Supporting Information for

Engineering a long-acting, potent exendin-4 analog for microstructure-based transdermal delivery

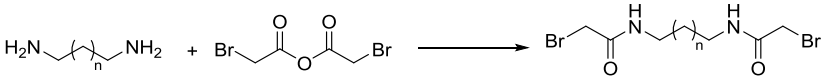
Peng-Yu Yang[1,2], Huafei Zou[2], Elizabeth Chao[2], Lance Sherwood[2], Vanessa Nunez[2], Michael Keeney[3], Esi Ghartey-Tagoe[3], Zhongli Ding[3], Herlinda Quirino[2], Xiaozhou Luo[1], Gus Welzel[2], Guohua Chen[3], Parminder Singh[3], Ashley Woods[2], Peter G. Schultz[1,2], Weijun Shen[2]

[1]Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, United States. [2]Institute for Biomedical Research, 11119 North Torrey Pines Road, Suite 100, La Jolla, California 92037, United States. [3]Corium International, Inc., 235 Constitution Drive, Menlo Park, California 94025, United States

SI Appendix

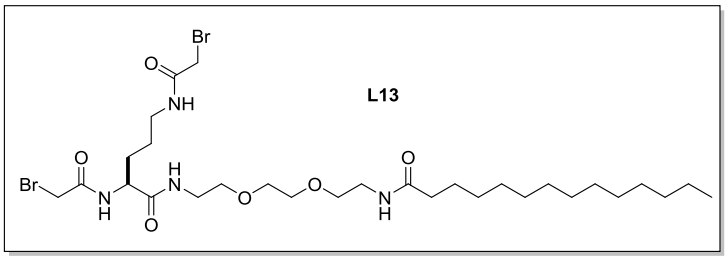
1. Synthesis of cross-linkers

General procedure for non-branched linkers **L1-L12**



To a solution of diamines (1 mmol) in DCM (10 mL) was added bromoacetic anhydride (0.52 g, 2 mmol) and DIEA (0.35 mL, 2 mmol) at -20 ^{o}C. The reaction mixture was stirred for 2 h and the solvent was removed under reduced pressure. The residue was taken up in EtOAc (20 mL) and transferred to a separatory funnel. The organic phase was washed (1 N HCl, brine, sat. $NaHCO_3$, brine), dried (Na_2SO_4), filtered, and concentrated to afford the product as white solids. The products were used in the next steps without further purification.

A first branched cross-linker having the structure



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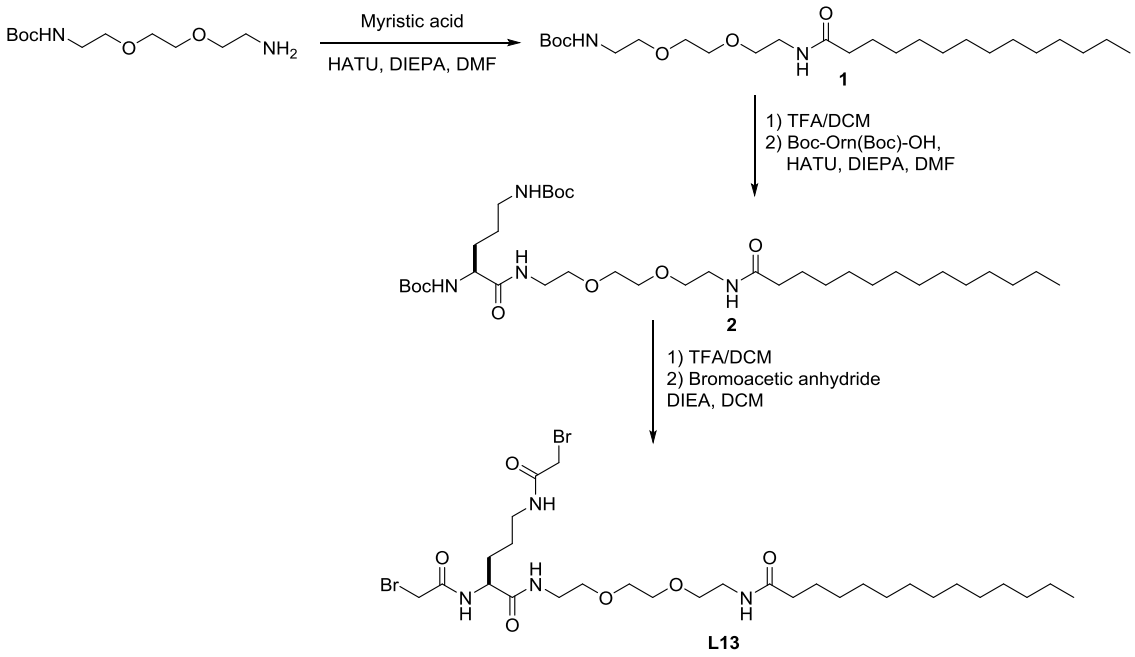


tert-butyl (2-(2-(2-tetradecanamidoethoxy)ethoxy)ethyl)carbamate (**1**).

To a solution of myristic acid (0.46 g, 2 mmol) in 5 mL of DMF was added HATU (0.8 g, 2.1 mmol), DIEPA (0.4 mL, 2.2 mmol) and *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (0.5 g, 2 mmol). The reaction mixture was stirred for 6 h and the solvent was removed under reduced pressure. The residue was taken up in EtOAc (50 mL) and transferred to a separatory funnel. The organic phase was washed (1 N HCl, brine, sat. $NaHCO_3$, brine), dried (Na_2SO_4), filtered, and concentrated to afford **1** as white solids in 90% yield. ESI-MS: calcd MW 458.4; found 459.6 $[M+1]^+$. The product was used in the next step without further purification.

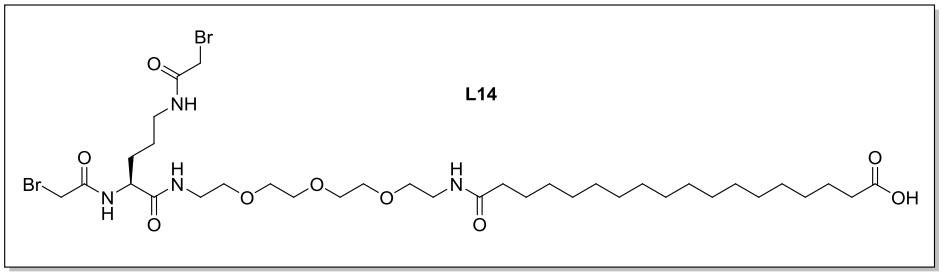
di-*tert*-butyl (5,16-dioxo-9,12-dioxa-6,15-diazanonacosane-1,4-diyl)dicarbamate (**2**).

A solution of **1** (0.46 g, 1 mmol) in DCM (10 mL) was added TFA (5 mL) at 0^{o}C. The reaction mixture was stirred for 2 h and the solvent was removed under reduced pressure. The residue was washed with ether twice (2 x 10 mL) and dried to afford the Boc-deprotected product. The product was used in the next steps without further purification. To a solution of Boc-deprotected product in 10 mL of DMF was added DIEPA (0.4 mL, 2.2 mmol), Boc-Orn(Boc)-OH (0.33 g, 1 mmol) and HATU (0.41 g, 1.1 mmol). The reaction mixture was stirred for 6 h and the solvent was removed under reduced pressure. The residue was taken up in EtOAc (50 mL) and transferred to a separatory funnel. The organic phase was washed (1 N HCl, brine, sat. $NaHCO_3$, brine), dried

(Na_2SO_4), filtered, and concentrated to afford **2** as a white solid in 87% yield. ESI-MS: calcd MW 672.9; found 673.6 $[M+1]^+$. The product was used in the next step without further purification.

(*S*)-*N*,*N'*-(5,16-dioxo-9,12-dioxa-6,15-diazanonacosane-1,4-diyl)bis(2-bromoacetamide) (**L13**).
A solution of **2** (0.33 g, 0.5 mmol) in DCM (10 mL) was added TFA (5 mL) at 0°C. The reaction mixture was stirred for 2 h and the solvent was removed under reduced pressure. The residue was washed with ether twice (2 x 10 mL) and dried to afford the Boc-deprotected product. The product was used in the next steps without further purification. To a solution of Boc-deprotected product in 10 mL of DCM was added DIEPA (0.4 mL, 2.2 mmol) and bromoacetic anhydride (0.26 g, 1 mmol). The reaction mixture was then stirred for 6 h, and the solvent was removed under reduced pressure. The residue was taken up in EtOAc (50 mL) and transferred to a separatory funnel. The organic phase was washed (1 N HCl, brine, sat. $NaHCO_3$, brine), dried (Na_2SO_4), filtered, and concentrated to afford **L13** as white solids in 83% yield. ESI-MS: calcd MW 714.6; found 715.4 $[M+1]^+$. The product was used to prepare the cross-linked peptide **E3** without further purification.

A second branched cross-linker having the structure



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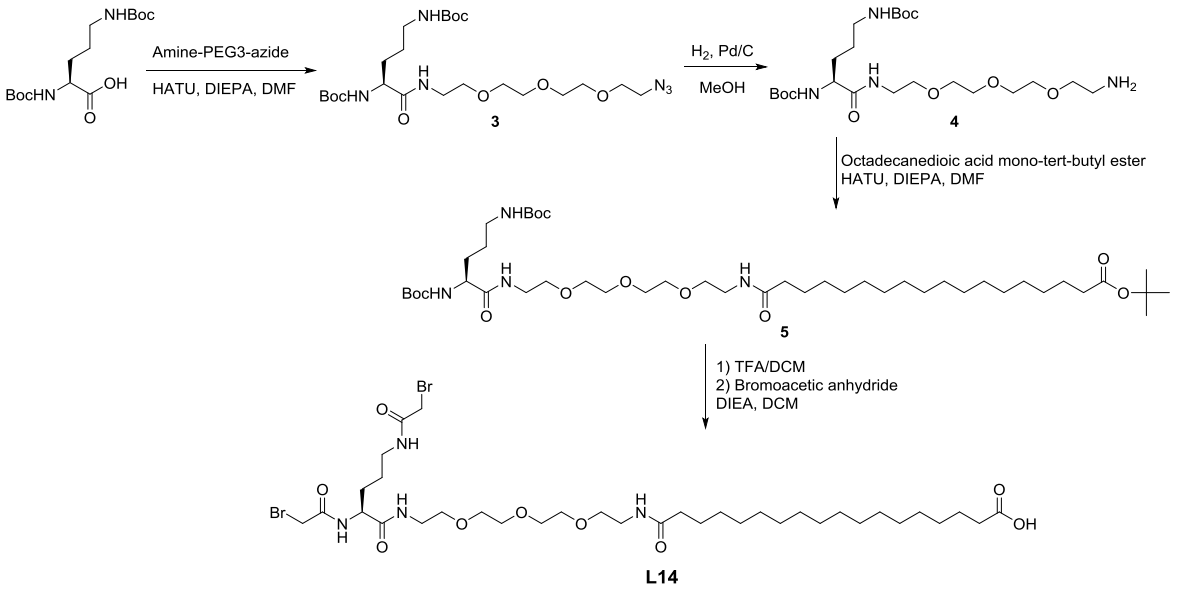


(*S*)-*di*-*tert*-butyl (1-azido-13-oxo-3,6,9-trioxa-12-azaheptadecane-14,17-diyl)dicarbamate (**3**)

To a solution of Boc-Orn(Boc)-OH (1.32 g, 4 mmol) in 10 mL of DMF was added HATU (1.7 g, 4.4 mmol), DIEPA (0.75 mL, 4.4 mmol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine (amine-Peg3-azide, 0.88 g, 4 mmol). The reaction mixture was stirred for 16 h and the solvent was removed under reduced pressure. The residue was taken up in EtOAc (100 mL) and transferred to a separatory funnel. The organic phase was washed (1 N HCl, brine, sat. $NaHCO_3$, brine), dried (Na_2SO_4), filtered, and concentrated to afford **3** as white solids in 75% yield. The product was used in the next step without further purification.

(*S*)-*di*-*tert*-butyl (1-amino-13-oxo-3,6,9-trioxa-12-azaheptadecane-14,17-diyl)dicarbamate (**4**)

To a solution of **3** (1.06 g, 2 mmol) in MeOH (20 mL) was added Pd/C (0.11 g) and a hydrogen balloon (1 atm of H_2). The reaction mixture was stirred for 6 h and filtered through celite twice to remove Pd/C. The solvent was removed under reduced pressure. The crude product was used immediately in the next step without further purification.

(*S*)-*tert*-butyl 9-((tert-butoxycarbonyl)amino)-2,2-dimethyl-4,10,24-trioxo-3,14,17,20-tetraoxa-5,11,23-triazahentetracontan-41-oate (**5**)

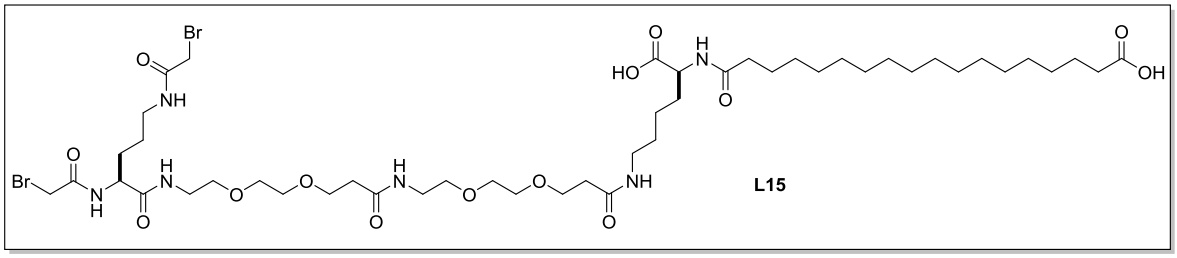
To a solution of above hydrogenated product (0.31 g, 0.6 mmol) in 10 mL of DMF was added octadecanedioic acid mono-tert-butyl ester (0.22 g, 0.6 mmol), HATU (0.23 g, 0.6 mmol) and

DIEPA (0.1 mL, 0.6 mmol). The reaction mixture was stirred for 16 h and the solvent was removed under reduced pressure. The residue was taken up in EtOAc (100 mL) and transferred to a separatory funnel. The organic phase was washed (1 N HCl, brine, sat. $NaHCO_3$, brine), dried (Na_2SO_4), filtered, and concentrated to afford **5** as white solids in 75% yield. The product was used in the next step without further purification.

(*S*)-1-bromo-7-(2-bromoacetamido)-2,8,22-trioxo-12,15,18-trioxa-3,9,21-triazanonatriacontan-39-oic acid (**L14**)

To a solution of **5** (0.26 g, 0.3 mmol) in DCM (10 mL) was added TFA (5 mL) at 0°C. The reaction mixture was stirred for 2 h and the solvent was removed under reduced pressure. The residue was washed with ether twice (2 x 15 mL) and dried to afford the Boc-deprotected product. The product was used in the next steps without further purification. To a solution of Boc-deprotected product in 10 mL of DCM was added DIEPA (0.2 mL, 1.2 mmol) and bromoacetic anhydride (0.16 g, 0.6 mmol). The reaction mixture was then stirred for 6 h, and the solvent was removed under reduced pressure. The product was dissolved in 10 mL of ACN/H_2O/TFA (1:1:0.1) and purified by preparative HPLC column (Phenomenex, Prep C18, 300A, 50 x 250 mm) equilibrated with 40% CH_3CN (0.05% TFA) in water (0.05% TFA). The composition of the eluent then was ramped to 40% CH_3CN-water (0.05%TFA) over 1 min, and a linear gradient was initiated at a rate of 2%/min of CH_3CN (0.05% TFA) into water (0.05% TFA) and run for 25 min. Fractions containing the product were combined and freeze-dried to afford **L14** as white solids in 45% yield over two steps. ESI-MS: calcd MW 844.7; found 845.4 $[M+1]^+$.

A third branched cross-linker having the structure



L15

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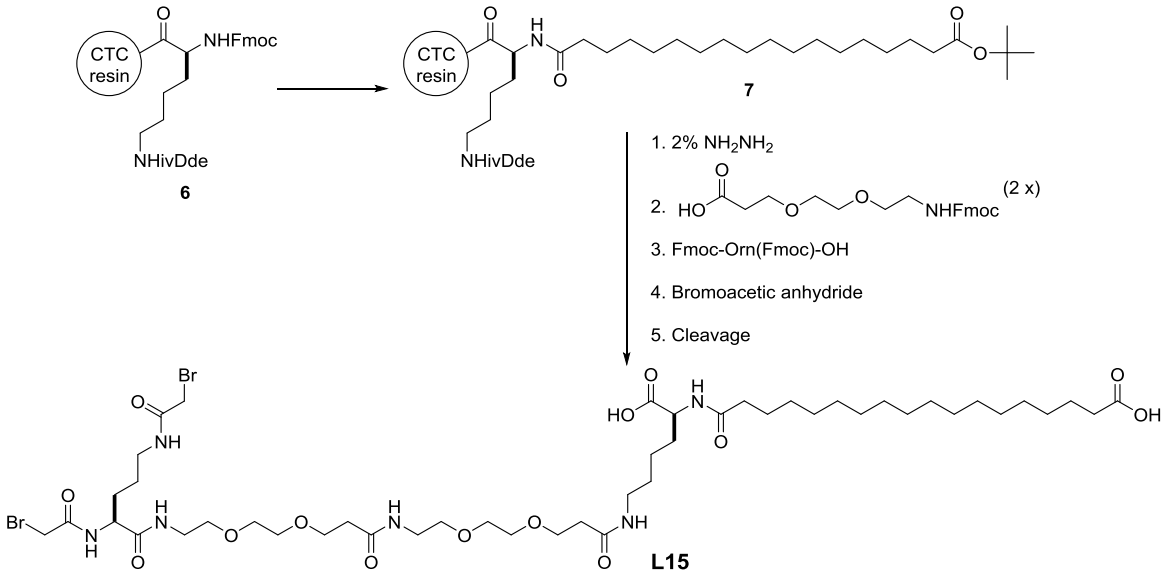


Preparation of Fmoc-Lys(ivDde)-2-chlorotrityl resin (**6**)

2-Chlorotrityl chloride resin (Novabiochem, 100-200 mesh, 1 % DVB, loaded 1.0 mmol/g) (5.0 g, 5 mmol) was swollen in DCM (80 mL) for 1 h and then drained. A solution of Fmoc-Lys(ivDde)-OH (Novabiochem, 1 equiv) was suspended in DMF (30 mL) and DCM (30 mL) and DIPEA (1.1 equiv). This suspension was added to the resin and shaken for 2 h. The resin was drained and washed with DMF (3x), DCM (3x) and treated with CH_3OH/DCM/DIPEA (8:1:1) for 30 min to cap the unreacted trityl chloride sites, washed with DCM (3x), and dried under vacuum, and stored in a desiccator until use.

C18-diacid-Lys(ivDde)-2-chlorotrityl resin (**7**)

Solid-supported **6** (5 mmol) was added a solution of 20% piperidine in DMF (100 mL). The mixture was shaken for 30 min and drained. Another 50 mL of 20% piperidine in DMF was added and the mixture was shaken for another 30 min. Positive ninhydrin test was observed. The resin was then washed as described above for **6**. The resin was then treated with octadecanedioic acid mono-*tert*-butyl ester (AstaTech) (3 equiv, 15 mmol), HATU (3.3 equiv), and DIPEA (3.3 equiv) in DMF (100 mL) for 2 h and repeated until a negative ninhydrin test was observed. The resin was washed with DMF and DCM as described for **6** and used directly in the next step.

(7*S*,34*S*)-1-bromo-7-(2-bromoacetamido)-34-carboxy-2,8,18,28,36-pentaoxo-12,15,22,25-tetraoxa-3,9,19,29,35-pentaazatripentacontan-53-oic acid (**L15**)

Solid-supported **7** was treated twice with 2% hydrazine in DMF (100 mL, 15 min). Positive ninhydrin test was observed. The resin was then washed as described for **6** and treated with Fmoc-PEG2-propionic acid (Quanta BioDesign) (3 equiv) using HATU (3.3 equiv), and DIPEA (3.3 equiv) in DMF (100 mL) for 2 h. The resin was then washed as described above for **6**. Then Fmoc group was removed and washing steps were repeated as described above for **6**. The resin was then treated with Fmoc-Orn(Fmoc)-OH (Novabiochem, 3 equiv), HATU (3.3 equiv), and DIPEA (3.3 equiv) in DMF (100 mL) for 2 h. The resin was again washed as described above for **6**. Then Fmoc group was removed and washing steps were repeated as described above for **6**. The resin was then treated with bromoacetic anhydride (4 equiv), and DIPEA (4.4 equiv) in 200 mL of DCM for 30 min. After washing with DCM (3x), the product was cleaved from the resin using 5 mL of 80% TFA in DCM containing 10% H_2O and 10% triisopropylsilane for 1 h. After cleavage, TFA was removed under reduced pressure. The resulting yellow residue was washed twice with cold diethyl ether and was finally dried to a crude product as yellow powder. The crude peptide was dissolved in DMSO (5 mL) and this solution was diluted to a final volume of 100 mL with 50% CH_3CN-water. The solution was filtered. The filtered solution (10 mL) was loaded onto the preparative HPLC column (Phenomenex, Prep C18, 300A, 50 x 250 mm) equilibrated with 10% CH_3CN (0.05% TFA) in water (0.05% TFA), and the column was eluted with 10% CH_3CN (0.05% TFA) in water (0.05% TFA) to wash DMSO from the column. The composition of the eluent then was ramped to 35% CH_3CN-water (0.05%TFA) over 1 min, and a linear gradient was initiated at a rate of 3%/min of CH_3CN (0.05% TFA) into water (0.05% TFA) and run for 30 min. Eluted fractions were checked for purity on an analytical scale reversed phase C18 column (Phenomenex, C18, 120A, 4.6 x 50 mm) and fractions containing the product in >95% purity were combined and lyophilized to afford **L15** in 31% product yield. The molecular weight of product was analyzed by ESI-MS: calcd MW 1117.0; found 1118.3 $[M+1]^+$, 1119.2 $[M+2]^+$.

2. Peptide cross-linking

The cross-linking reaction was carried out by incubating the dicysteine-containing peptides (2 mM) (Innopep, San Diego, CA; greater than 95% purity) with 1.5 equiv of cross-linkers in a mixed solvent solution (1:3 CH_3CN/30 mM NH_4HCO_3 buffer, pH 8.5). The mixture was stirred at RT for 2-4 hours. Under ice cooling, acetic acid was then added dropwise to pH 5. Crude cross-linked peptide was then purified by preparative HPLC column (Phenomenex, Prep C18, 300A, 50 x 250 mm) with a linear gradient from 10% to 70% acetonitrile/water containing 0.1% trifluoroacetic acid for 30 min at a flow rate of 15 mL/min. The fractions containing the products were collected and lyophilized to afford the products as a powder with >95% purity and in >70% product yield. Their identity was confirmed by ESI-MS and MALDI-TOF. Analytical HPLC was performed using an Agilent 1100 series LC/MS system with a ZORBAX C18 column (5 μm, 150 × 4.6 mm) from Agilent with a linear gradient of 10-70% acetonitrile/water containing 0.05% trifluoroacetic acid for 20 min at a flow rate of 1.0 mL/min with UV-vis detection wavelength set at 220 and 254 nm.

3. Generation of CRE-Luc stable cell line over-expressing GLP-1 receptor

HEK293 cells were infected with lentivirus encoding firefly luciferase gene under the control of cAMP responsive element (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 GLP-1R. In brief, GLP-1R plasmid was transfected into CRE-HEK293 cells using Lipofectamine 2000 and selected with 400 μg/mL geneticin (Life technologies, Carlsbad, CA). Single colony stable cell line over-expressing both CRE-luciferase and GLP-1R (HEK293-GLP-1R-CRE) was then established for in vitro activity assay.

4. In vitro GLP-1 receptor activation reporter assay (receptor-mediated cAMP synthesis)

HEK293-GLP-1R-CRE cells were seeded in 384-well plates at a density of 5000 cells per well and cultured for 18 hours in DMEM with 10% FBS at 37°C and 5% CO_2. Cells were treated with peptides in a dose dependent manner for 24 hours, and receptor activation was reported by luminescence intensities, using One-Glo (Promega, WI) luciferase reagent following

manufacturer's instruction. The EC_{50} of each peptide was determined using GraphPad Prism 6 software (GraphPad, San Diego, CA).

5. Circular dichroism (CD) measurements

CD spectra were recorded with an AVIV model 420SF CD spectrometer at 25 °C in a 2 mm path-length cuvette. The spectra were recorded in the wavelength range of 190-260 nm and averaged over 3 scans with a resolution of 0.5 nm, a bandwidth of 1.0 nm and a response time of 3 s. All peptides were dissolved in water to reach final concentrations of 40 μM. The mean residue ellipticity was plotted vs wavelength.

6. Pharmacokinetics of peptides in mice

Female CD-1 mice (N=4) from Charles River Laboratory were fasted overnight and administrated with 100 μL of each peptide in phosphate buffered saline by intravenous (*i.v.*) or subcutaneous (*s.c.*) route. Food was provided to mice immediately after bleeding at 30 minute. Blood was extracted into heparin tubes and centrifuged at 3,000x g for 15 min. The resulting supernatant plasma were then stored at -80 ^{o}C for peptide concentration determination. The concentrations of peptides in plasma at each time point were determined by in vitro cell based activity assay. Briefly, HEK293-GLP-1R-CRE cells were treated with plasma samples at different time points (5 point dose response, starting from 1:10 to 1:100 dilution of each plasma sample) and incubated for 16 hours in DMEM with 10% FBS at 37°C with 5% CO_2, and the firefly luciferase activity was then measured. At the same time, same peptides were used to obtain standard curves and parameters for Bottom, Top, EC_{50}, and 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).

7. Oral glucose tolerance test (OGTT) and intraperitoneal glucose tolerance test (IPGTT)

Female Charles River CD-1 mice were fasted overnight and then administrated with 100 μl of each peptide in PBS (pH = 7.3) by *i.v.* or *s.c.* route. After 6 hours, mice were orally or intraperitoneally administrated with 2 g of glucose solution per kg body weight and their tail blood glucose levels were measured before (0 min) and after glucose challenge for 2 to 3 hours.

8. Body weight, food intake, and visceral fat mass measurement

Charles River Diet Induced Obese (DIO) mice were administrated by daily subcutaneous injections of peptides. Mouse body weight and food intake were monitored daily throughout the study. At the end of the experiment, mice were sacrificed and visceral fat mass were taken out and weighed.

9. PPARγ mRNA level measurement

Total RNA from mouse white adipose tissue was extracted with TRIZOL (Life technology, Carlsbad, CA) and cDNA was synthesized using high-capacity cDNA reverse transcription kit (ThermoFisher scientific, Pittsburgh, PA).Quantitative RT-PCR reactions were conducted and analyzed on a ViiA 7 Real-time PCR System (ThermoFisher scientific, Pittsburgh, PA). Endogenous PPARγ and was determined using the SYBR qPCR premix (Clontech,Mountain View, CA). GAPDH was also measured as the standard gene.

10. Cholesterol level determination

Collected plasma was used for cholesterol level determination according to the manufacturer's guide (cholesterol assay kit, Abcam, Cambridge, England). Briefly, plasma was diluted using cholesterol assay buffer and then reacted with the same volume of reaction mix containing cholesterol assay buffer, cholesterol probe, enzyme mix and cholesterol esterase. After incubation at 37 ^{o}C for 1 hour, the absorbance at 560 nm was measured using an Envision multilabel plate reader (PerkinElmer, Waltham, MA). Subsequently, the concentration of cholesterol in plasma was calculated according to a standard curve.

11. Triglyceride level measurement

Collected plasma was used for triglyceride level determination using a triglyceride colorimetric assay kit (Cayman chemical, Ann Arbor, Michigan). 5μL of plasma samples or standard were plated into a 384 well plate and followed by adding 75 μL of diluted enzyme buffer to each well. The mixture was incubated at room temperature for 15 min, and the absorbance was read at 560 nm using an Envision plate reader (PerkinElmer, Waltham, MA). The concentration of triglyceride in plasma was calculated using a standard curve.

12. Oil red O staining (Lipid droplet staining)

Frozen tissue sections of liver were cut at 10μm and air dried onto the slides. After fixation in 10% formalin for 5 min, the slides were briefly washed with running tap water for 10 min, followed by rinse with 60% isopropanol. Subsequently, oil red O working solution (0.3% oil red O) was used to stain lipid for 15 min. Slides were again rinsed with 60% isopropanol and then nuclei were lightly stained with alum haematoxylin, followed by rinse with distilled water and mounted in glycerin jelly. Pictures were taken under microscopy.

SI Appendix Tables

Table S1. Structure of synthetic cross-liners used in this study

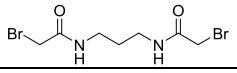
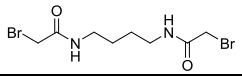
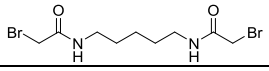
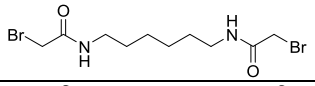
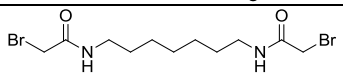
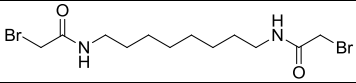
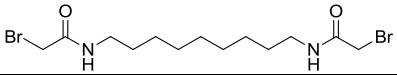
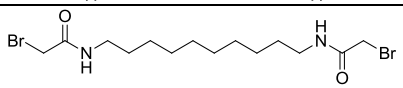
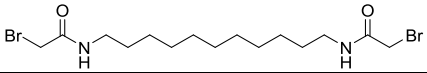
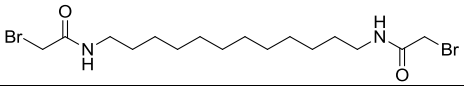
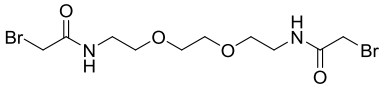
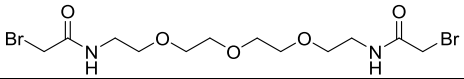
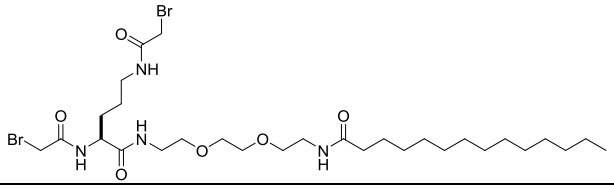
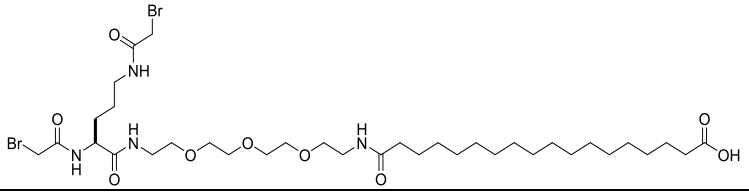
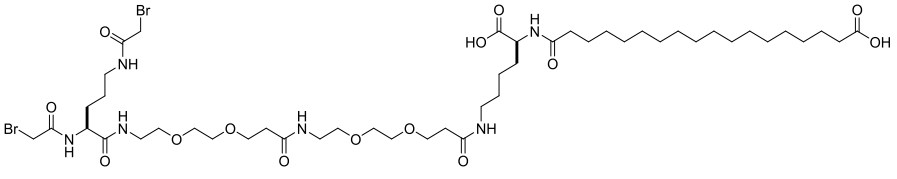
Entry	Name	Structure
1	**L1**	
2	**L2**	
3	**L3**	
4	**L4**	
5	**L5**	
6	**L6**	
7	**L7**	
8	**L8**	
9	**L9**	
10	**L10**	
11	**L11**	
12	**L12**	
14	**L13**	
15	**L14**	
16	**L15**	

Table S2. Characterization of the cross-linked exendin-4 analogs

Entry	Peptide	Mass expected	Mass found	GLP-1R (EC_{50}, pM)	Half-life (hours) *i.v., s.c.*
1.	**Exendin-4**	4186.6	1047.6 ($[M+4H]^{4+}$), 838.2 ($[M+5H]^{5+}$)	16	$t_{1/2}$ = 0.5 h (*i.v.*)
2.	**SEQ-1-L1**	4288.9	1073.2 ($[M+4H]^{4+}$), 858.8 ($[M+5H]^{5+}$)	28	ND
3.	**SEQ-1-L2**	4302.9	1076.2 ($[M+4H]^{4+}$), 861.4 ($[M+5H]^{5+}$)	18	$t_{1/2}$ = 0.8 h (*i.v.*)
4.	**SEQ-1-L3**	4316.9	1080.2 ($[M+4H]^{4+}$), 864.4 ($[M+5H]^{5+}$)	24	ND
5.	**SEQ-1-L13**	4687.5	1172.8 ($[M+4H]^{4+}$), 938.4 ($[M+5H]^{5+}$)	20	ND
6.	**SEQ-1-L14**	4817.6	1205.3 ($[M+4H]^{4+}$), 964.4 ($[M+5H]^{5+}$)	19	$t_{1/2}$ = 5.2 h (*i.v.*), C_{max} = 4h (*s.c.*)
7.	**SEQ-1-L15**	5089.9	1273.2 ($[M+4H]^{4+}$), 1018.7 ($[M+5H]^{5+}$)	16	$t_{1/2}$ = 13.8 h (*i.v.*), C_{max} = 4h (*s.c.*)
8.	**SEQ-2-L4**	4331.5	1444.7 ($[M+3H]^{3+}$), 1083.8 ($[M+4H]^{4+}$)	31	ND
9.	**SEQ-2-L5**	4345.5	1449.5 ($[M+3H]^{3+}$), 1087.4 ($[M+4H]^{4+}$)	22	ND
10.	**SEQ-2-L6**	4359.5	1454.1 ($[M+3H]^{3+}$), 1090.9 ($[M+4H]^{4+}$)	49	ND
11.	**SEQ-2-L7**	4373.5	1458.9 ($[M+3H]^{3+}$), 1094.4 ($[M+4H]^{4+}$)	43	ND
12.	**SEQ-2-L11**	4353.0	1452.0 ($[M+3H]^{3+}$), 1089.2 ($[M+4H]^{4+}$)	620	ND
13.	**SEQ-3-L8**	4403.2,	1101.8 ($[M+4H]^{4+}$), 881.6 ($[M+5H]^{5+}$)	530	ND
14.	**SEQ-3-L9**	4417.2	1105.4 ($[M+4H]^{4+}$), 884.4 ($[M+5H]^{5+}$)	150	ND
15.	**SEQ-3-L10**	4431.2	1108.8 ($[M+4H]^{4+}$), 887.2 ($[M+5H]^{5+}$)	340	ND
16.	**SEQ-3-L12**	4423.1	1106.8 ($[M+4H]^{4+}$), 885.6 ($[M+5H]^{5+}$)	850	ND

Table S3. *In vitro* functional profile of **E6** against 168 different GPCRs.[a]

Receptor	Control Ligand	Replicate 1 (RLU)	Replicate 2 (RLU)	Mean RLU	SD	%CV	% Activity of Control
ADCYAP1R1	PACAP-27	155600	159600	157600	2828	2%	3%
ADORA3	2-Cl-IB-MECA	157000	150800	153900	4384	3%	0%
ADRA1B	Phenylephrine	324800	304000	314400	14708	5%	0%
ADRA2A	UK 14,304	415600	409000	412300	4667	1%	0%
ADRA2B	UK 14,304	210400	203600	207000	4808	2%	-1%
ADRA2C	UK 14,304	331800	327600	329700	2970	1%	-1%
ADRB1	Isoproterenol	171000	180400	175700	6647	4%	2%
ADRB2	Isoproterenol	32600	34800	33700	1556	5%	0%
AGTR1	Angiotensin II	544600	545800	545200	849	0%	-1%
AGTRL1	Apelin-13	412600	425400	419000	9051	2%	1%
AVPR1A	Vasopressin	29800	27000	28400	1980	7%	0%
AVPR1B	Vasopressin	34200	32600	33400	1131	3%	0%
AVPR2	Vasopressin	654400	697400	675900	30406	4%	1%
BDKRB1	LDA-Bradykinin	25800	28800	27300	2121	8%	0%
BDKRB2	Bradykinin	510800	535600	523200	17536	3%	1%
BRS3	TAPN-Bombesin	195400	195400	195400	0	0%	3%
C3AR1	C3A Receptor Agonist (Short Fragment)	72600	64800	68700	5515	8%	1%
C5AR1	Complement C5a	152600	146400	149500	4384	3%	1%
C5L2	Complement C5a	787600	785600	786600	1414	0%	-3%
CALCR	Calcitonin	46600	50200	48400	2546	5%	-2%
CALCRL-RAMP1	beta CGRP	74400	69400	71900	3536	5%	1%
CALCRL-RAMP2	Adrenomedullin	242000	222600	232300	13718	6%	-1%
CALCRL-RAMP3	Adrenomedullin	552400	535200	543800	12162	2%	3%
CALCR-RAMP2	Calcitonin	132000	133400	132700	990	1%	3%
CALCR-RAMP3	Calcitonin	52600	47800	50200	3394	7%	-1%
CCKAR	CCK-8	21400	21200	21300	141	1%	0%
CCKBR	CCK-8	763800	759000	761400	3394	0%	2%
CCR1	CCL27	702400	743600	723000	29133	4%	-3%
CCR10	CCL3	70400	66000	68200	3111	5%	-1%
CCR2	CCL2	213400	183800	198600	20930	11%	4%
CCR3	CCL13	142800	150600	146700	5515	4%	4%
CCR4	CCL22	227000	223000	225000	2828	1%	4%
CCR5	CCL3	63200	67400	65300	2970	5%	1%
CCR6	CCL20	48800	57000	52900	5798	11%	0%
CCR7	CCL19	454600	465200	459900	7495	2%	0%
CCR8	CCL1	17600	19000	18300	990	5%	0%
CCR9	CCL25	139000	128800	133900	7212	5%	1%
CHRM1	Acetylcholine	173400	185200	179300	8344	5%	0%
CHRM2	Acetylcholine	37600	44200	40900	4667	11%	0%
CHRM3	Acetylcholine	33400	34600	34000	849	2%	1%
CHRM4	Acetylcholine	1176200	1081200	1128700	67175	6%	2%
CHRM5	Acetylcholine	1067600	1040800	1054200	18950	2%	2%
CMKLR1	Chemerin	70200	66200	68200	2828	4%	0%
CNR1	CP55940	98200	93200	95700	3536	4%	2%
CNR2	CP55940	449000	385400	417200	44972	11%	10%
CRHR1	Sauvagine	326000	285800	305900	28426	9%	1%
CRHR2	Sauvagine	113000	107200	110100	4101	4%	0%
CRTH2	PGD2	95000	98400	96700	2404	2%	-2%
CX3CR1	Fractalkine	307400	325200	316300	12587	4%	0%
CXCR1	CXCL8	248200	212400	230300	25314	11%	1%
CXCR2	CXCL8	133600	117600	125600	11314	9%	2%
CXCR3	CXCL11	243800	253800	248800	7071	3%	-1%
CXCR4	CXCL12	152600	139400	146000	9334	6%	3%
CXCR5	CXCL13	790200	815400	802800	17819	2%	0%
CXCR6	CXCL16	47600	50400	49000	1980	4%	4%
CXCR7	CXCL12	347800	374800	361300	19092	5%	0%
DRD1	Dopamine	54600	52600	53600	1414	3%	-1%
DRD2L	Dopamine	85800	88200	87000	1697	2%	0%
DRD2S	Dopamine	197400	185400	191400	8485	4%	3%
DRD3	Dopamine	792800	774400	783600	13011	2%	0%
DRD4	Dopamine	19400	17600	18500	1273	7%	3%
DRD5	Dopamine	52200	45400	48800	4808	10%	4%
EBI2	7a,25-dihydroxycholesterol	144000	136600	140300	5233	4%	1%

EDG1	S-1-P	184000	170600	177300	9475	5%	1%
EDG3	S-1-P	775400	697600	736500	55013	7%	4%
EDG4	Oleoyl LPA	160400	148000	154200	8768	6%	-1%
EDG5	S-1-P	191400	193400	192400	1414	1%	0%
EDG6	S-1-P	935400	971800	953600	25739	3%	-4%
EDG7	Oleoyl LPA	80800	64600	72700	11455	16%	-1%
EDNRA	Endothelin I	26800	29400	28100	1838	7%	0%
EDNRB	Endothelin 3	61200	70600	65900	6647	10%	1%
F2R	TFLLR-NH2	163200	183600	173400	14425	8%	0%
F2RL1	SLIGRL-NH2	574200	517200	545700	40305	7%	1%
F2RL3	AYPGKF-NH2	687000	748200	717600	43275	6%	0%
FFAR1	GW9508	405000	356800	380900	34083	9%	-4%
FPR1	WKYMVm-NH2	1009600	943200	976400	46952	5%	4%
FPRL1	WKYMVm-NH2	72600	72000	72300	424	1%	0%
FSHR	FSH	181000	171800	176400	6505	4%	4%
GALR1	Galanin	307800	313200	310500	3818	1%	1%
GALR2	Galanin	442400	415200	428800	19233	4%	0%
GCGR	Glucagon	673400	672000	672700	990	0%	4%
GHSR	Ghrelin	249600	252400	251000	1980	1%	1%
GIPR	GIP	51400	46600	49000	3394	7%	6%
GLP1R	Exendin-4	2439200	2477200	2458200	26870	1%	95%
GLP2R	GLP II (1-33)	335600	318600	327100	12021	4%	14%
GPR1	Chemerin	37400	34800	36100	1838	5%	0%
GPR103	QRFP-26	92800	85600	89200	5091	6%	1%
GPR109A	Nicotinic Acid	667400	666600	667000	566	0%	-4%
GPR109B	3-Hydroxyoctanoic acid	1032000	1044200	1038100	8627	1%	-1%
GPR119	Oleoyl Ethanolamide	335200	334000	334600	849	0%	3%
GPR120	GW9508	25600	27200	26400	1131	4%	4%
GPR35	Zaprinast	386800	394600	390700	5515	1%	3%
GPR92	Oleoyl LPA	186800	163200	175000	16688	10%	-3%
GRPR	GRP	48400	49000	48700	424	1%	0%
HCRTR1	Orexin A	37200	41400	39300	2970	8%	0%
HCRTR2	Orexin A	62400	62600	62500	141	0%	0%
HRH1	Histamine	216800	206200	211500	7495	4%	0%
HRH2	Histamine	82600	79000	80800	2546	3%	4%
HRH3	R-a methylhistamine	76800	82800	79800	4243	5%	2%
HRH4	Histamine	635400	620600	628000	10465	2%	-1%
HTR1A	Serotonin / 5-HT	606400	603200	604800	2263	0%	2%
HTR1B	Serotonin / 5-HT	1901200	1860200	1880700	28991	2%	6%
HTR1E	Serotonin / 5-HT	118200	123800	121000	3960	3%	8%
HTR1F	Serotonin / 5-HT	326600	327400	327000	566	0%	3%
HTR2A	Serotonin / 5-HT	300200	299800	300000	283	0%	3%
HTR2C	Serotonin / 5-HT	419800	438600	429200	13294	3%	1%
HTR5A	Serotonin / 5-HT	566600	530400	548500	25597	5%	0%
KISS1R	Kisspeptin-10	47000	46000	46500	707	2%	-1%
LHCGR	hCG	101800	90600	96200	7920	8%	-2%
LTB4R	Leukotriene B4	131000	154400	142700	16546	12%	0%
MC1R	Melanotan II	11600	15800	13700	2970	22%	-3%
MC3R	Melanotan II	99000	85600	92300	9475	10%	-1%
MC4R	Melanotan II	28400	32800	30600	3111	10%	2%
MC5R	Melanotan II	116000	112000	114000	2828	2%	-2%
MCHR1	MCH	36600	38400	37500	1273	3%	0%
MCHR2	MCH	71400	71600	71500	141	0%	0%
MLNR	Motilin	224800	235200	230000	7354	3%	1%
MRGPRX1	BAM(8-22)	718000	676400	697200	29416	4%	0%
MRGPRX2	Cortistatin 14	298000	323400	310700	17961	6%	2%
MTNR1A	2-Iodomelatonin	71400	67600	69500	2687	4%	-4%
NMBR	Neuromedin B	85400	72800	79100	8910	11%	1%
NMU1R	Neuromedin U-25	96800	94000	95400	1980	2%	2%
NPBWR1	Neuropeptide W23	138800	119800	129300	13435	10%	1%
NPBWR2	Neuropeptide W23	188600	179800	184200	6223	3%	3%
NPFFR1	RFRP-3	148200	146400	147300	1273	1%	0%
NPSR1B	Neuropeptide S	55400	49200	52300	4384	8%	1%
NPY1R	Peptide YY	36400	32800	34600	2546	7%	0%
NPY2R	Peptide YY	217600	253000	235300	25032	11%	1%
NTSR1	[Lys 8,9] Neurotensin	397600	396000	396800	1131	0%	-2%

OPRD1	DADLE	109400	103800	106600	3960	4%	0%
OPRK1	Dynorphin A	20000	19600	19800	283	1%	0%
OPRL1	Orphanin FQ	164400	162600	163500	1273	1%	1%
OPRM1	[Met] Enkephalin	97400	100800	99100	2404	2%	0%
OXER1	5-OxoETE	176400	175000	175700	990	1%	0%
OXTR	Oxytocin	27000	28800	27900	1273	5%	0%
P2RY1	2-methylthio-ADP	97200	99000	98100	1273	1%	-2%
P2RY11	ATP	63400	64200	63800	566	1%	0%
P2RY12	2-methylthio-ADP	210000	212200	211100	1556	1%	2%
P2RY2	UTP	343800	356800	350300	9192	3%	-2%
P2RY4	UTP	168600	165600	167100	2121	1%	2%
P2RY6	UTP	421800	412600	417200	6505	2%	1%
PPYR1	Pancreatic Polypeptide	48600	49800	49200	849	2%	1%
PRLHR	PrRP-31	56400	59000	57700	1838	3%	0%
PROKR1	EG VEGF	19200	25000	22100	4101	19%	1%
PROKR2	EG VEGF	47800	39800	43800	5657	13%	1%
PTAFR	PAF	722800	689200	706000	23759	3%	0%
PTGER2	Prostaglandin E2	24000	25200	24600	849	3%	0%
PTGER3	Prostaglandin E2	398600	402600	400600	2828	1%	0%
PTGER4	Prostaglandin E2	186800	183200	185000	2546	1%	3%
PTGFR	Cloprostenol	11400	11800	11600	283	2%	0%
PTGIR	Beraprost	289200	299800	294500	7495	3%	2%
PTHR1	PTH(1-34)	125600	114800	120200	7637	6%	0%
PTHR2	TIP-39	115000	118800	116900	2687	2%	1%
RXFP3	Relaxin-3	93200	84800	89000	5940	7%	-3%
SCTR	Secretin	607400	623400	615400	11314	2%	5%
SSTR1	Somatostatin 28	50000	49000	49500	707	1%	-4%
SSTR2	Somatostatin 28	13000	9000	11000	2828	26%	0%
SSTR3	Tyr-SST 14	72200	63200	67700	6364	9%	1%
SSTR5	Somatostatin 28	298000	310800	304400	9051	3%	-1%
TACR1	Substance P	457000	471000	464000	9899	2%	-1%
TACR2	Substance P	362000	380200	371100	12869	3%	1%
TACR3	Substance P	133400	140600	137000	5091	4%	0%
TBXA2R	I-BOP	175000	181000	178000	4243	2%	-3%
TRHR	TRH	17200	15600	16400	1131	7%	0%
TSHR(L)	TSH	10000	7800	8900	1556	17%	-2%
UTR2	Urotensin II	25400	26200	25800	566	2%	-2%
VIPR1	VIP	277800	248200	263000	20930	8%	1%
VIPR2	VIP	366600	327000	346800	28001	8%	0%

[a] E6 was screened against 168 GPCRs in a panel of function cell-based assays (gpcrMAX℠, DiscoveRx). Results are expressed as % activity of control, calculated by [100 − (E6 agonist activity / control agonist activity) x 100]. E6 was tested in duplicate at a concentration of 1 μM and the control ligands were tested in duplicate with 10 concentrations.

SI Appendix Figures

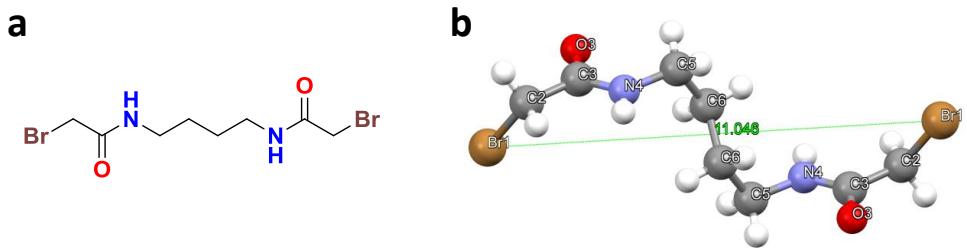


Figure S1. Chemical structure (**a**) and crystal structure (**b**) of the cross-linker L2. The green line indicates the Br-Br distance of 11.05 Å.

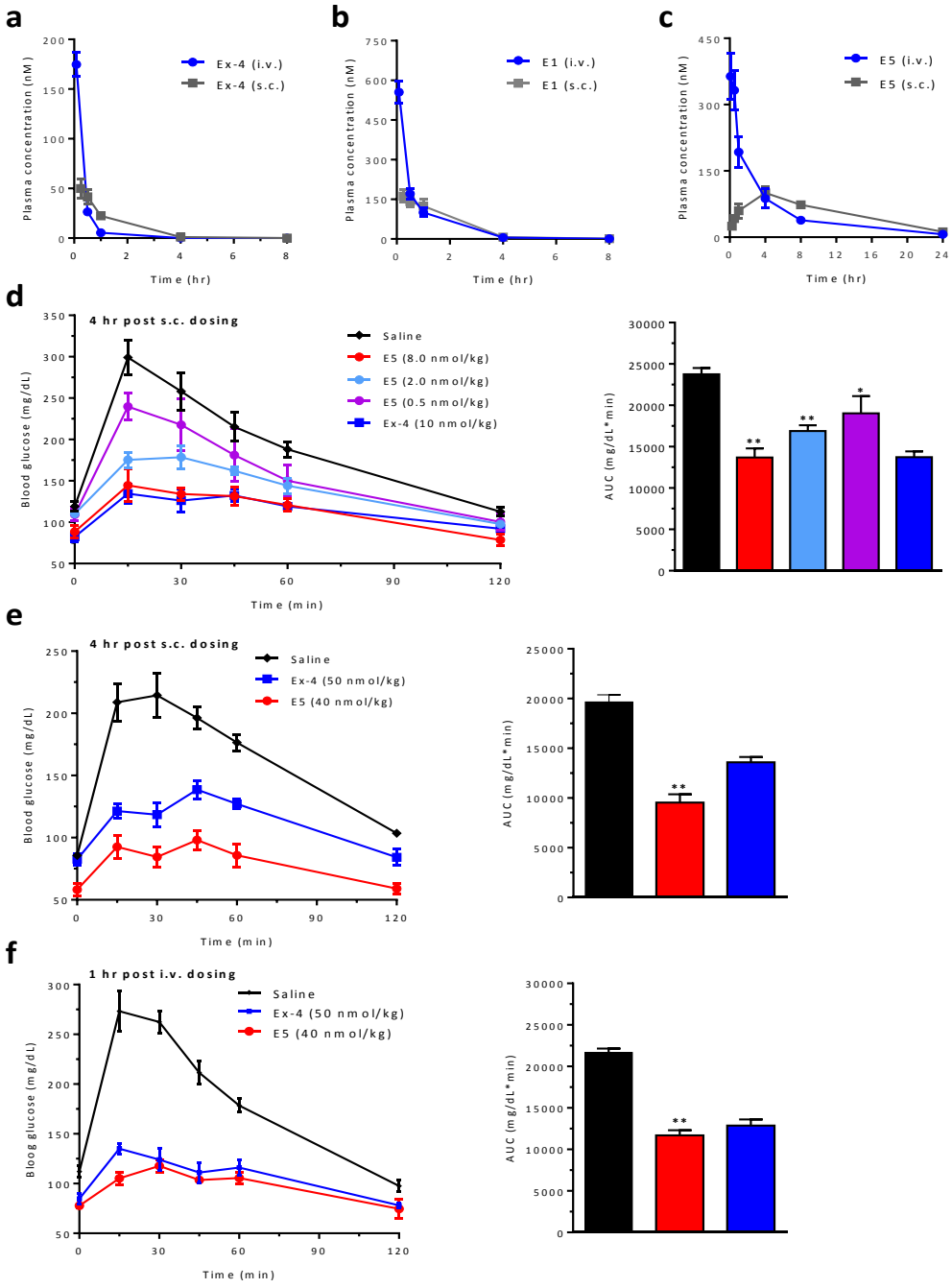


Figure S2. Effects of cross-linking on pharmacokinetics and OGTT in CD1 mice. Plasma concentrations of Ex-4 (10 nmol/kg) (**a**), E1 (7.5 nmol/kg) (**b**), E5 (7.5 nmol/kg) (**c**) in mice (n = 4 per group) after intravenous or subcutaneous administration. The peptide concentrations in plasma at various time points were determined by *in vitro* GLP-1R activity assay. Assay was performed in triplicate. (**d–f**) Plasma glucose excursion during an oral glucose tolerance test (OGTT) in mice (n = 5 per group). Mice were subcutaneously injected with vehicle, E5, or Ex-4 for 4 hours in a dose-dependent manner (**d**,**e**) or intravenously injected for 1 hour (**f**) prior to the glucose challenge. Bar graph showing the total amount of glucose in the mice obtained by measuring the area under curve (AUC). *P < 0.05, **P < 0.01, E6 versus vehicle.

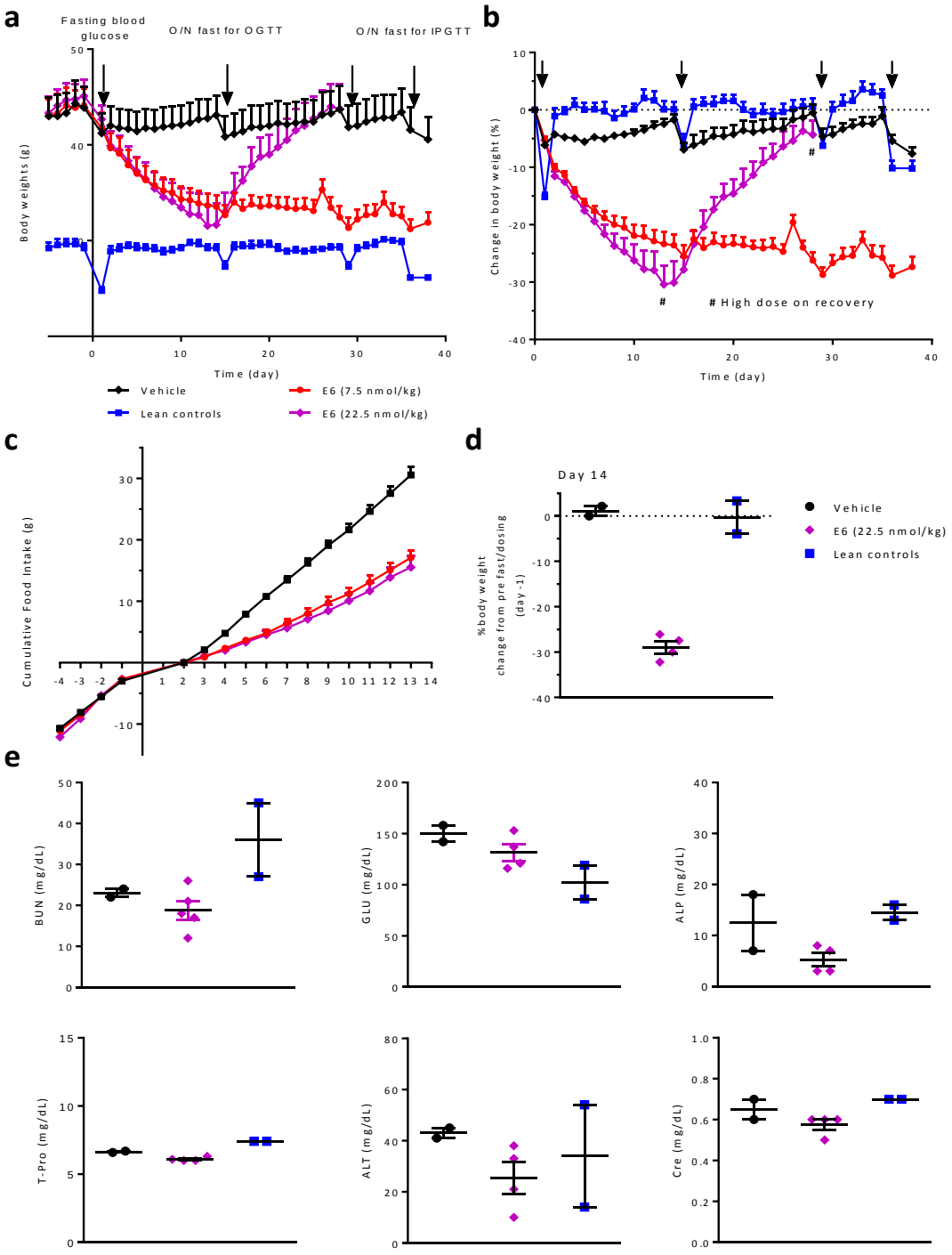
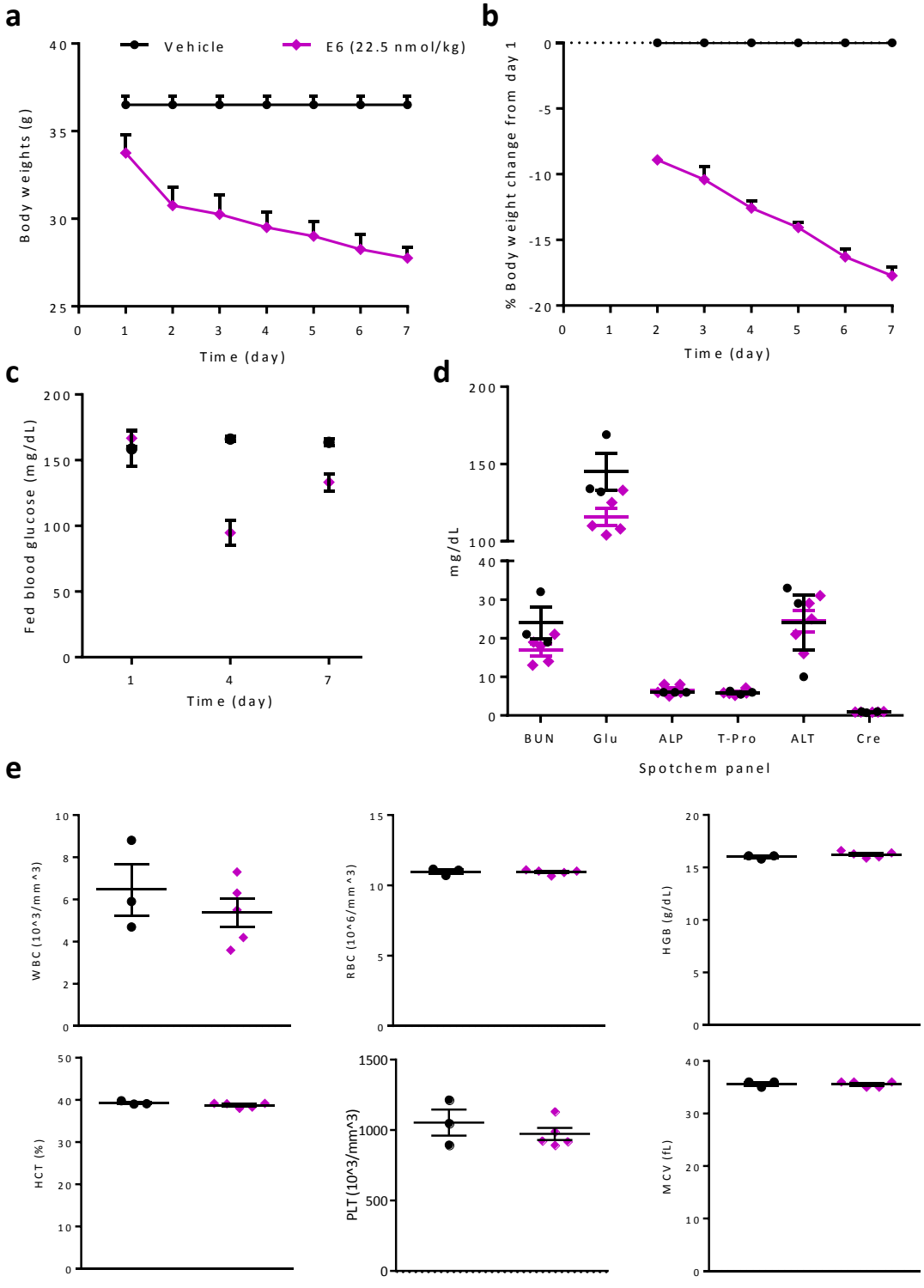


Figure S3. High-dose reversal study after E6 withdrawal in diet-induced obese (DIO) mice. Effects on (**a**–**b**) body weight regain after treatment cessation, cumulative food intake (**c**), body weight change on day 14 (**d**), (**e**) Effect of E6 on liver function and kidney function on day 14. Data are presented as mean ± SD (n = 4). BUN = blood urea nitrogen; GLU = glucose, ALP = alanine phosphatase, T-Pro = total protein, ALT = alanine aminotransferase; Cre = Creatinine. All DIO mice were treated by daily subcutaneous injections at a dose of 22.5 nmol/kg or vehicle. Lean mice were fed control.


a
Vehicle
E6 (22.5 nmol/kg)
Body weights (g)
Time (day)
b
% Body weight change from day 1
Time (day)
c
Fed blood glucose (mg/dL)
Time (day)
d
mg/dL
BUN
Glu
ALP
T-Pro
ALT
Cre
Spotchem panel
e
WBC (10^3/mm^3)
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HGB (g/dL)
HCT (%)
PLT (10^3/mm^3)
MCV (fL)

Continued

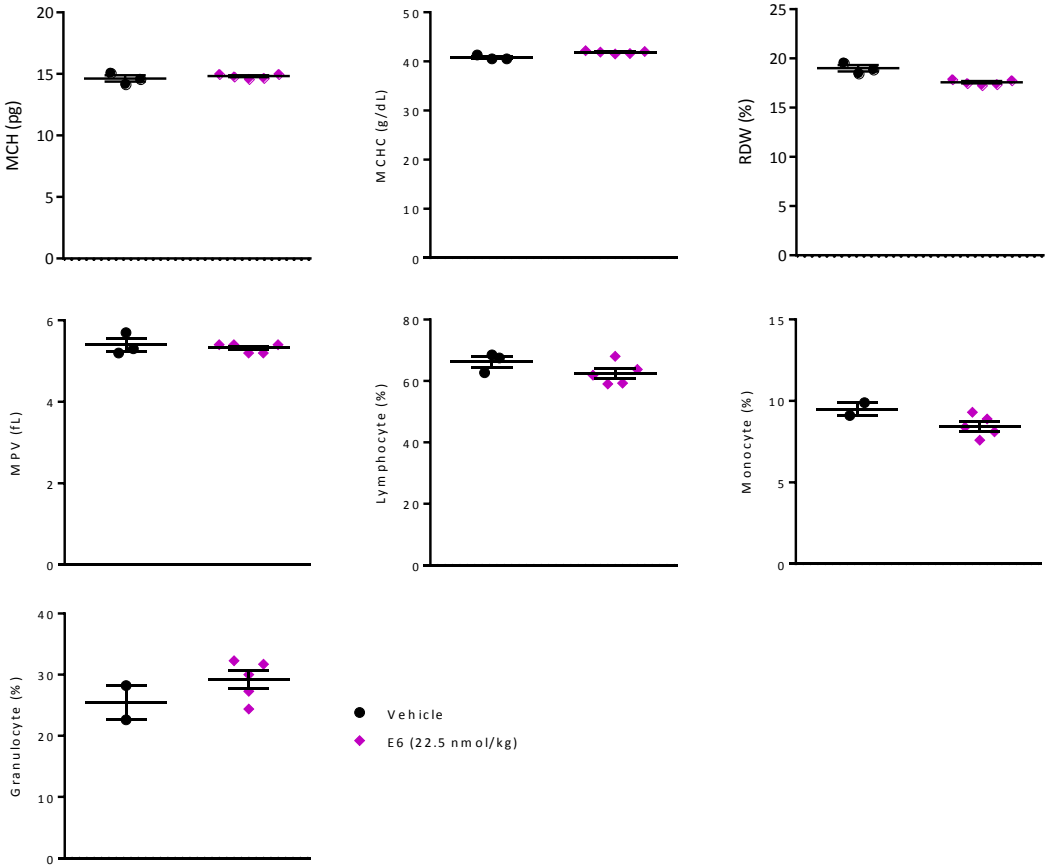

Figure S4. One-week repeated subcutaneous dose toxicity studies of E6 in diet-induced obese (DIO) mice. Effects on (**a**–**b**) body weight change, fed blood glucose (**c**), liver function and kidney function (**d**) and (**e**) hematological parameters. Data are presented as mean ± SD (n = 5). WBC = white blood cell (leukocyte), RBC = red blood cell (erythrocyte), HGB = hemoglobin, HCT = hematocrit, PLT = platelet, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, RDW = red cell distribution width, MPV = mean platelet volume.

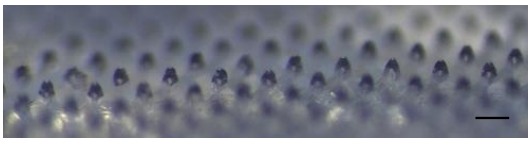

Figure S5 Representative photo of E6 MSA after a 5 min application to skin. (Scale bar 200 μm). E6-containing microstructure tips dissolve rapidly within the skin, as evidenced by the absence of sharp tips. The difference in coloration between the upper portion of the microstructures (dark colored tips and stumps) and their bases (light colored) is caused by the lighting technique used to enhance visibility of the microstructures. Under normal lighting conditions, the entire microstructure is translucent.

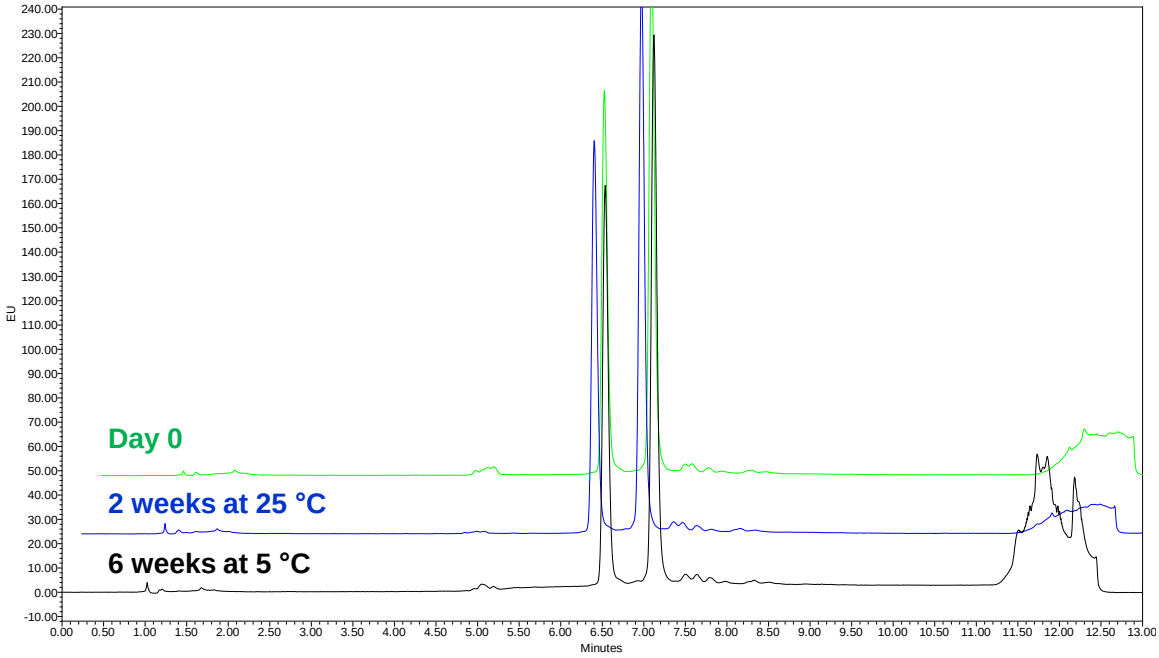

Figure S6 Stability of E6 in MicroCor MSAs stored at 25 °C for 2 weeks and 5 °C for 6 weeks. There were no extra peaks compared to Day 0.

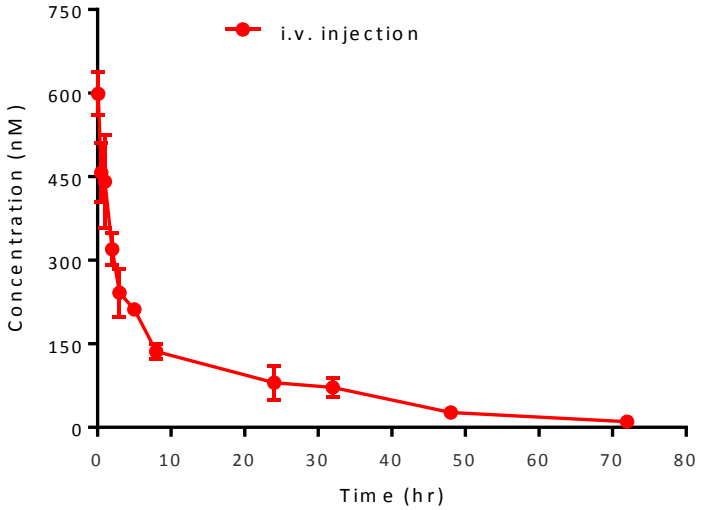

Figure S7 Pharmacokinetics of E6 in male Dunkin-Hartley guinea pigs (n = 4 per group) by *i.v.* injection. The peptide concentrations in plasma at various time points were determined by in vitro GLP-1R activity assay. Assay was performed in triplicate. The elimination half-life with *i.v.* injection was about 16.5 h.