Supporting Information for

Original article

Design of a highly potent GLP-1R and GCGR dual-agonist for recovering hepatic fibrosis

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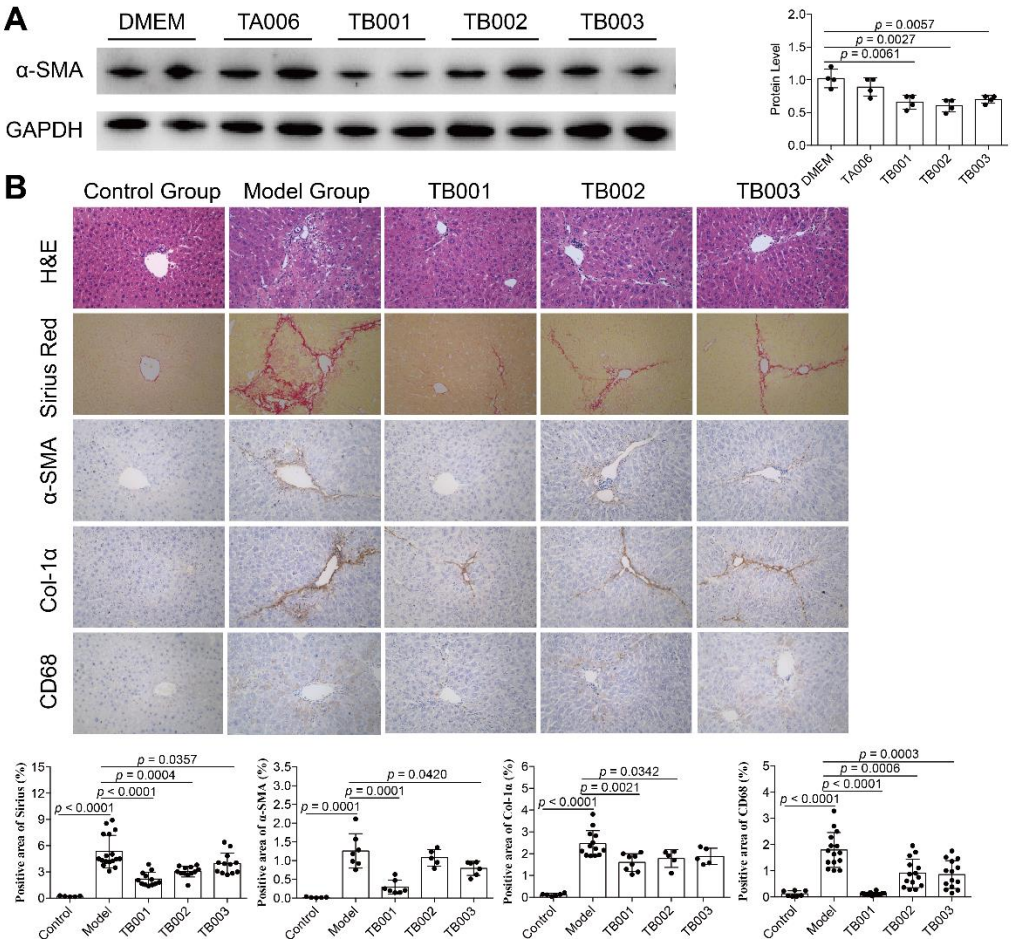


Figure S1 Peptide screening *in vitro* or *in vivo*. (A) Western blotting for *α*-SMA expression in LX2 cells after peptides treated. Three separated experiments were performed. *α*-SMA expression was normalized to that of GAPDH. Positive areas were analyzed with ImageJ. (B) Representative images of HE staining, sirius red staining and IHC for *α*-SMA, Col-1*α* and CD68 of liver sections on mice treated with Corn Oil, CCl_4 or CCl_4 plus TB001, TB002 and TB003 therapy. Here and later, for each assay, three separated experiments were performed. And for each experiment (n = 4 cell samples/group or n = 6 mice/group). For the in vivo experiment, the doses of TB001-TB003 are 60 μg/kg. All the above data are presented as mean values ± SEM using unpaired Student's t test.

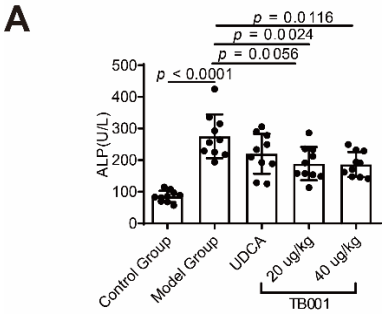


Figure S2 Serum ALP levels in ANIT-induced rat liver fibrosis. (A) Serum ALP of mice in the indicated groups were measured (n = 10).

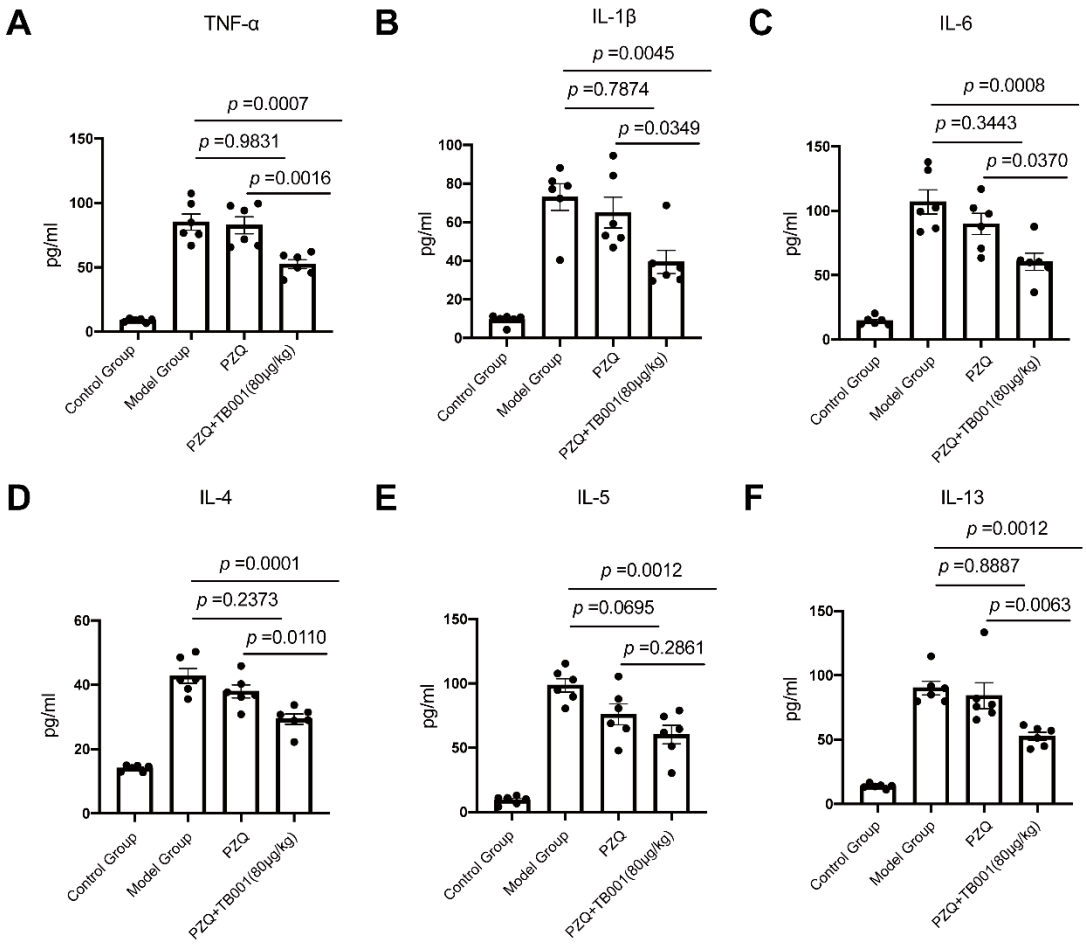


Figure S3 TB001 alleviated hepatocytes damage and inflammation in *Schistosoma japonicum*–induced mouse hepatic fibrosis model. (A–C) measurement of serum TNF-*α*, IL-1*β*, IL-6, IL-4, IL-5, and IL-13 levels were lower in mice treated with TB001 by ELISA assays (n = 5). All the above data are presented as mean values ± SEM using unpaired Student's t test.

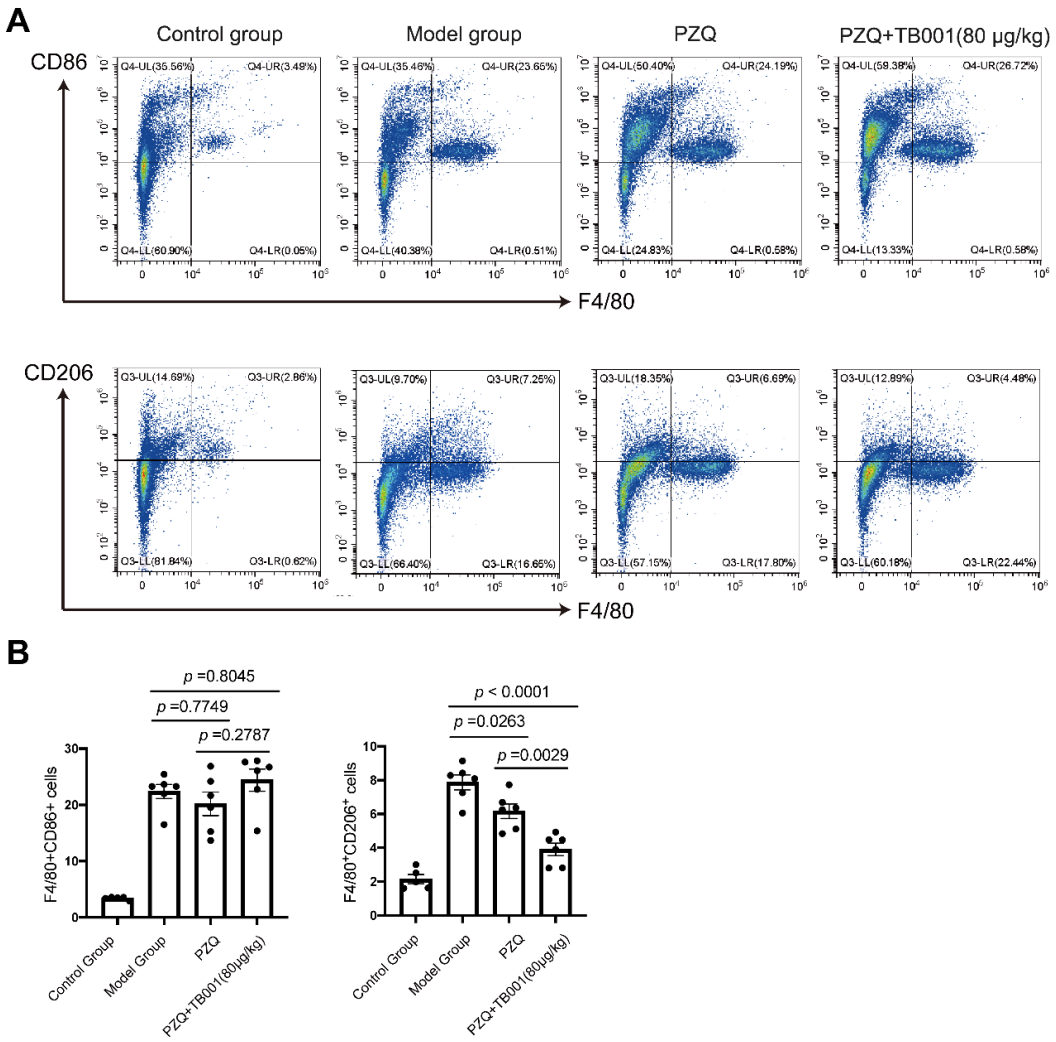


Figure S4 TB001 restore the immune microenvironment homeostasis in schistosomiasis (A–B) The number of F4/80+CD86+ M1 cells (n = 5) and F4/80+CD206+ M2 cells (n = 5) in each of mice liver were analyzed by flow cytometry. All the above data are presented as mean values ± SEM using unpaired Student's t test.

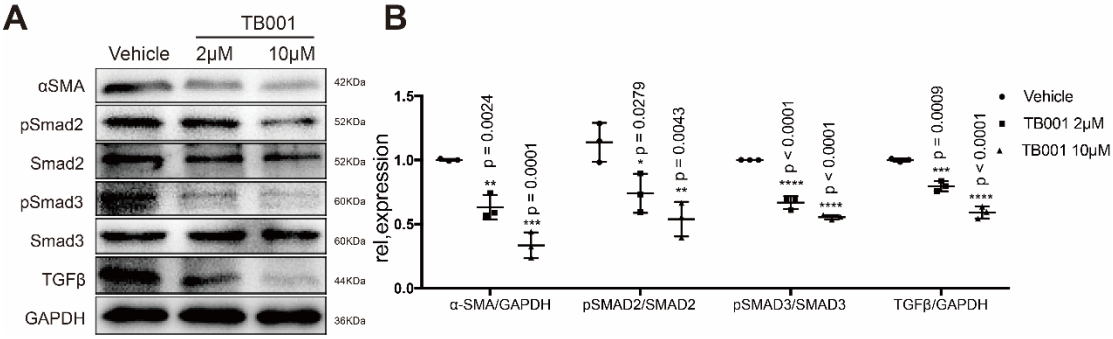


Figure S5 TB001 ameliorates hepatic fibrosis via TGFβ/Smad signal pathway in LX2 cell model. (A–B) Western blotting assay of Smad2/3, pSmad2/3 and TGFβ measured in HSCs with or without TB001 treating. GAPDH serves as the loading control. All the above data are presented as mean values ± SEM using unpaired Student's t test.

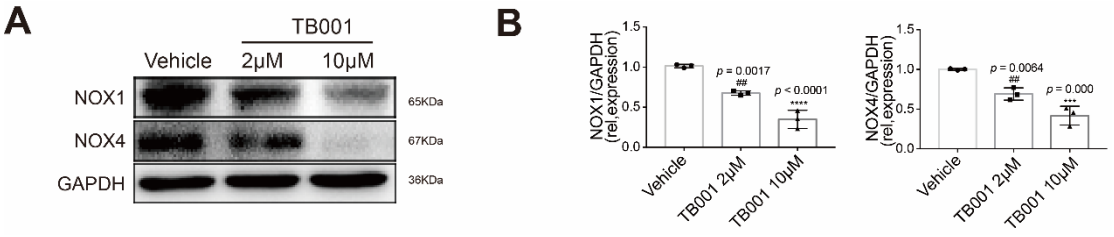


Figure S6 TB001 resolves inflammation caused by hepatic fibrosis in LX2 cell model. (A–B) LX2 cells were treated with TB001 and the expression of NOX1/4 were measured by Western blot. All the above data are presented as mean values ± SEM using unpaired Student's *t* test.

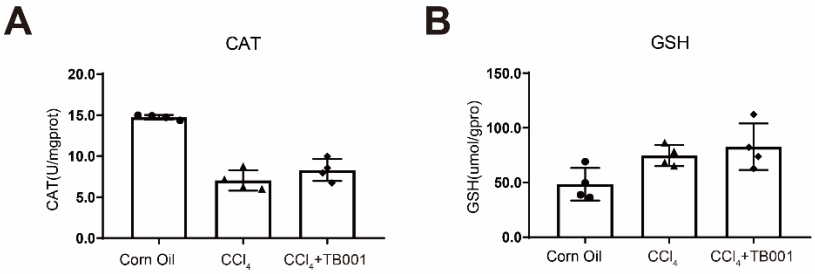


Figure S7 TB001 resolves oxidative stress caused by hepatic fibrosis in primary hepatocytes. (A) anti-oxidative stress function of primary hepatocytes manifested by CAT and GSH (n = 4). All the above data are presented as mean values ± SEM using unpaired Student's *t* test.

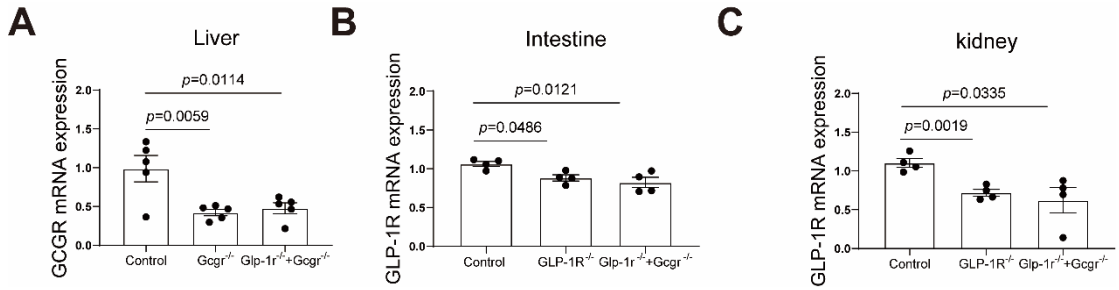


Figure S8 Evaluation of GCGR or GLP-1R knock-down effect in mice. (A) The mRNA levels of GCGR of liver in GCGR or GLP-1R knock-down mice (n = 5). (B–C) The mRNA levels of GLP-1R of intestines and kidney in GCGR or GLP-1R knock-down mice (n = 4). All the above data are presented as mean values ± SEM using unpaired Student's *t* test.

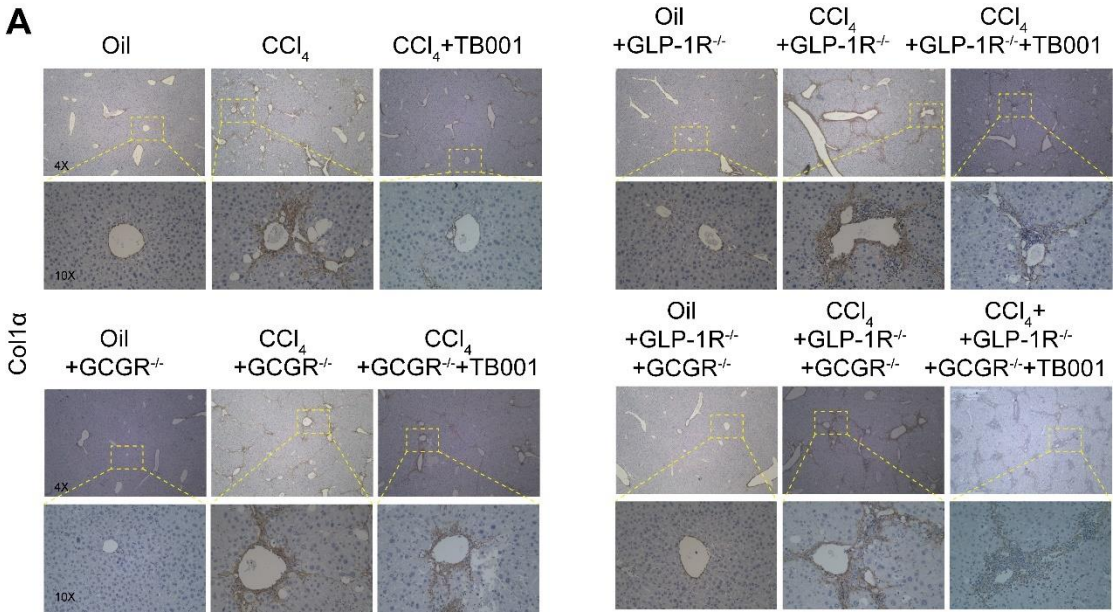


Figure S9 TB001 ameliorate the progress of CCl_4-induced mouse liver fibrosis by targeting GLP-1R/GCGR. (A) Sirius Red staining of mice liver sections representative images from each group (n = 5).

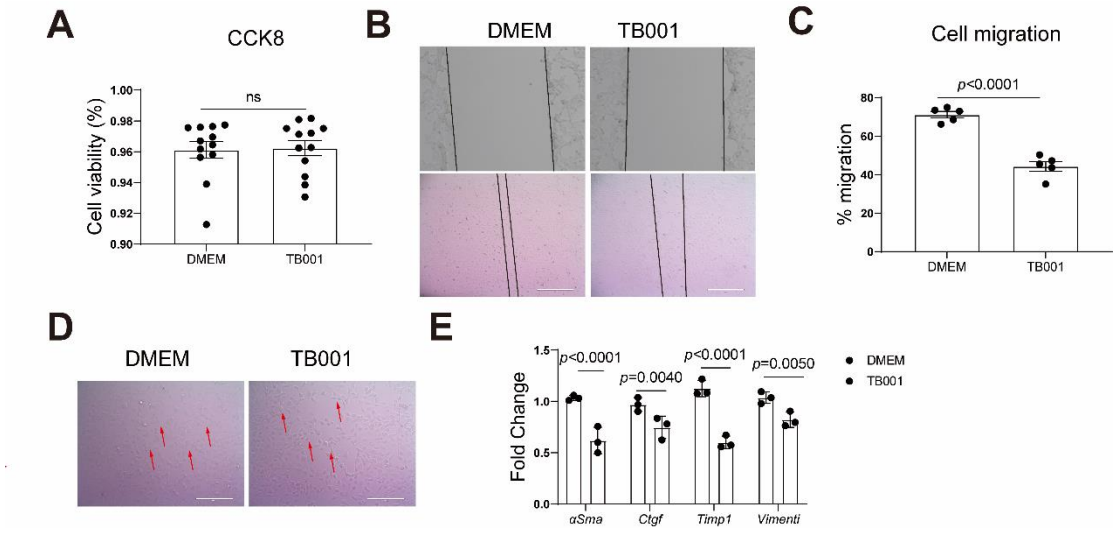
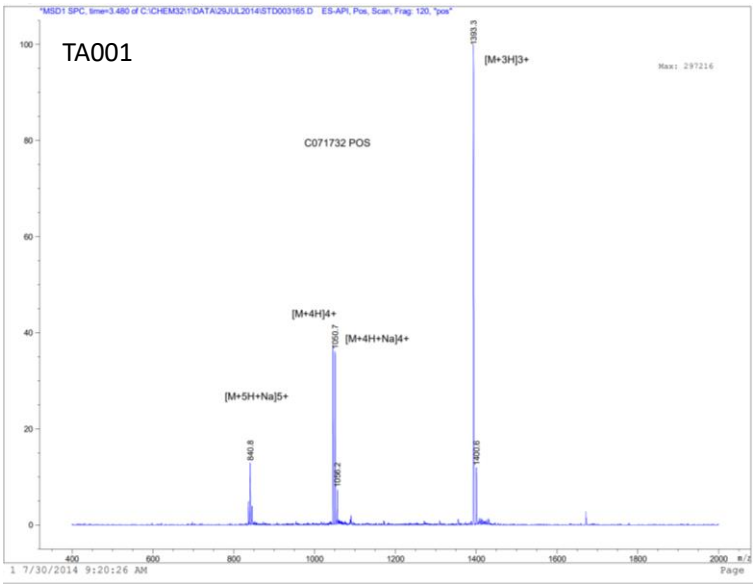


Figure S10 TB001 ameliorates hepatic fibrosis via regulating specific phenotypic changes of LX2 cells. (A) Evaluation of TB001 on LX2 cell proliferation (n = 3). (B–C) Results of the scratch wound-healing experiment demonstrate that TB001 treatments led to fewer cells migrating (n = 3). (D) TB001 promote LX2 contraction (n = 3). (E) qPCR analysis of genes related to HSC activation (*αSma*, *Ctgf*, *Timp1*, *Vimenti*) (n = 3). All the above data are presented as mean values ± SEM using unpaired Student's t test.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840.8
1056.2
1400.6
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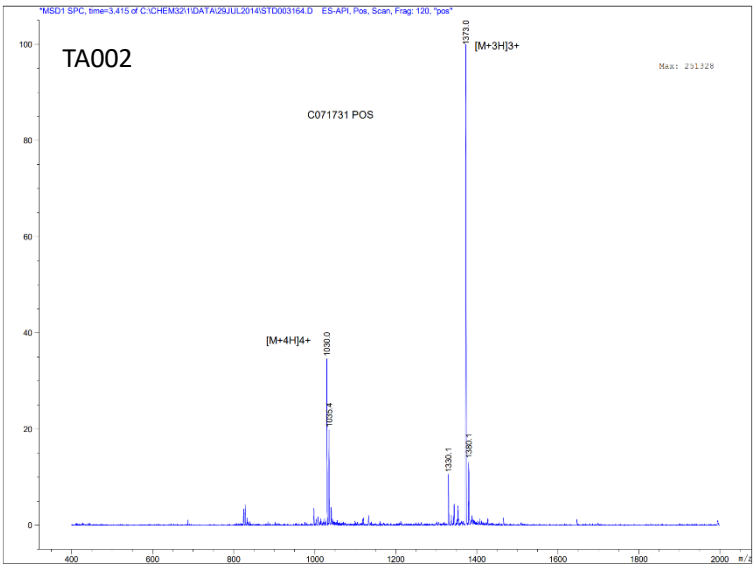
Page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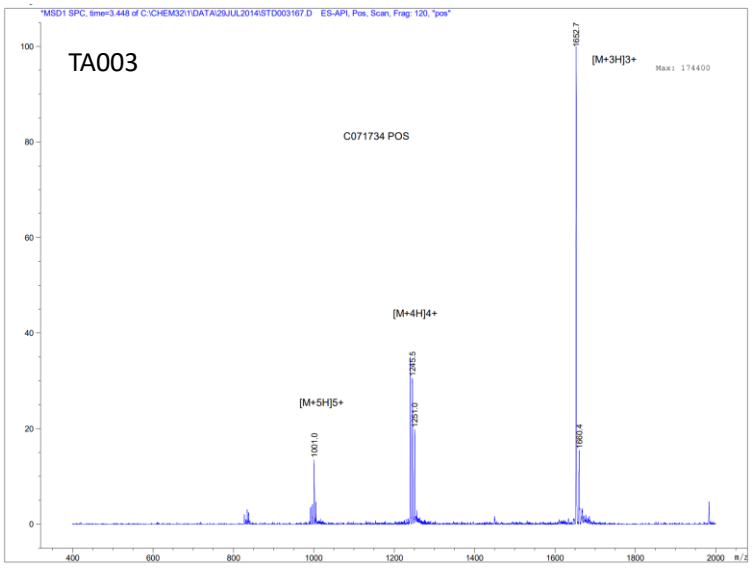
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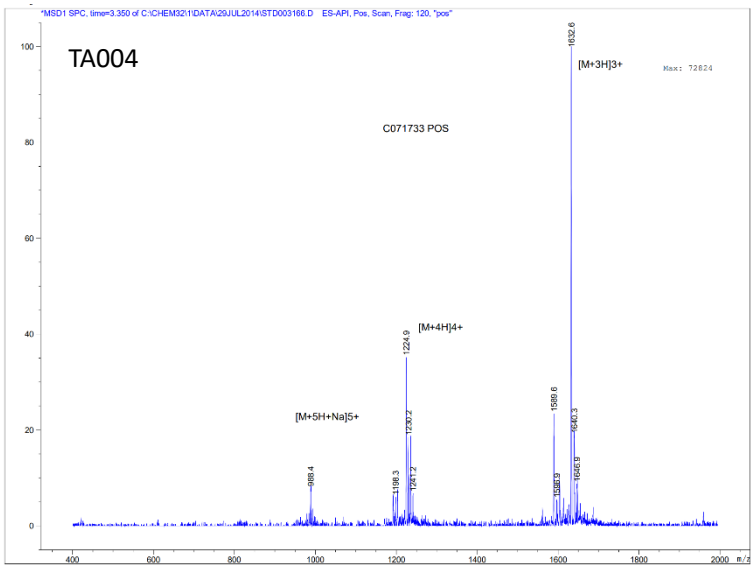
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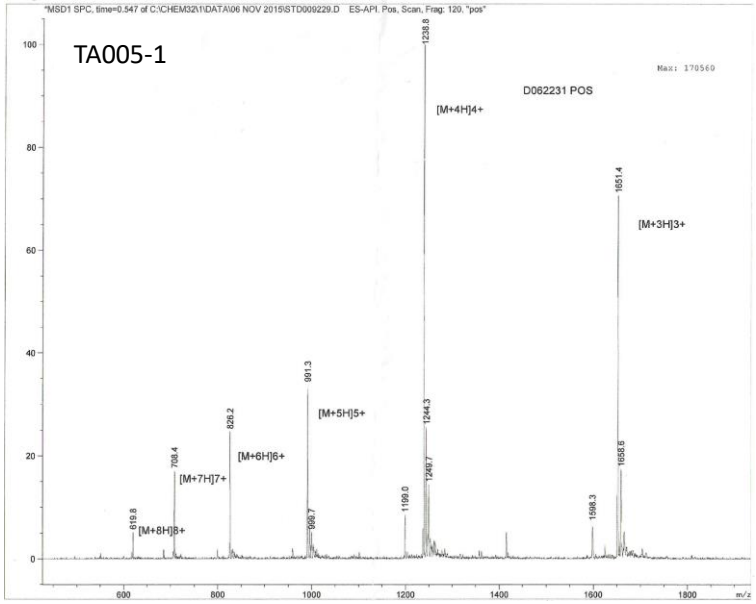
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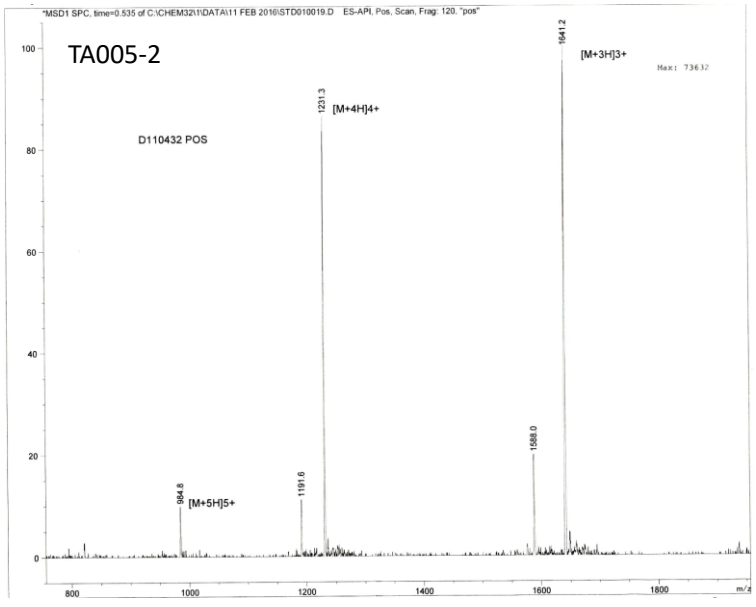
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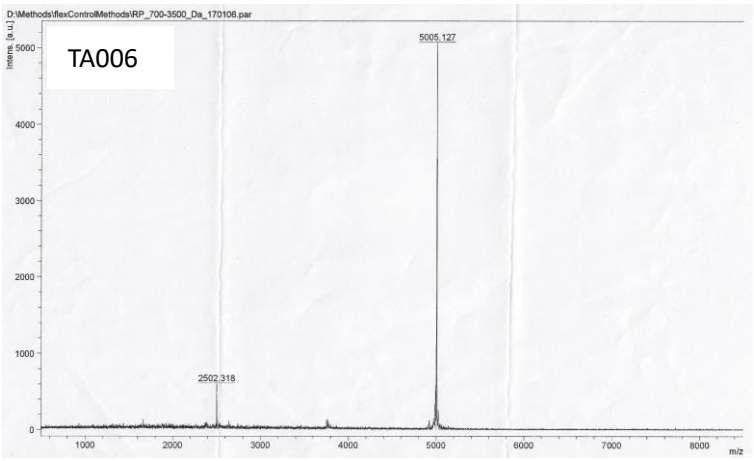
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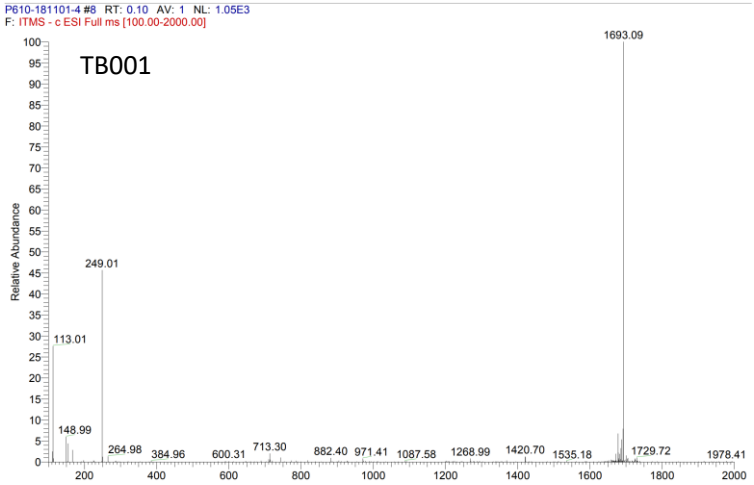
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F: ITMS - c ESI Full ms [100.00-2000.00]
TB001
Relative Abundance
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113.01
148.99
264.98
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882.40
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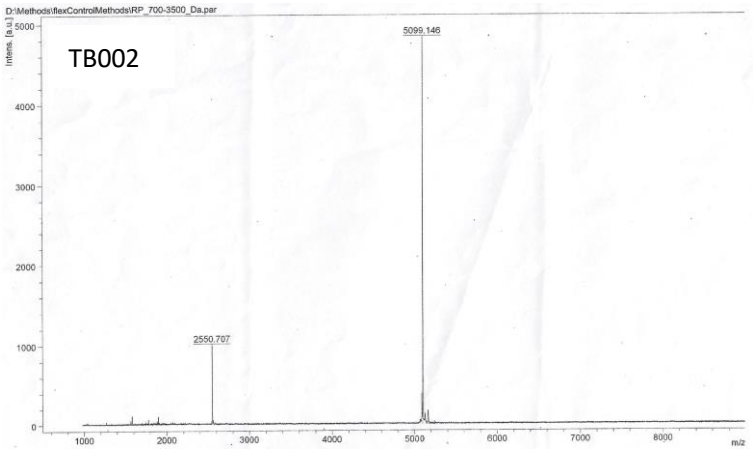
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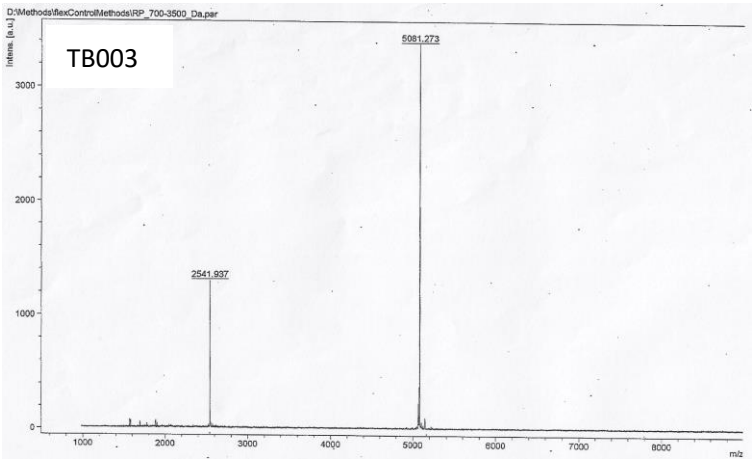
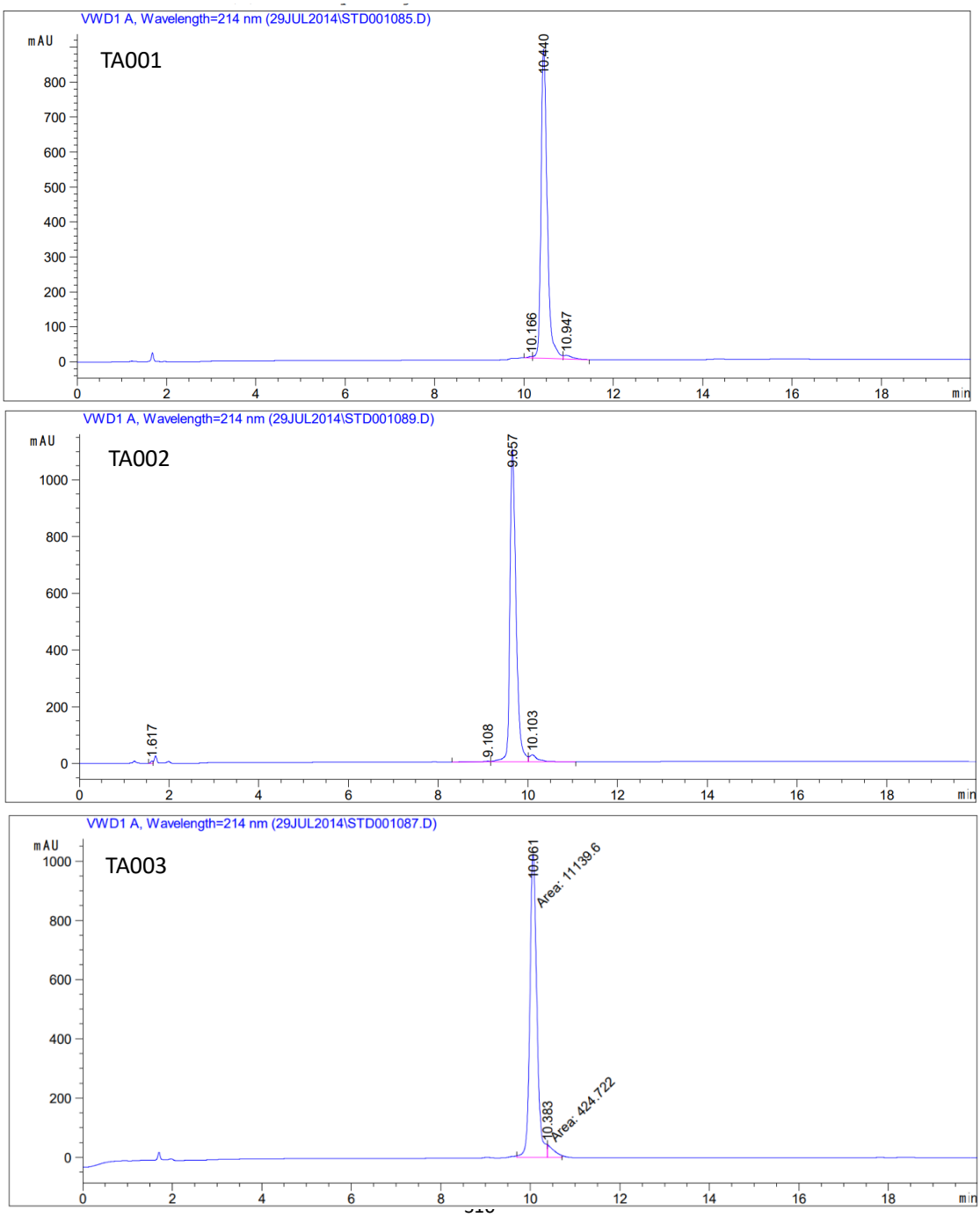
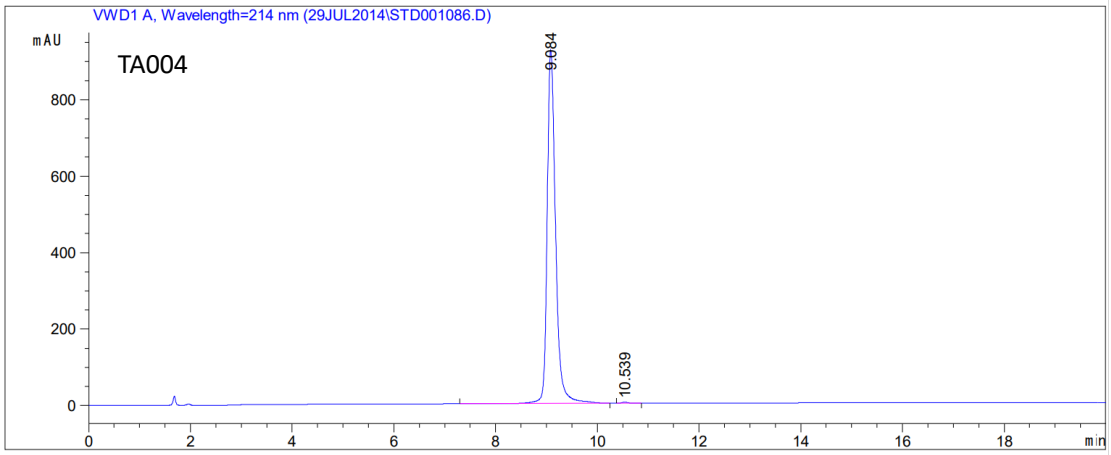


Figure S11 Confirmation of atomic accumulation of candidate peptides with electrospray ionisation-mass spectrometry.

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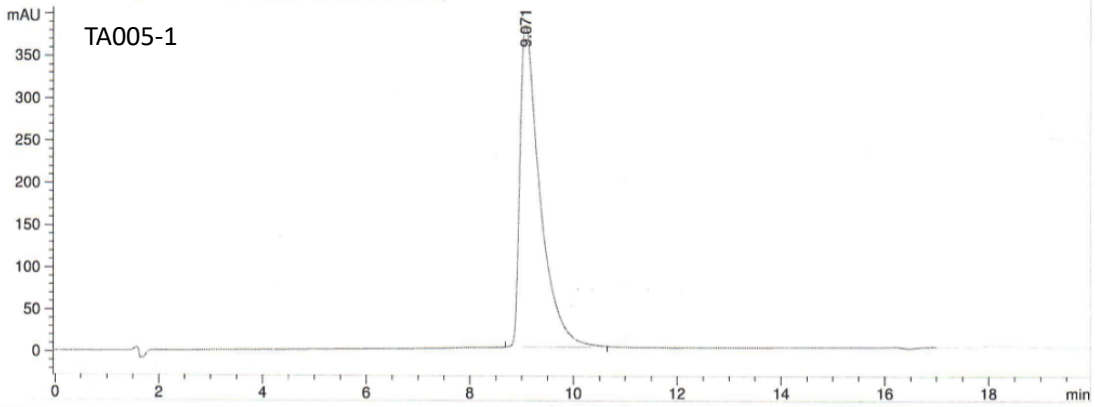
min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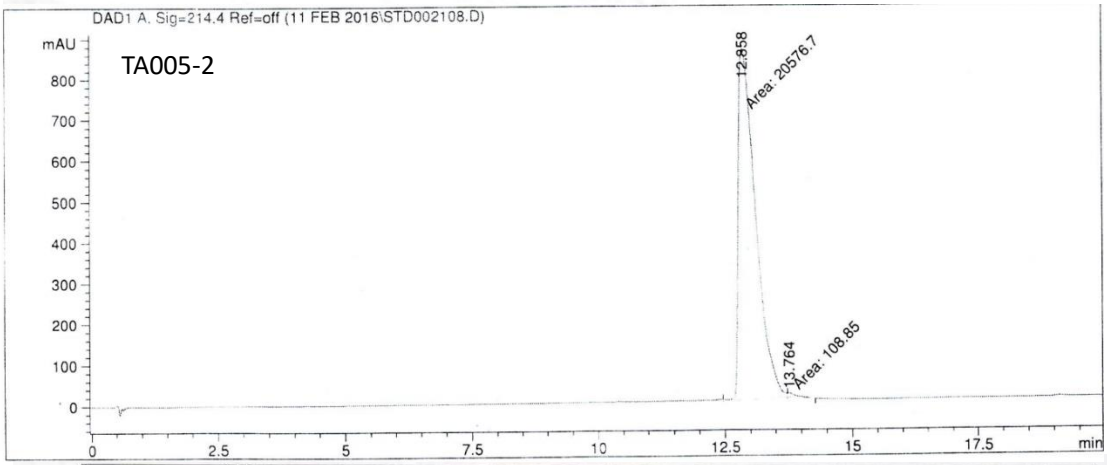
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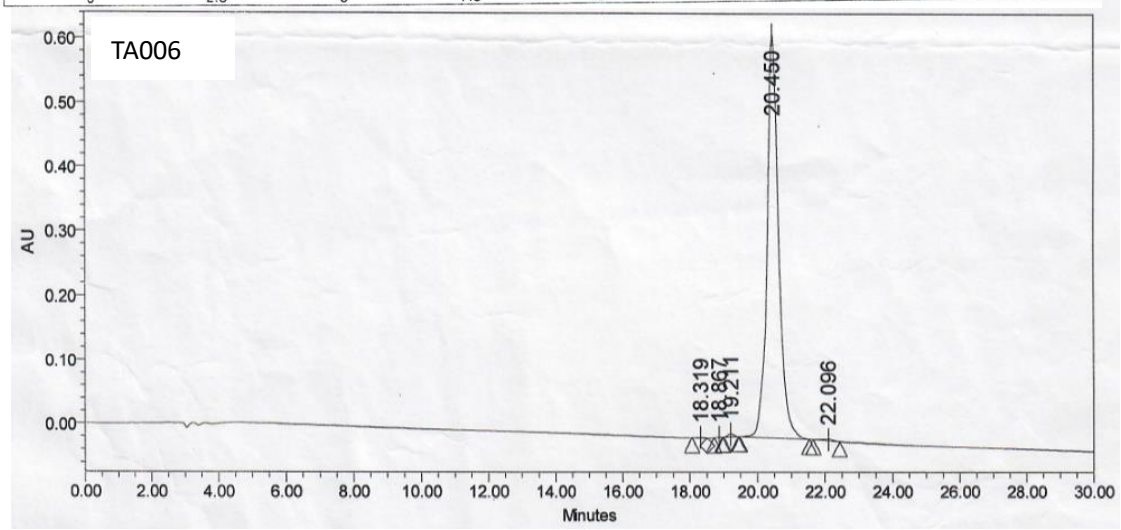
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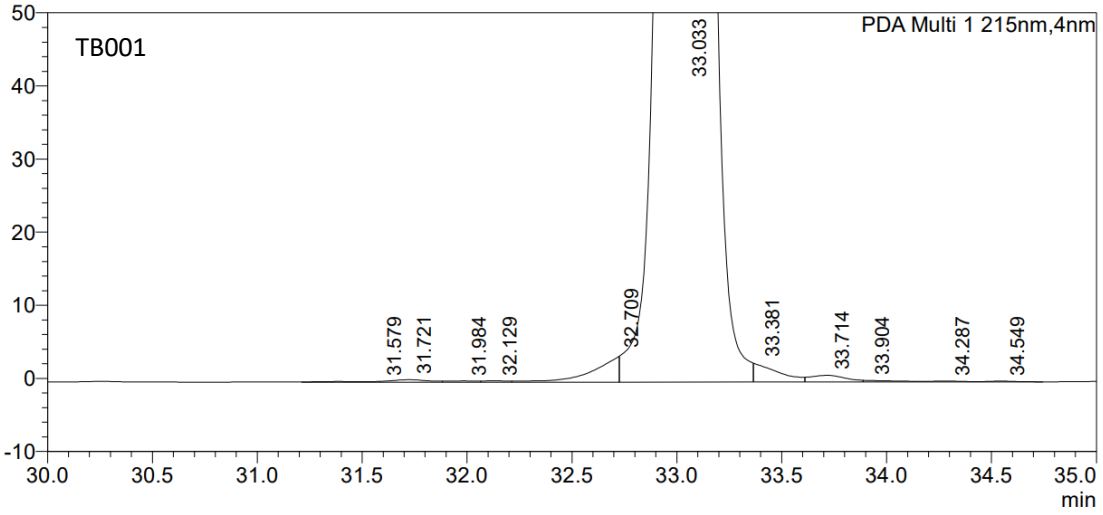

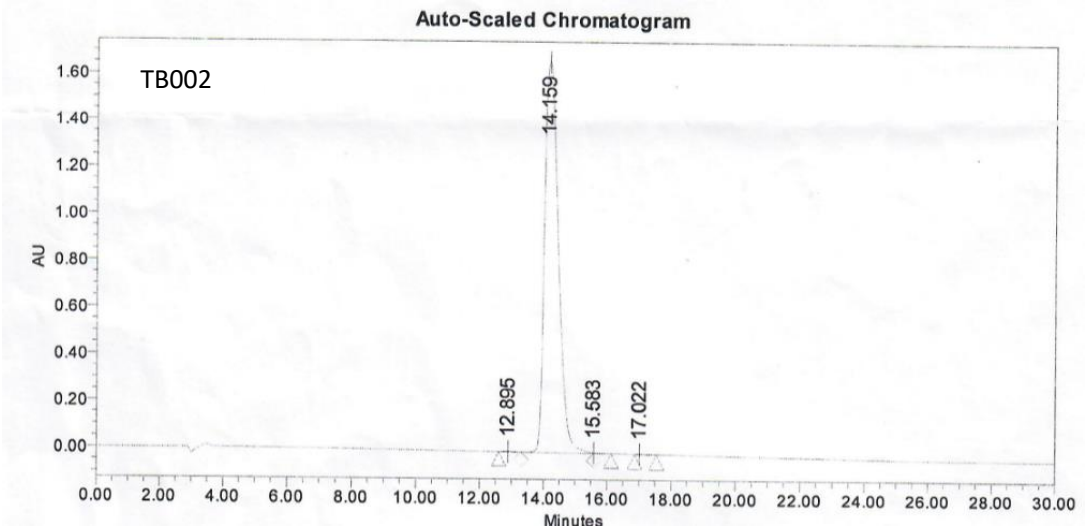

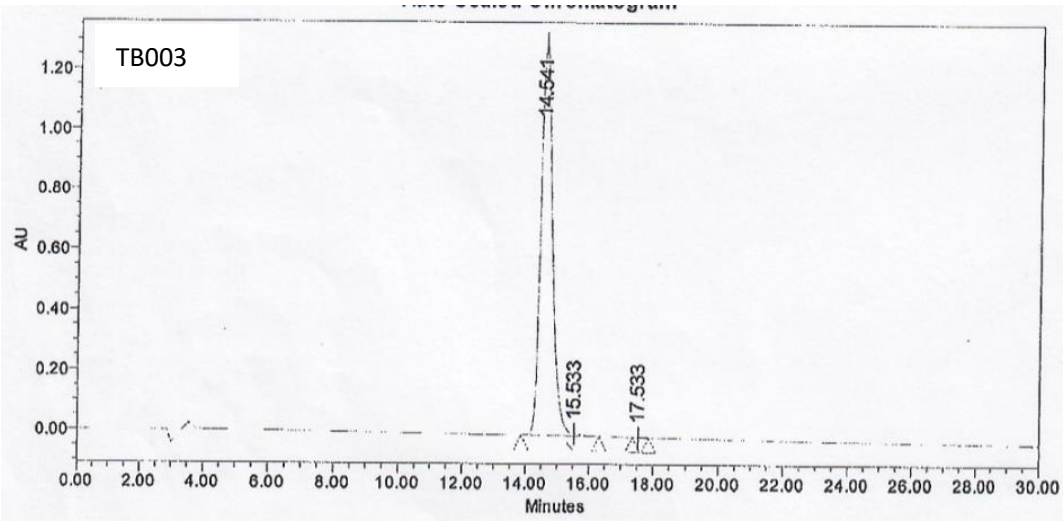

Figure S12 Purification of candidate peptides with reverse phase high performance liquid chromatography mass spectrometric (RP–HPLC–MS).

Table S1 Glucagon and GLP-1 receptor-mediated cAMP synthesis by chimeric peptide analogs.

Peptide	GCGR	GLP-1R	GCGR	GLP-1R	Selectivity ratio
	EC50(nmol/L)	EC50(nmol/L)	Relative %	Relative %	GCGR: GLP-1R
Glucagon	0.97	131.10	100.00	1.00	100.00
Liraglutide	>1000	0.04	<0.1	100.00	<0.1
TA001	3197.00	1.13	0.03	3.54	0.01
TA002	433.00	0.32	0.22	12.50	0.02
TA003	31.99	0.68	3.03	5.88	0.52
TA004	103.22	0.19	0.94	21.05	0.04
TA005-1	8.68	1.55	11.18	2.58	4.33
TA005-2	5.32	0.93	18.23	4.30	4.24
TA006	3.22	2.50	30.12	1.60	18.83
TB001	0.01	0.04	970.00	100.00	9.70
TB002	1.19	0.98	81.51	4.08	19.97
TB003	4.23	1.14	22.93	3.51	6.54

Table S2 Summary of the pharmacokinetic parameters of TB001 in rhesus monkey (n =6).

	Single dose				Multiple dose, sc. 20 ug/kg	
	iv. 15.3 ug/kg	sc. 5 ug/kg	sc. 20 ug/kg	sc. 60 ug/kg	Day 1	Day 7
$t_{1/2}$ (h)	0.498 ± 0.0100	3.33 ± 1.57	2.83 ± 1.42	2.47 ± 0.571	3.07 ± 1.28	1.94 ± 0.305
T_{max} (h)	NA	1.67 ± 0.516	1.83 ± 0.408	2.67 ± 1.03	2.00	2.00
C_{max} (ng/mL)	NA	1.96 ± 0.570	12.0 ± 3.90	43.4 ± 19.6	9.82 ± 4.72	14.8 ± 5.35
$AU_{C0\rightarrow t}$ (h·ng/mL)	289 ± 46.2	9.19 ± 2.73	75.5 ± 21.3	301 ± 92.2	62.3 ± 19.5	90.7 ± 28.3
$AUC_{0\rightarrow\infty}$ (h·ng/mL)	290 ± 46.4	11.8 ± 2.99	83.0 ± 19.9	314 ± 95.5	68.3 ± 17.6	93.7 ± 29.2
CL (mL/min/kg)	0.897 ± 0.132	NA	NA	NA	NA	NA
Vss (mL/kg)	37.9 ± 3.47	NA	NA	NA	NA	NA
MRT (h)	0.669 ± 0.136	5.68 ± 2.00	5.85 ± 1.57	5.70 ± 0.708	6.04 ± 1.51	4.72 ± 0.386
F (%)	NA	13.1 ± 2.79	22.0 ± 4.95	27.5 ± 6.06	NA	NA

NA, not applicable; data are presented as mean±SD.

Table S3 Selected biochemistry parameters in rats for repeated dose toxicity in Sprague–Dawley rat (n =10).

Group	Male (n =5)				Female (n =5)			
	Control	sc. 20 ug/kg	sc. 100 ug/kg	sc. 500 ug/kg	Control	sc. 20 ug/kg	sc. 100 ug/kg	sc. 500 ug/kg
AST (U/L)	112.7 ± 39.5	111.2 ± 22.9	131.6 ± 37.7	150.8 ± 16.0	86.3 ± 16.7	78.5 ± 10.3	120.7 ± 36.1	103.9 ± 36.0
ALT (U/L)	35.2 ± 3.8	34.6 ± 6.1	45.0 ± 17.3	42.2 ± 8.8	26.0 ± 3.2	30.6 ± 4.0	26.6 ± 3.7	25.8 ± 6.7
ALP (U/L)	169.5 ± 63.4	150.2 ± 30.7	174.4 ± 95.5	174.3 ± 38.7	90.9 ± 23.6	69.1 ± 9.3	78.8 ± 10.2	86.3 ± 23.8
TBIL (μmol/L)	0.8 ± 0.3	1.0 ± 0.3	1.4 ± 0.1*	1.4 ± 0.2*	1.0 ± 0.4	0.9 ± 0.5	1.3 ± 0.7	2.2 ± 0.4*
CHOL (mmol/L)	1.46 ± 0.28	1.25 ± 0.21	0.61 ± 0.14*	0.45 ± 0.10*	1.40 ± 0.19	1.41 ± 0.30	0.72 ± 0.13*	0.61 ± 0.18*
Crea (μmol/L)	24.4 ± 2.1	22.8 ± 1.8	23.4 ± 1.8	22.4 ± 1.5	25.2 ± 1.9	24.8 ± 1.9	27.6 ± 3.0	28.2 ± 3.6
Urea (mmol/L)	5.34 ± 1.08	3.85 ± 0.66	3.74 ± 0.95	4.02 ± 0.38	4.56 ± 0.58	3.87 ± 0.73	3.96 ± 0.68	3.73 ± 0.95

CK (U/L)	742 ± 382	622 ± 159	492 ± 108	594 ± 193	475 ± 176	368 ± 169	752 ± 440	525 ± 343

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; CHOL, total cholesterol; Crea, creatinine; CK, creatine kinase; Data are presented as Mean±SD, measured after 14-day treatment, * $P < 0.05$, vs. the respective control group.

Table S4 Selected biochemistry parameters of toxicity in rhesus monkey (n =40).

	male				female			
	control	20 μg/kg	60 μg/kg	300 μg/kg	control	20 μg/kg	60 μg/kg	300 μg/kg
AST (U/L)	33.2 ± 6.6	37.6 ± 5.9	45.9 ± 9.2	47.9 ± 15.5	35.5 ± 2.1	37.1 ± 5.9	46.6 ± 13.4	42.6 ± 7.1
ALT (U/L)	40.6 ± 4.7	53.3 ± 32.2	47.3 ± 13.3	34.3 ± 6.6	31.9 ± 4.1	41.0 ± 7.4	56.9 ± 23.8*	34.3 ± 6.6
TBIL (μmol/L)	4.0 ± 2.5	4.0 ± 1.3	5.4 ± 1.1	9.4 ± 6.0	3.1 ± 0.9	3.5 ± 1.2	5.4 ± 2.1	4.7 ± 1.7
CK (U/L)	144 ± 27	161 ± 57	129 ± 47	123 ± 49	169 ± 32	123 ± 11	130 ± 32	102 ± 38 *
CHOL (mmol/L)	3.37 ± 0.67	2.75 ± 0.34	2.21 ± 0.44 *	2.11 ± 0.26 *	3.14 ± 0.43	3.02 ± 0.45	2.74 ± 0.54	2.16 ± 0.80
Crea (μmol/L)	46.8 ± 2.0	49.4 ± 4.0	46.6 ± 8.4	45.4 ± 6.3	44.6 ± 12.4	47.4 ± 8.6	53.6 ± 9.6	45.4 ± 4.7
Urea (mmol/L)	6.48 ± 1.54	5.82 ± 0.82	6.56 ± 2.44	5.20 ± 1.13	5.60 ± 1.57	6.44 ± 0.81	6.54 ± 1.17	4.88 ± 1.37
ALP (U/L)	367.8 ± 94.8	335.4 ± 92.5	299.8 ± 106.8	268.6 ± 79.1	508.6 ± 121.5	329.4±104.6*	260.2 ± 65.2*	191.8 ± 37.4*

Data are presented as mean±SD, measured after 28-day treatment; *$P < 0.05$, *vs*. the respective control group.

Table S5 Summary of the toxicokinetics parameters of TB001 in rhesus monkey (n =30).

dose (μg/kg)	day	AUC_{last} (h·ng/mL)				C_{max} (ng/mL)				T_{max} (h)					
		Female (n =5)		Male (n =5)		Female (n =5)		Male (n =5)		Female (n =5)			Male (n =5)		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Median	Minimal	Maximum	Median	Minimal	Maximum
20	1	106	13.1	136	20.8	23.7	3.85	33.9	5.30	1.0	1.0	2.0	1.0	1.0	2.0
	28	102	6.98	93.9	14.4	25.1	2.16	25.4	5.05	1.0	1.0	2.0	1.0	1.0	2.0
60	1	565	89.3	422	87.3	117	19.7	91.7	21.5	2.0	1.0	2.0	2.0	2.0	2.0
	28	841	934	369	173	180	130	97.9	38.8	2.0	1.0	2.0	1.0	1.0	2.0
300	1	4440	1660	3890	1480	562	203	602	186	4.0	2.0	4.0	2.0	2.0	4.0
	28	3370	1310	2890	1010	533	201	590	174	2.0	1.0	2.0	2.0	1.0	2.0

Table S6 Antibodies used for western blotting and IHC.

Antibody	Company	Dilution	Catalogue number
AMPK*α*	Cell Signaling Technology	1:1000	#2532S
pSMAD2	Cell Signaling Technology	1:1000	#2455
SMAD2	Cell Signaling Technology	1:1000	#5339
pSMAD3	Cell Signaling Technology	1:1000	#9520
SMAD3	Cell Signaling Technology	1:1000	#9523
TGF-*β*	Abcam	1:1000	ab269279
GAPDH	TRANS	1:1000	HC301
pIKB*α*	Abcam	1:1000	ab133462
IKB*α*	Abcam	1:1000	ab109509
pNFκB	Cell Signaling Technology	1:1000	#3036
NFκB	Abcam	1:2000	ab209795
pJNK	Cell Signaling Technology	1:1000	#4668
JNK	Abcam	1:2500	ab199380
Cle-caspase3	Abcam	1:500	ab2302
XBP-1	affbiotech	1:1000	AF5110
pIRE1*α*	affbiotech	1:1000	13872-AF7150
IRE1*α*	affbiotech	1:1000	11445-DF7709
GRP78	Proteintech	1:1000	11587
caspase3	Abcam	1:5000	ab32351
Col1*α*	Cell Signaling Technology	1:1000	#72026
NOX1	Abcam	1:1000	ab131088
NOX4	Abcam	1:1000	ab79971
CD68	Boster	1:100	BM4593
α-SMA	Abcam	1:1000	ab7817
Goat anti-Rabbit lgG	Zsbio	1:5000	ZDR–5118
Goat anti-Mouse lgG	Zsbio	1:5000	ZDR–5307

Table S7 Primer sequences of mouse gene for real-time PCR.

Mouse gene	Forward primer	Reverse primer
Pdgf	TCCATGCTAGACTCAGAAGTCA	TCCCGGTGGACACAATTTTTC
Timp1	CCTGGTCATAAGGGCTAAATTCA	TTAGTCATCTTGATCTTATAACGCTGC
Fn	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG
Tnf-α	AAAGCATGATCCGAGATGTG	AGCAGGAATGAGAAGAGGCT
F4/80	CCCCAGTGTCCTTACAGAGTG	GTGCCCAGAGTGGATGTC T
α-Sma	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
Tgf-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Mmp2	CCAGAAGGCGAACAGACTG	TGGGCCGGAGACCTAAAGAG
Mmp9	GCGCCACCACAGCCAACTATG	TGGATGCCGTCTATGTCGTCTTTA
Tnf-α	AAAGCATGATCCGAGATGTG	AGCAGGAATGAGAAGAGGCT
Il-1	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC

Pai-1	TTCAGCCCTTGCTTGCCTC	ACACTTTTACTCCGAAGTCGGT
Fas	TATCAAGGAGGCCCATTTTGC	TGTTTCCACTTCTAAACCATGCT
Fasl	TCCGTGAGTTCACCAACCAAA	GGGGGTTCCCTGTTAAATGGG
Bid	GAGATGGAGGCAACCAAACTG	CTGCGCGTTGTACTGATGT
Bim	GCTCCTGTGCAATCCGTATC	GCCCCTACCTCCCTACAGAC
Bcl2	GTCGCTACCGTCGTGACTTC	CAGACATGCACCTACCCAGC
Gapdh	AATGTGTCCGTCGTGGATCT	AGACAACCTGGTCCTCAGTG
Il-1β	CTCACAAGCAGAGCACAAGC	TCCAGCCCATACTTTAGGAA
Tnf-α	CCCTCACACTCAGATCATCT	GCTACGACGTGGGCTACAG
Il-4	CATGCACGGAGATGGATGTG	ACCTTGGAAGCCCTACAGAC
Il-5	AAAGAGAAGTGTGGCGAGGA	ACCAAGGAACTCTTGCAGGT
Il-13	GCAGCATGGTATGGAGTGTG	GGAATCCAGGGCTACACAGA
Caspase-1	CACAGCTCTGGAGATGGTGA	GGTCCCACATATTCCCTCCT
Caspase-3	GTCATCTCGCTCTGGTACGG	CACACACACAAAGCTGCTCC
Caspase-4	CCGAGACAAAACAGGAGGCT	GGTGGGCATCTGGGAATGAA
Bax	CAAGAAGCTGAGCGAGTGTC	GTCCACGTCAGCAATCATCC
Gpr78	GGTGGGCAAACCAAGACATT	TCAGTCCAGCAATAGTGCCA
Xbp-1	GCTGCGGAGGAAACTGAAA	CAAATCCACCACTTGCTGCT
Perk	CGTCCGAAGCTTCTCCCTAT	CTCTGGGCTCCTCCTTACTG

Table S8 Target sequences for AAV9-RNAi mediated GCGR and GLP-1R knockdown.

AAV9-RNAi	Target sequence
AAV9-Control-shRNA	CGCTGAGTACTTCGAAATGTC
AAV9-GLP-1R-shRNA	CAGCGCATCTTCAAGCTGTAT
AAV9-GCGR-shRNA	TCCCAACACCACTGCCAACAT