate of 5 mL/min and a gradient of 10-90% ACN/H_2O while monitoring at 220 nm and 254 nm. Analytical HPLC was performed using Phenomenex Luna C18 or Kinetex C18 column (250 × 4.6 mm) with the flow rate set at 1.0 mL/min and UV detection set at 220 and 254 nm. Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system or using an Agilent 6520 accurate-mass quadrupole-time-of-light (Q-TOF) instrument equipped with reverse phase liquid chromatography and an electrospray ionization (ESI) source.

Cross-linking reactions: Cross-linking reactions were carried out by incubating the purified cysteine-containing peptides with 1.5 equivalent of cross-linkers (Bph/Bpy/Alk/Phe/mBph) in a mixed acetonitrile/water (1:4 to 2:3 depending on solubility) containing 30 mM NH_4HCO_3 buffer, pH 8.5, to obtain a final peptide concentration of 1 mM. The mixture was stirred at room temperature for 1.5-2 h. Afterwards the reaction mixture was lyophilized, and the lyophillized powder was washed with diethyl ether to remove excess cross-linker. The residue was dissolved in ACN/H_2O, 0.05% TFA and purified by preparative HPLC.

Generation of CREB responsive luciferase stable cell lines: A CREB responsive luciferase stable HEK 293 cell line overexpressing either human glucagon receptor (GCGR) or glucagon-like peptide 1 receptor (GLP-1R) was generated as follows: HEK293 cells were infected with Lentivirus encoding firefly luciferase gene under control of the CRE promoter (Qiagen, Netherlands) and then were selected using 1 μg/mL puromycin (Life technologies, Carlsbad) for 1 week. The surviving cells (referred to as CRE-HEK293) were expanded and then transfected with a G418 selective mammalian expression plasmid encoding human GCGR or GLP-1R, followed by geneticin selection (Life technologies, Carlsbad, CA). Single-colony stable cell lines expressing both CRE-luciferase and GCGR/GLP-1R were then established for *in vitro* activity assays.

***In vitro* receptor activation reporter assays:** HEK293-GCGR-CRE and HEK293-GLP-1R-CRE cells were seeded in a white 384-well plate at a density of 5,000 cells per well and cultured for 24 hours in DMEM with 10% FBS at 37°C with 5% CO_2. Cells were treated with different peptides in a dose dependent manner. After 24 hours, 10 μL of Bright-Glo reagent (Promega, Madison, WI) was added to each well and luminescence was determined using an Envision multilabel plate reader (PerkinElmer, Waltham, MA). The EC_{50} of each peptide was calculated using GraphPad Prism 6 software (GraphPad, San Diego, CA).

Circular dichroism (CD) measurement: CD spectra were recorded with an AVIV model 202SF CD spectrometer at 25 °C in a 0.2-cm path-length cuvette. The spectra were recorded in the wavelength range 185-250 nm and averaged over 2 scans with a resolution of 0.5 nm, a bandwidth of 1.0 nm and a response time of 4 s. The sensitivity and scan rate of the

spectrophotometer were set to 100 mdeg and 50 nm/min, respectively. All peptides were dissolved in 0.1× PBS to reach final concentrations of 0.2 mg/mL. The mean residue ellipticity was plotted vs wavelength, and the percent helicity of each peptide was calculated based on $[\theta]_{222}/[\theta]_{max}$. $[\theta]_{max}$ was calculated according to the formula: $[\theta]max = -39500 (1-3/n)$ where n is the number of amide bonds.

Molecular Modeling Studies: The coordinates for GLP-1 complexed with GLP-1R was obtained from the PDB Code: 3C589. Glucagon (PDB code: 1GCN) was aligned with GLP-1 and the two L-Cys substitutions were introduced to replace Arg-17 and Gln-24 of Glucagon and the resulting peptide was energy-minimized using the Amber 99 force field in Hyperchem 8 and Bpy cross-linker was constructed and connected to sulfhydryl groups of the cysteines. Then, the cross-linked peptide-GLP-1 complex was subjected to energy minimization in Hyperchem 8 and the geometry optimization was obtained by the molecular mechanics force field method using the Polak–Ribière conjugate gradient algorithm with a root mean square gradient of 0.1 kcal/(Å × mol) as stop criterion before calculating the distance between the pyridyl nitrogen and the Glu-128 of GLP-1R ECD (Figure S4).

***In vivo* pharmacokinetic (PK) studies:** All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of California Institute for Biomedical Research and strictly followed the NIH guidelines for humane treatment of animals. Female CD-1 mice obtained from Charles River Limited were used after overnight food deprivation for *in vivo* PK studies. Peptides were dissolved in pH adjusted Phosphate buffered saline (PBS). 100 µL of each peptide (0.3 mg/kg) was administered to each mouse by either *i.v.* or *s.c* routes. Food was provided to mice immediately after bleeding at 30 minutes. Blood was extracted into heparinized tubes and centrifuged at 3,000x *g* for 15 min. The resulting supernatant plasma was then stored at -80 ºC for peptide concentration determination. Peptide concentration in the plasma was determined by *in vitro* GLP1R activation reporter assay.

PK determination: HEK 293 cells overexpressing GLP1R and CRE-Luc reporter were treated with plasma samples at different time points (5 point dose response, starting from 1:20 dilution of each plasma sample), incubated for 16 hours in DMEM with 10% FBS at 37°C with 5% CO_2,

and the firefly luciferase activity was measured. At the same time, injected peptides were used to obtain standard curves and parameters for Bottom, Top, EC_{50}, Hill Slope. Random luciferase unit (RLU) for each plasma sample was used to calculate the peptide concentrations in plasma (nmol/L), using parameters derived from the standard curve (RLU = Bottom + (Top-Bottom) / (1 + 10^((LogEC_{50}-Conc.)*Hill Slope)). Peptide concentrations in plasma were obtained and plotted against time points to obtain *in vivo* half-life of each peptide, using WinNonLin Phoenix software (Pharsight Corp, St. Louis, MO).

Oral glucose tolerance tests (OGTT): CD1 mice were fasted overnight and then administered a subcutaneous dose of 10ug/mouse of peptide or vehicle control respectively. After 4 hours, mice were orally administered with 3 g of glucose solution per kg body weight and their blood glucose levels were measured before glucose administration (0 min) and after glucose challenge periodically for 2.5hrs.

Table S1. ESI-MS characterization of the cross-linked OXM analogs.

Peptide	Sequence	Mass calculated (Da)	Mass found (Da)
3	HsQGTFTSDYSKYLDSc[1]RAQDFVC[1]WLMNTKRNRNNIA	4548.99	4549.06
6	HsQGTFTSDYSKYLDEC[1]RAQDFVC[1]WLMNTKRNRNNIA	4591.02	4591.11
9	HsQGTFTSDYSKYLDEC[1]AAKEFIC[1]WLMNTKRNRNNIA	4533.01	4533.13
10	HsQGTFTSDYSKYLDEC[1]AVRLFIC[1]WLMNTKRNRNNIA	4574.19	4574.25
11	HsQGTFTSDYSKYLDEC[2]AAKEFIC[2]WLMNTKRNRNNIA[c]	4535.01	4535.10
12	HsQGTFTSDYSKYLDEC[3]AAKEFIC[3]WLMNTKRNRNNIA[c]	4509.12	4509.18
13	HsQGTFTSDYSKYLDEC[4]AAKEFIC[4]WLMNTKRNRNNIA[c]	4560.00	4560.12
14	HsQGTFTSDYSKYLDEC[5]AAKEFIC[5]WLMNTKRNRNNIA[c]	4533.01	4533.17

C^{1-5} denotes cysteine alkylated with cross-linkers (CL-1 to CL-5).

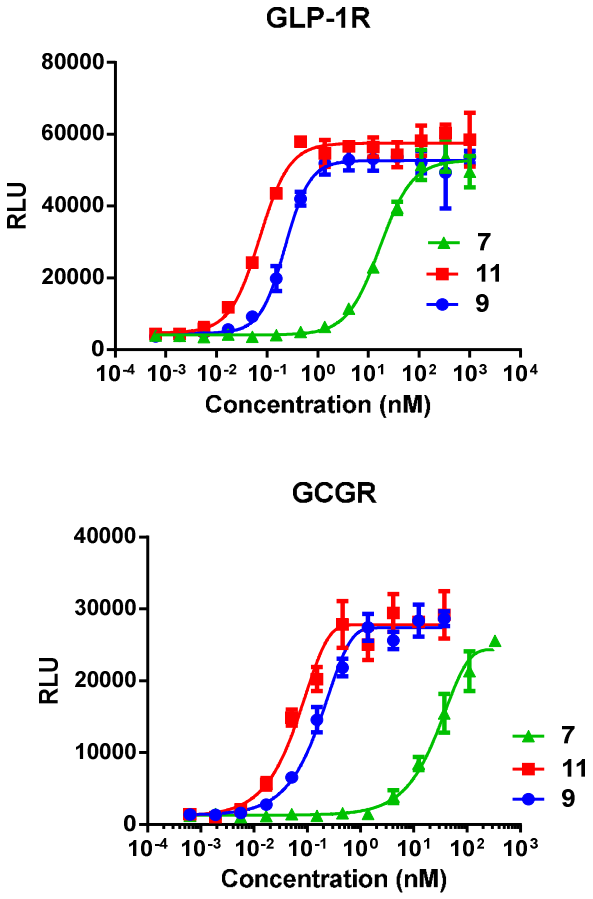


Figure S1. The cross-linked OXM analogs show potent dual-agonist activities. HEK293 cells with GLP1R and GCGR reporters were treated with peptides **7**, **9** and **11** at varying concentrations for 16 h, and the luminescent signals were acquired using the Bright-Glo™ Luciferase Assay System. Assays were performed in triplicate and the dose-response curves were fitted to log-agonist vs. response - variable slope in Prism to generate the EC_{50} values.

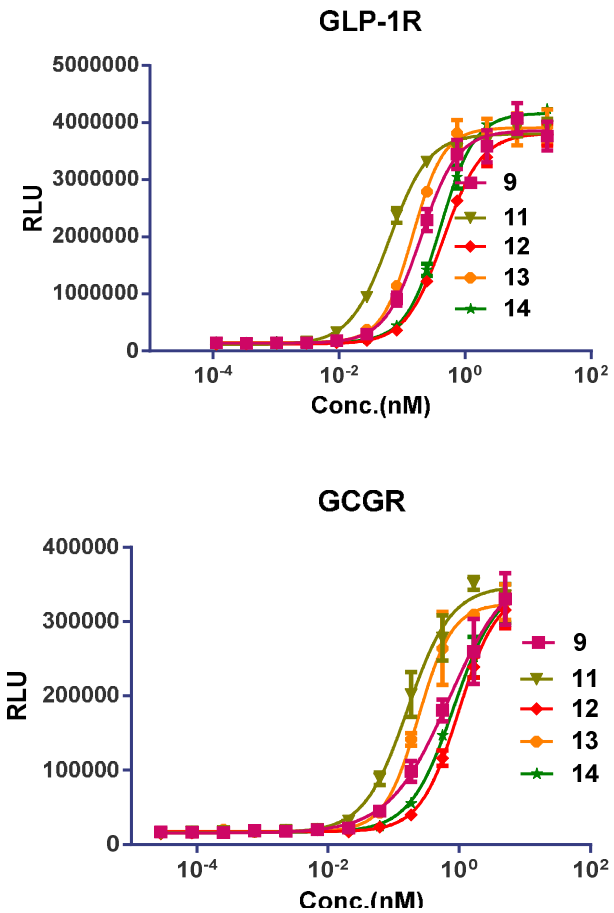
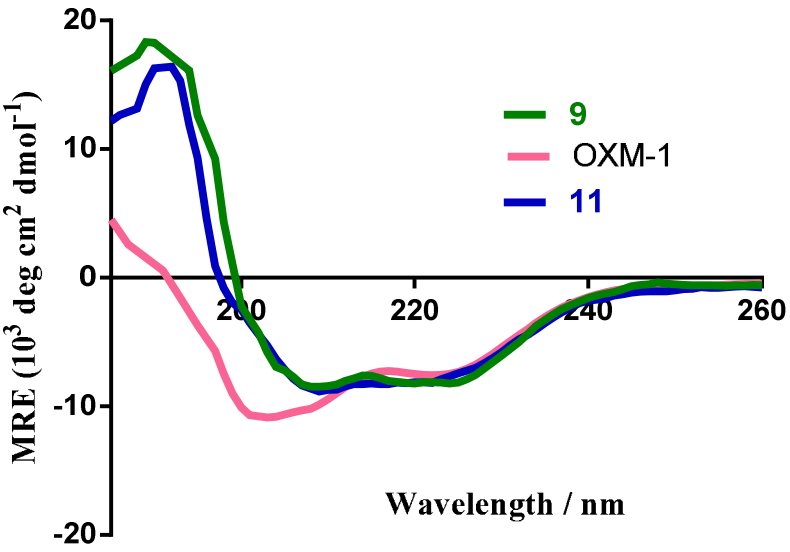


Figure S2. The dual-agonist activities of the cross-linked analogs of peptide **7** in the luciferase based reporter assay.

a



b

	$[\theta]_{222}$	$[\theta]_{208}$	$[\theta]_{222}/[\theta]_{208}$	% helicity
OXM-1	-7.6	-11.1	0.68	20
9	-8.05	-8.45	0.95	22
11	-8.32	-8.31	1.00	23

Figure S3. Circular dichroism analysis of the linear and cross-linked OXM analogs. (a) Far UV CD spectra of the OXM analogs. (b) Table summarizing the absorption minima $[\theta]_{222}$ and $[\theta]_{208}$, the ratios of $[\theta]_{222}/[\theta]_{208}$, and the percent helicities for all peptides. The peptides were dissolved in 0.1X PBS buffer to a final concentration of 50 μM. The percent helicity of each peptide was calculated based on $[\theta]_{222}/[\theta]_{max}$. $[\theta]_{max}$ was calculated according to the formula: $[\theta]_{max} = -39500(1-3/n)$, where n is the number of amide bonds.

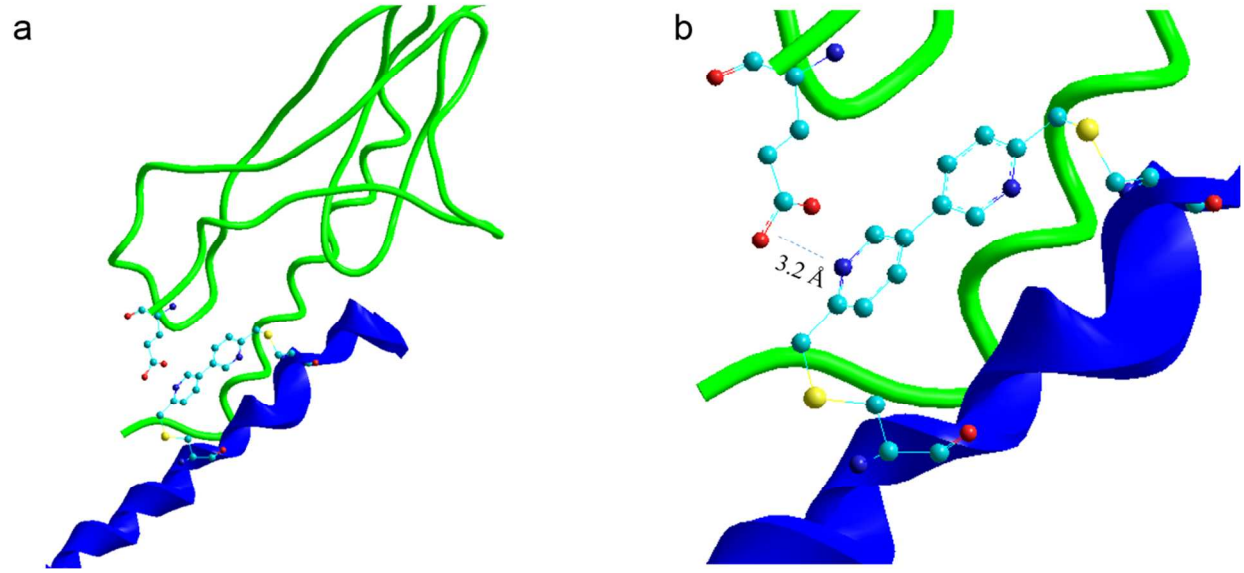


Figure S4. GLP-1R ECD (extracellular domain) is rendered as coil (green color) and the cross-linked OXM peptide is rendered as helical structure (blue color). (a) The binding interface of the OXM/GLP-1R ECD complex model showing a possible hydrogen bond between the pyridyl nitrogen of Bpy with the glutamate 128 of the GLP-1R ECD. The hydrogen bond distance was measured using Hyperchem 8.0. (b) The screenshot of Hyperchem session depicting the energy minimized cross-linked OXM model with GLP-1R.

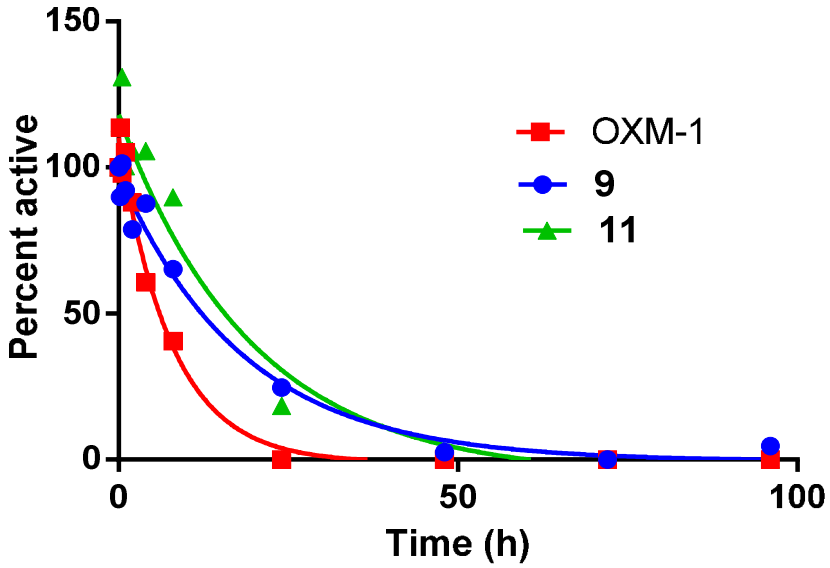


Figure S5. Chemical cross-linking extends OXM half-life *in vitro*. Percent active residual peptides (OXM-1, **9** and **11**) as determined by the GLP-1R activation assay. Aliquots were withdrawn at various times from mouse serum incubated with the peptides. Assay was performed in triplicate.