Supporting Information for

Original article

A novel and low-toxic peptide DR3penA alleviates pulmonary fibrosis by regulating the MAPK/miR-23b-5p/AQP5 signaling axis

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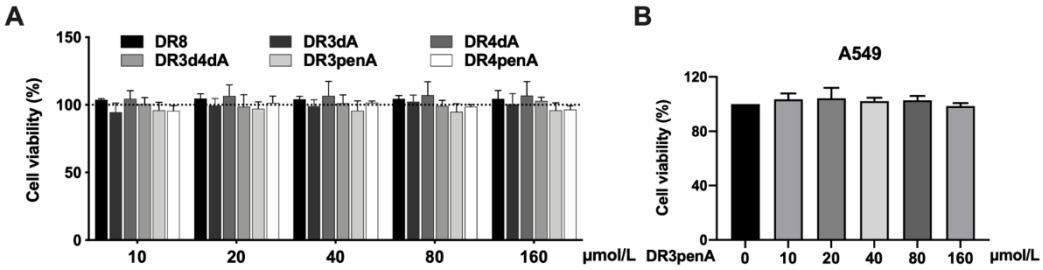


Figure S1 Viability of NIH3T3 and A549 cells after DR8 and DR8 analogs treatment. (A) Viability of NIH3T3 cells after treatment with DR8 and DR8 analogs at concentrations ranging from 10–160 μmol/L for 24 h. (B) Viability of A549 cells after treatment with DR3penA at a concentration range of 10–160 μmol/L for 48 h. The data are presented as the mean ± SD (n = 3).

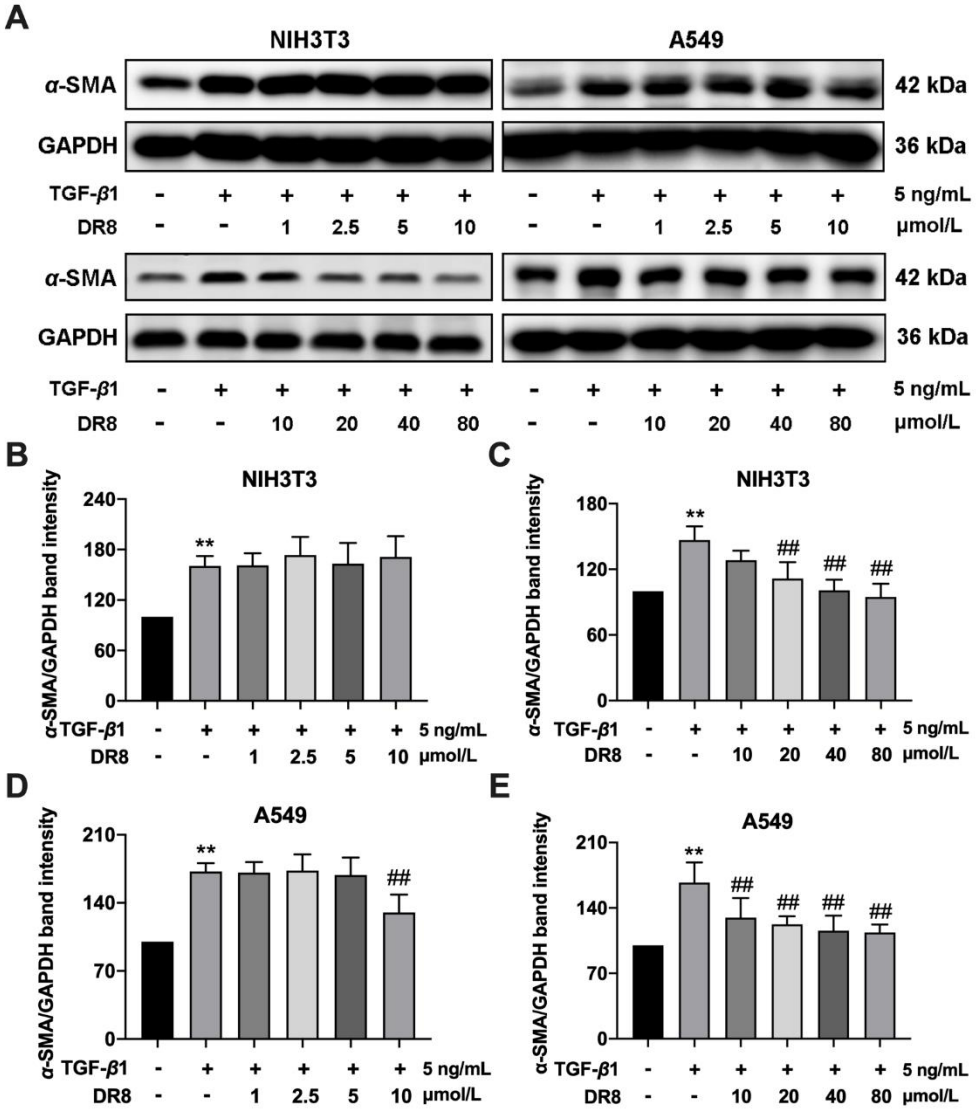


Figure S2 Activity study of peptide DR8. (A) α-SMA protein expression after DR8 treatment at concentrations from 1–80 μmol/L in NIH3T3 and A549 cells. (B–E) α-SMA protein expression bands were normalized to GAPDH expression levels in NIH3T3 and A549 cells. The data are presented as the mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. the TGF-β1 group.

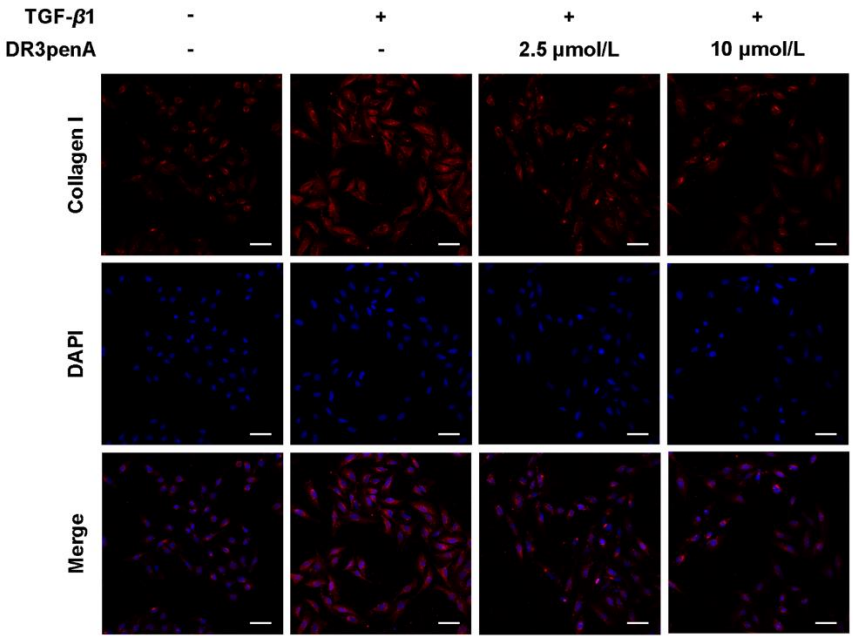


Figure S3 Immunofluorescence assay of collagen I expression in A549 cells treated with TGF-β1 and/or DR3penA (scale bars, 50 μm).

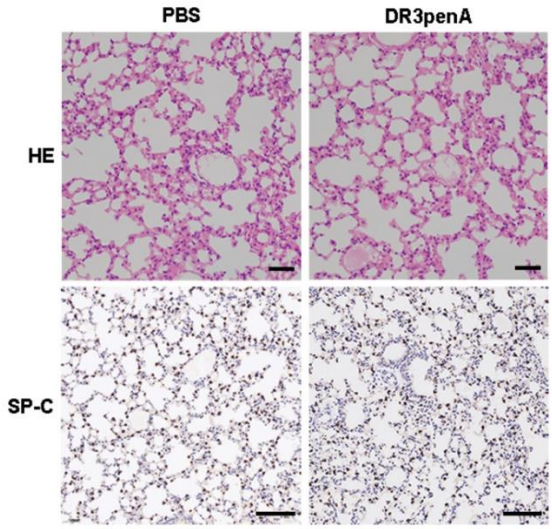


Figure S4 HE staining and immunohistochemistry of SP-C protein were used to assess lung histopathology in mice treated with PBS or DR3penA at a dose of 2.5 mg/kg (scale bars, 50 μm).

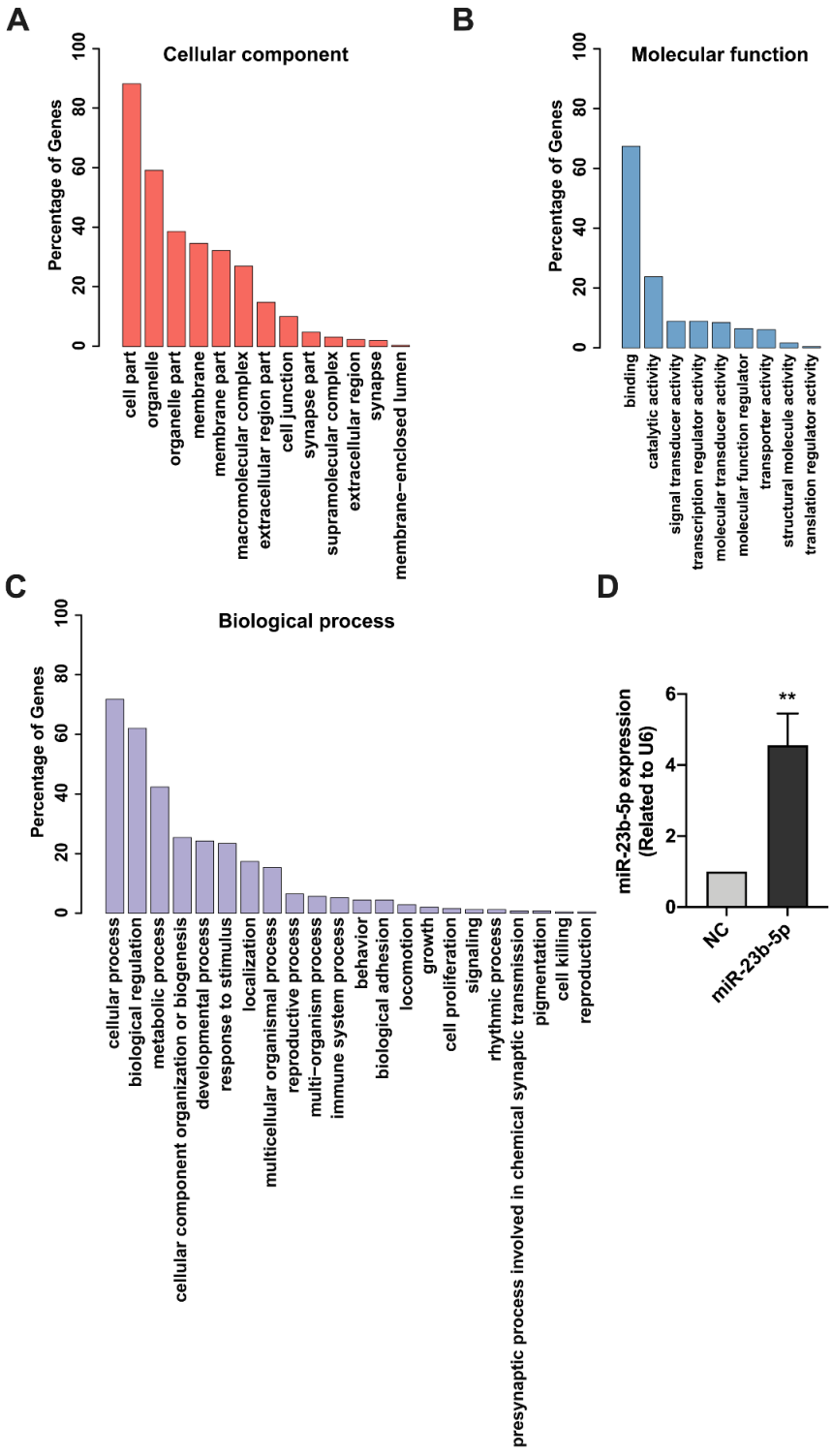


Figure S5 GO enrichment analysis and miR-23b-5p expression. (A) The functions of genes related to cellular components. (B) The functions of genes involved in molecular function. (C) The functions of genes involved in biological processes. (D) Overexpression efficiency of miR-23b-5p in A549 cells. The data are presented as the mean ± SD (n = 3). $^{**}P < 0.01$ *vs*. NC group.

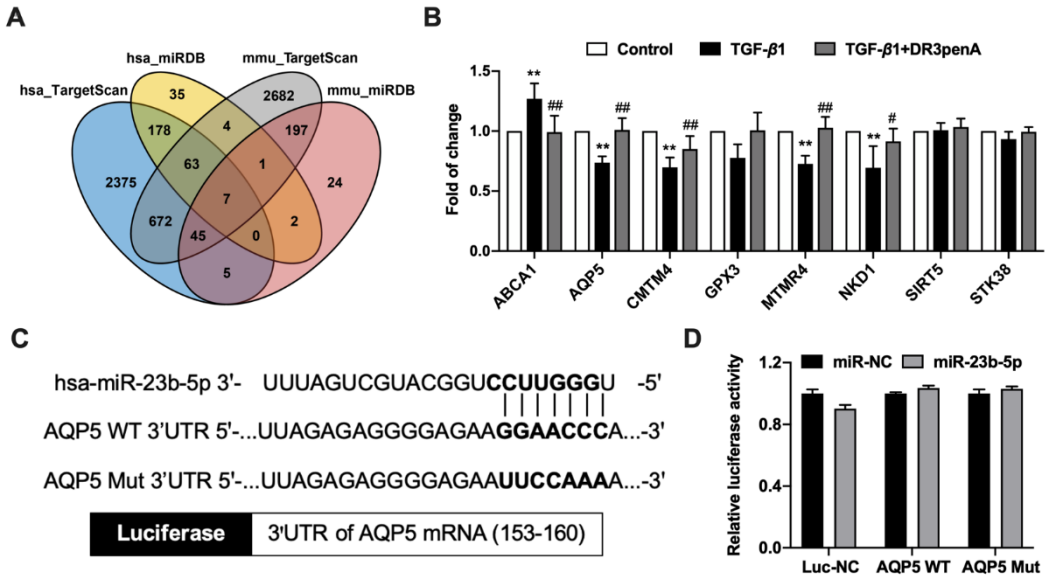


Figure S6 miR-23b-5p target gene screening and verification via dual-luciferase reporter assays. (A) Venn diagram showing the numbers of target genes of miR-23b-5p predicted by the miRDB and TargetScan databases. (B) The effect of DR3penA on the expression of potential miR-23b-5p target genes in A549 cells induced by TGF-β1. (C) The binding site between miR-23b-5p and AQP5 was predicted by the TargetScan database. (D) Relative luciferase activity was detected to verify the binding between miR-23b-5p and AQP5. The data are presented as the mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. the TGF-β1 group.

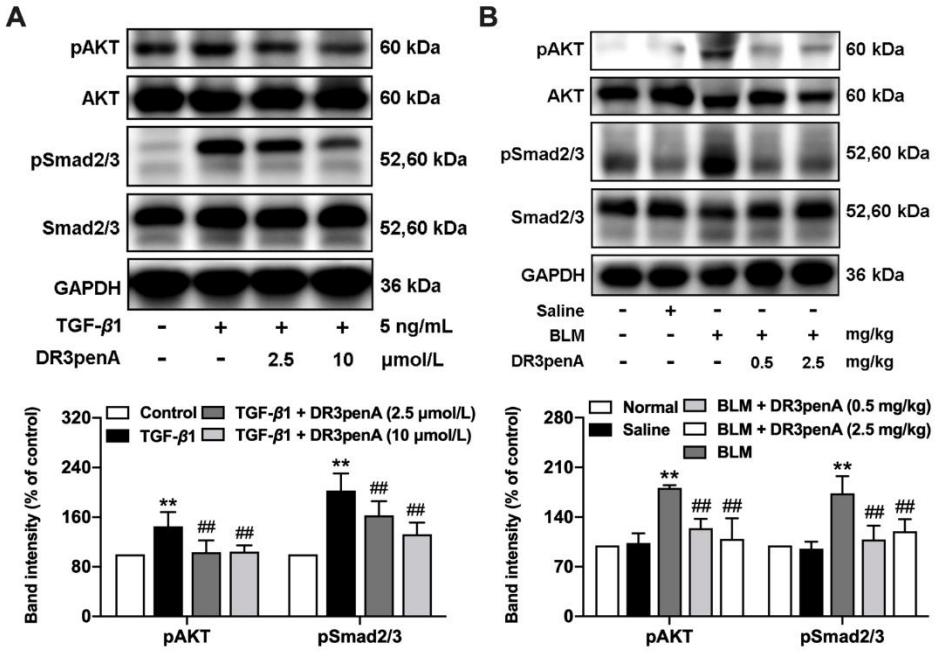


Figure S7 The effect of DR3penA on the AKT and Smad signaling pathways. Western blotting and statistical analysis of AKT and Smad2/3 phosphorylation in TGF-β1-induced A549 cells (A) and bleomycin-induced mice (B). pAKT and pSmad2/3 levels were normalized to total AKT and Smad2/3 levels, respectively. The data are presented as the mean ± SD (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. the control group or the normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. the TGF-β1 group or the BLM group.

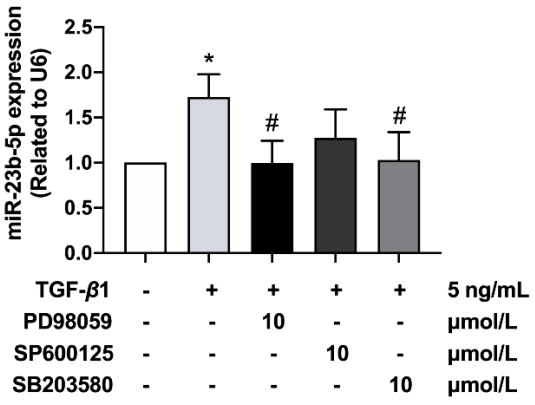


Figure S8 Real-time qPCR analysis of miR-23b-5p expression in TGF-β1-induced A549 cells pretreated with 10 μmol/L PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) or SB203580 (p38 inhibitor). The data are presented as the mean ± SD (n = 3). $^{*}P < 0.05$, $^{\#}P < 0.05$.

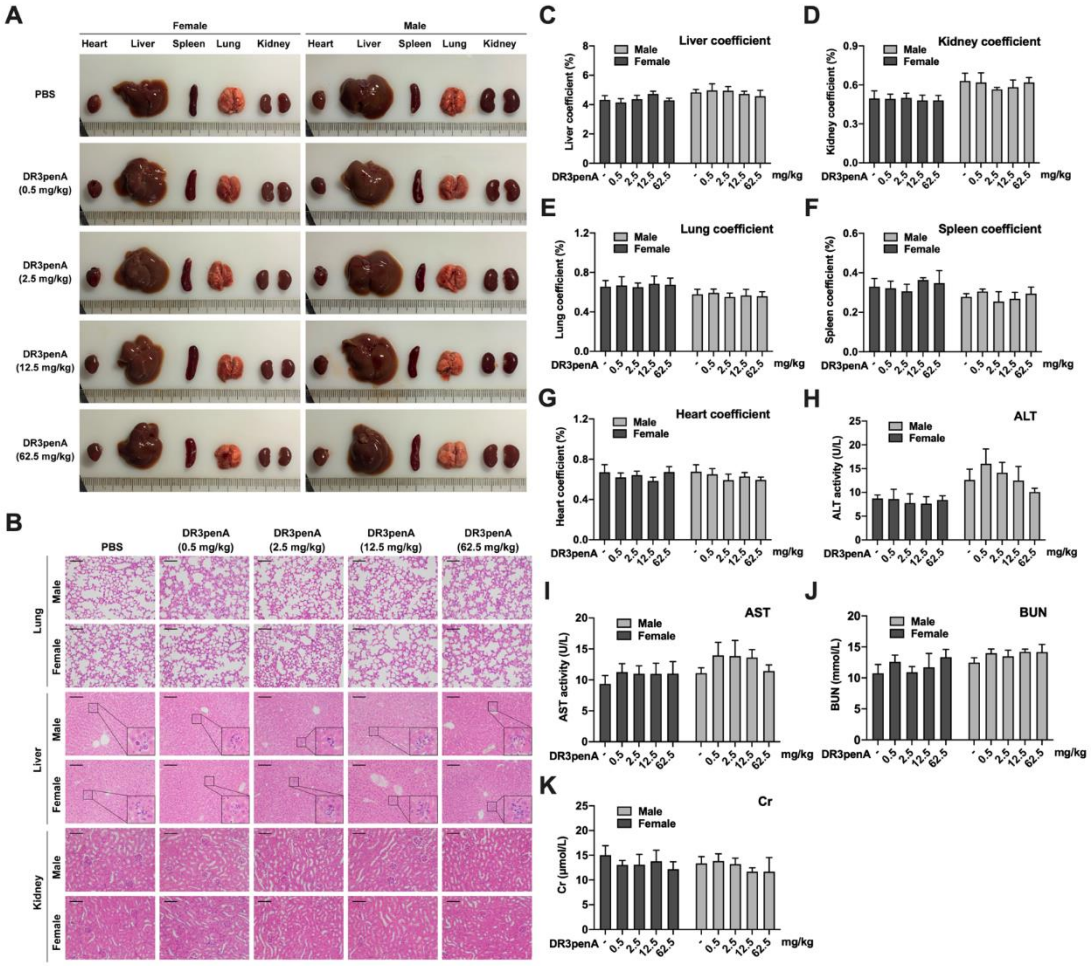


Figure S9 Acute toxicity evaluation of DR3penA at a dose of 0.5–62.5 mg/kg. (A) Gross analyses of hearts, livers, spleens, lungs and kidneys after DR3penA administration. (B) HE staining of livers, kidneys and lungs after DR3penA administration (scale bars, 100 μm). Liver (C), kidney (D), lung (E), spleen (F) and heart (G) coefficients in mice after DR3penA administration. ALT activity (H), AST activity (I), BUN level (J) and Cr level (K) in mice after DR3penA administration. The data are presented as the mean ± SD (n = 5).

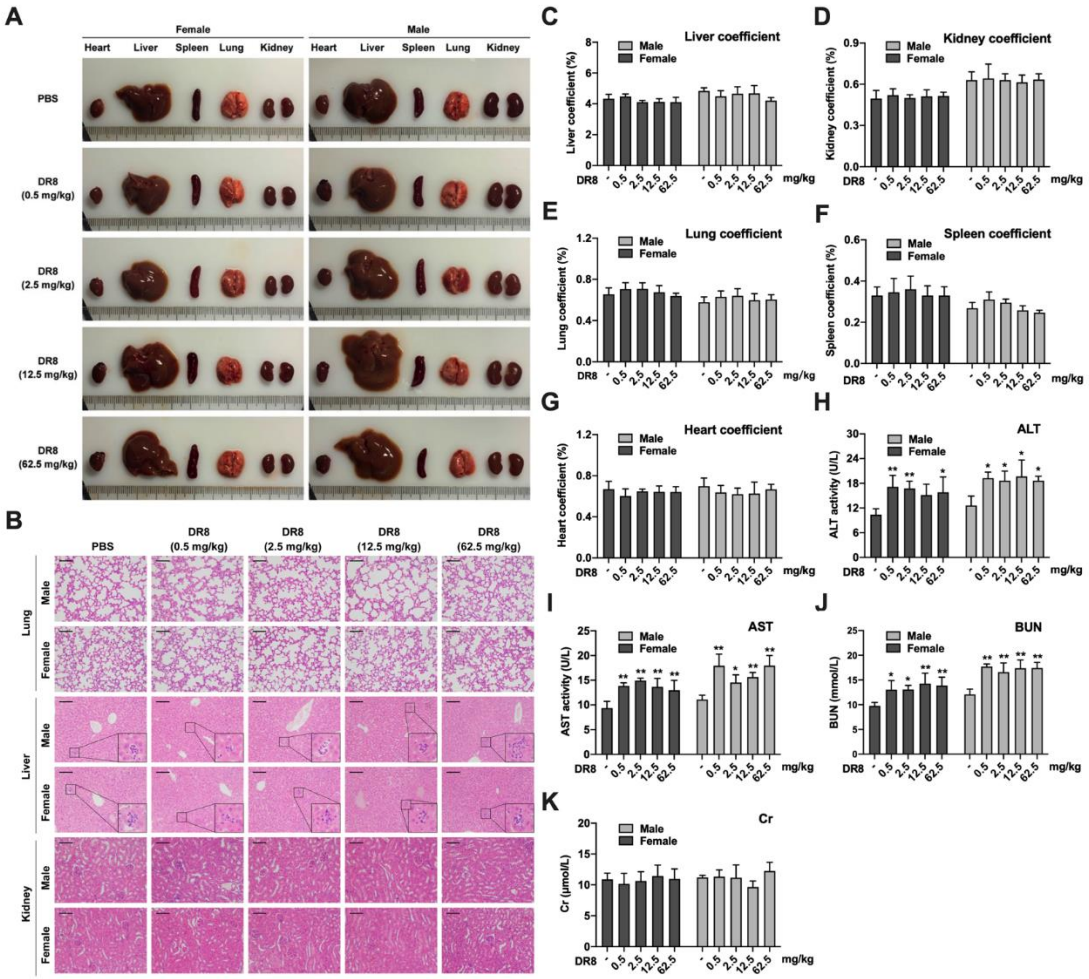


Figure S10 Acute toxicity evaluation of DR8 at a dose of 0.5–62.5 mg/kg. (A) Gross analyses of hearts, livers, spleens, lungs and kidneys after DR8 administration. (B) HE staining of livers, kidneys and lungs after DR8 administration (scale bars, 100 μm). Liver (C), kidney (D), lung (E), spleen (F) and heart (G) coefficients in mice after DR8 administration. ALT activity (H), AST activity (I), BUN level (J) and Cr level (K) in mice after DR8 administration. The data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. the control group. The data are presented as the mean ± SD ($n = 5$).

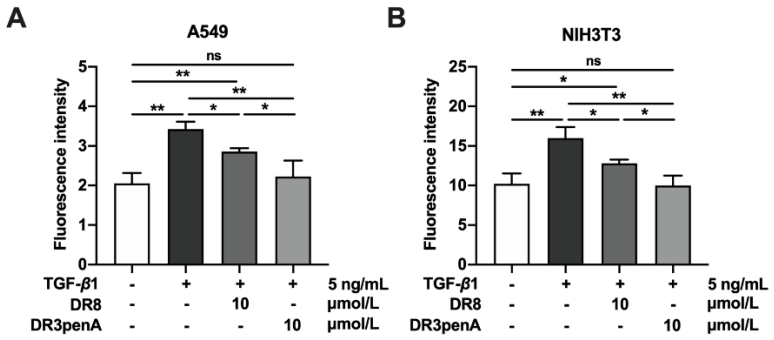


Figure S11 Antioxidant activity of DR8 and DR3penA. ROS levels were detected with DCFH-DA in TGF-β1-induced A549 cells (A) and NIH3T3 cells (B) after DR8 and DR3penA treatment. The data are presented as the mean ± SD (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$.

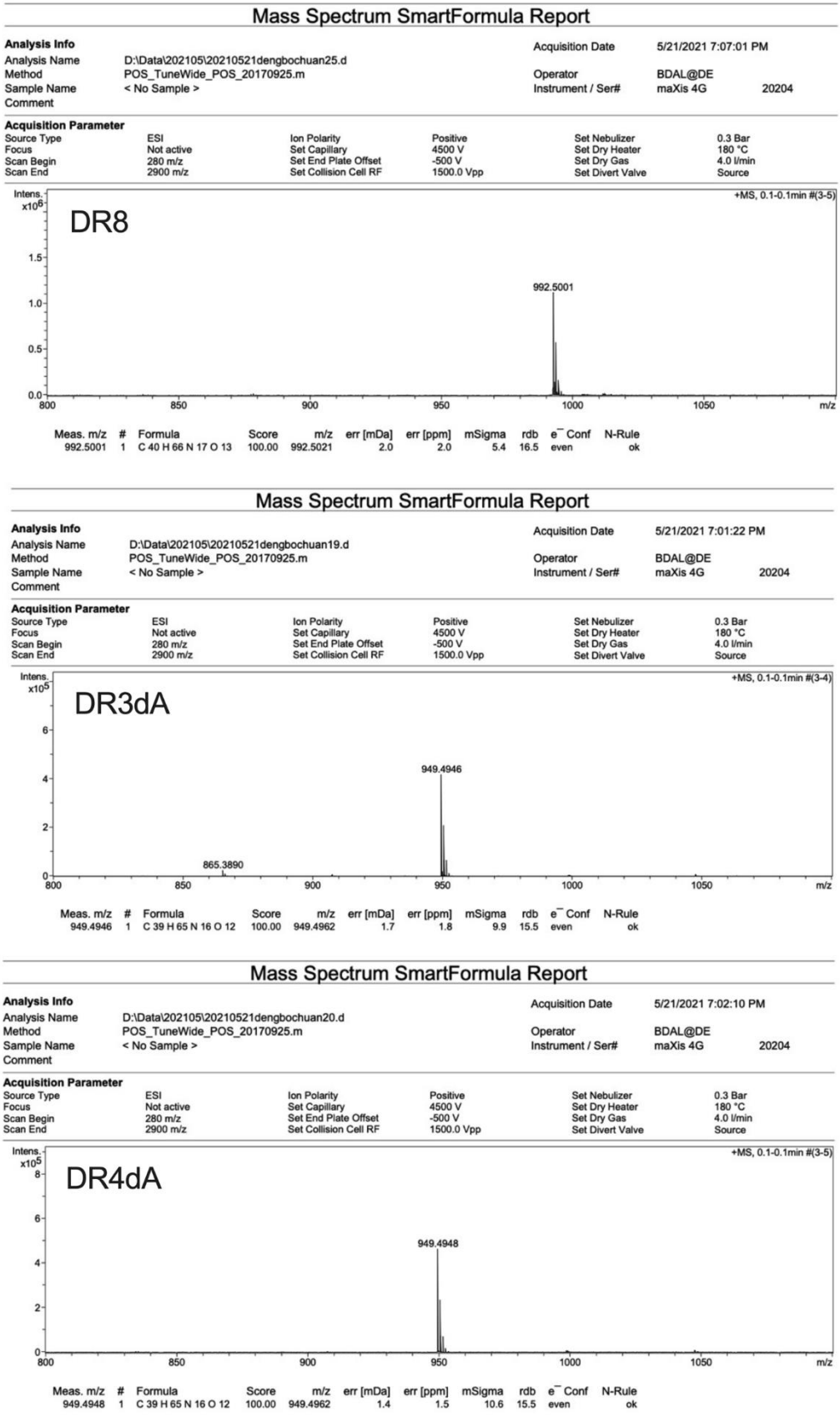


Figure S12 (continued):

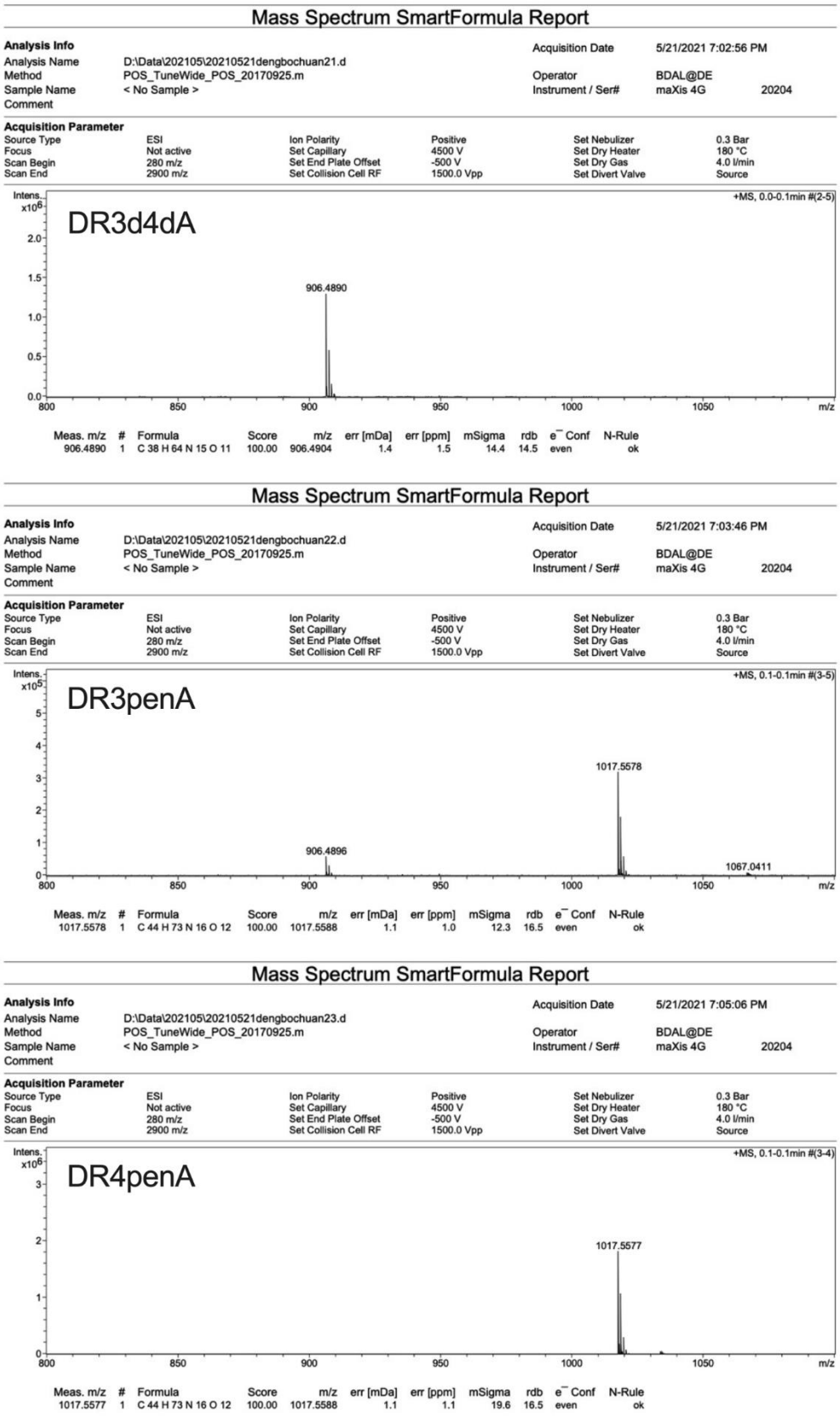
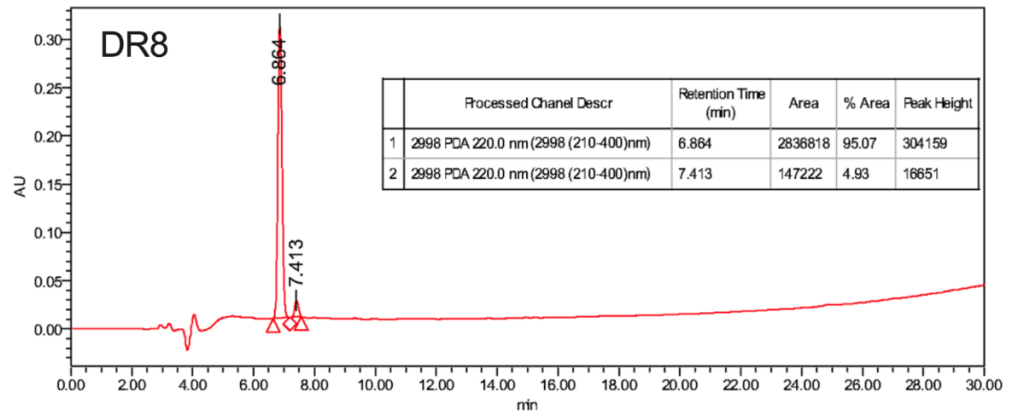
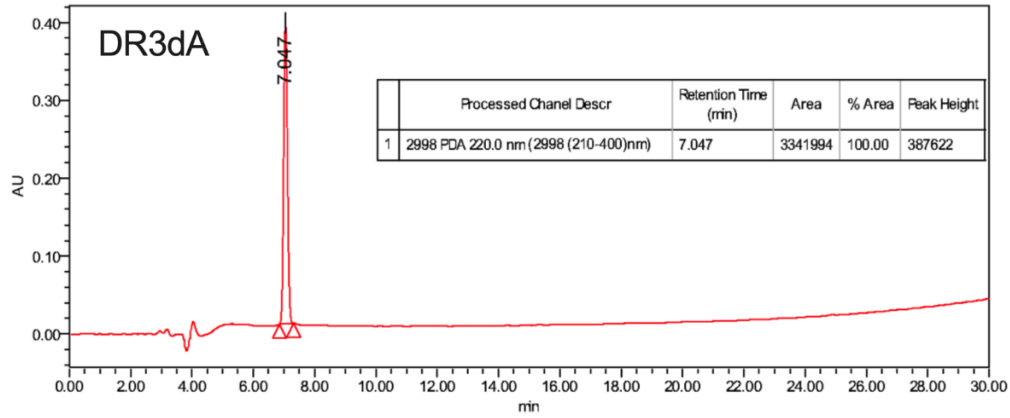


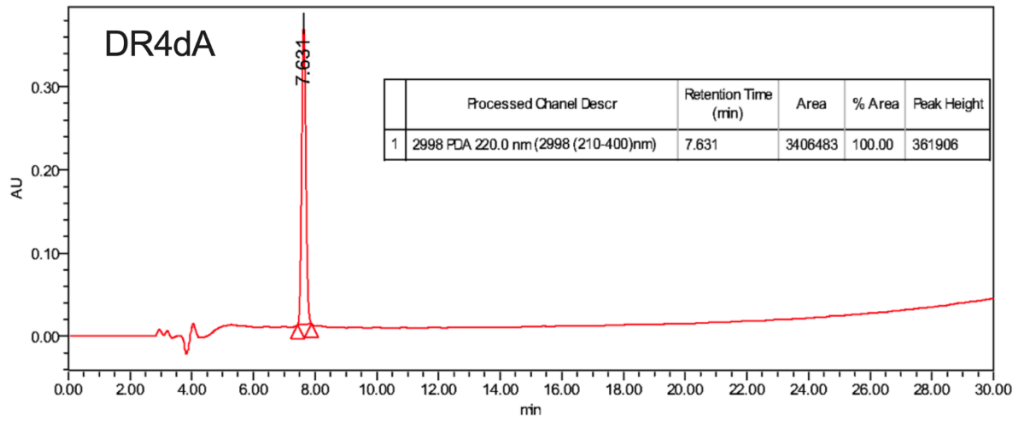
Figure S12 Identification of peptide DR8 and DR8 analogs with mass spectrometry.



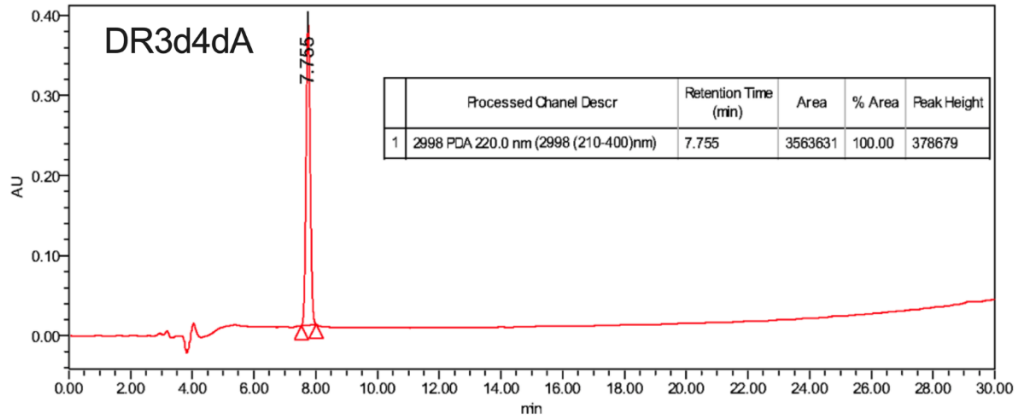
	Processed Chanel Descr	Retention Time (min)	Area	% Area	Peak Height
1	2998 PDA 220.0 nm (2998 (210-400)nm)	6.864	2836818	95.07	304159
2	2998 PDA 220.0 nm (2998 (210-400)nm)	7.413	147222	4.93	16651



	Processed Chanel Descr	Retention Time (min)	Area	% Area	Peak Height
1	2998 PDA 220.0 nm (2998 (210-400)nm)	7.047	3341994	100.00	387622



	Processed Chanel Descr	Retention Time (min)	Area	% Area	Peak Height
1	2998 PDA 220.0 nm (2998 (210-400)nm)	7.631	3406483	100.00	361906



	Processed Chanel Descr	Retention Time (min)	Area	% Area	Peak Height
1	2998 PDA 220.0 nm (2998 (210-400)nm)	7.755	3563631	100.00	378679

Figure S13 (continued):

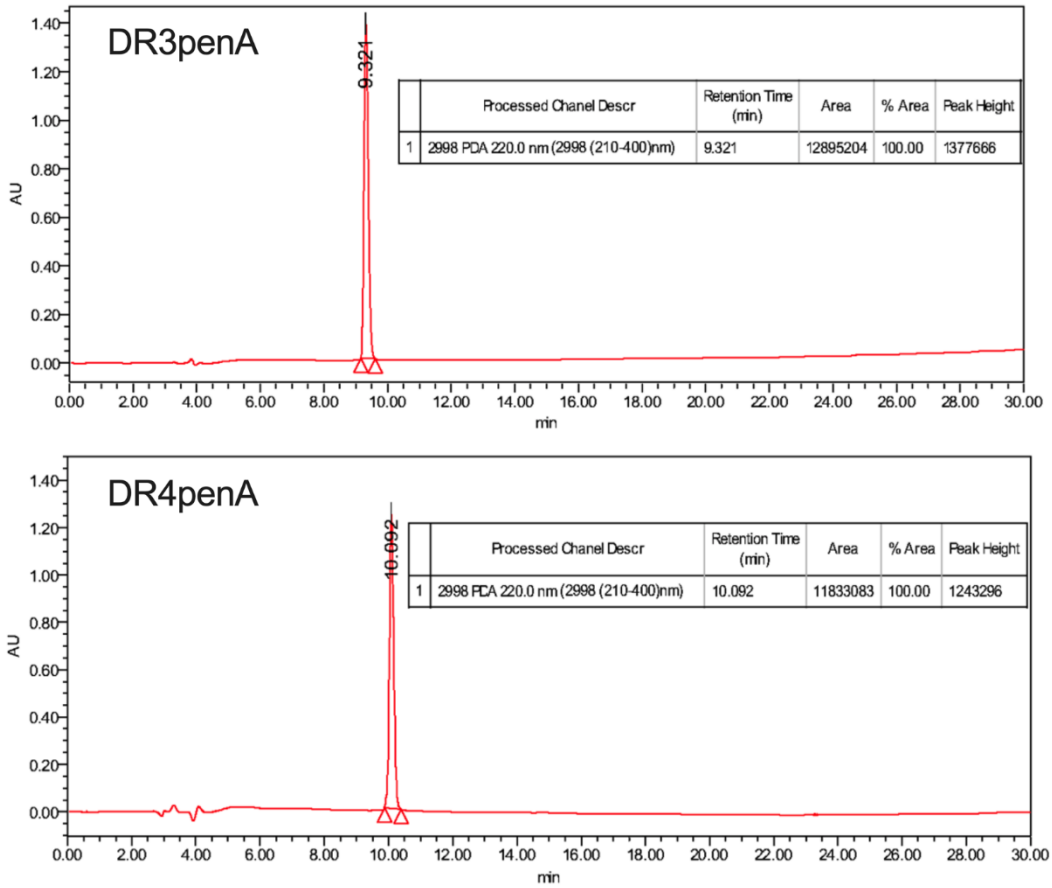


Figure S13 Purification of peptide DR8 and DR8 analogs with RP-HPLC.

Table S1 Body weight of mice after DR3penA administration (Mean ± SD, n = 5).

Day	Mice	Weight (g)				
		PBS	DR3penA (0.5 mg/kg)	DR3penA (2.5 mg/kg)	DR3penA (12.5 mg/kg)	DR3penA (62.5 mg/kg)
0	Female	25.33 ± 1.72	24.79 ± 1.68	24.87 ± 1.26	25.17 ± 1.08	25.27 ± 1.09
	Male	27.64 ± 1.90	26.76 ± 1.49	27.37 ± 1.19	27.51 ± 1.29	27.77 ± 1.60
1	Female	28.29 ± 1.97	27.63 ± 1.53	27.89 ± 1.12	27.05 ± 2.10	26.83 ± 1.32
	Male	31.69 ± 1.97	30.03 ± 0.97	31.03 ± 1.78	32.18 ± 1.44	31.11 ± 1.82
3	Female	30.96 ± 2.15	30.23 ± 1.70	30.89 ± 0.91	29.77 ± 2.36	30.15 ± 2.03
	Male	34.98 ± 2.18	33.14 ± 1.43	33.07 ± 1.79	34.78 ± 2.15	34.57 ± 1.81
5	Female	33.37 ± 2.48	32.35 ± 1.66	33.24 ± 1.01	32.54 ± 3.57	32.28 ± 2.31
	Male	40.09 ± 2.79	35.37 ± 1.22**	38.01 ± 2.87	38.82 ± 2.59	36.94 ± 2.02
7	Female	33.53 ± 2.14	32.97 ± 2.41	33.90 ± 2.00	32.65 ± 3.80	32.61 ± 2.67
	Male	38.77 ± 2.82	36.35 ± 1.66	37.22 ± 2.79	37.00 ± 2.98	37.37 ± 1.56
9	Female	33.38 ± 2.13	31.73 ± 1.47	34.01 ± 1.74	32.72 ± 3.64	33.02 ± 2.88
	Male	39.17 ± 2.67	37.50 ± 1.37	39.08 ± 2.62	40.53 ± 2.79	39.13 ± 1.46
11	Female	34.04 ± 2.53	32.98 ± 2.78	34.45 ± 1.76	33.71 ± 3.66	34.72 ± 2.53
	Male	41.43 ± 2.52	38.35 ± 1.26	40.53 ± 2.86	41.05 ± 3.28	40.87 ± 2.11
13	Female	35.78 ± 2.84	34.26 ± 2.33	35.99 ± 2.86	35.51 ± 3.60	35.39 ± 3.33
	Male	42.79 ± 2.78	39.15 ± 1.11*	42.54 ± 3.12	43.87 ± 3.61	42.50 ± 2.35

*P < 0.05, **P < 0.01 *vs*. the PBS control group.

Table S2 Body weight of mice after DR8 administration (mean ± SD, n = 5).

Day	Mice	Weight (g)				
		PBS	DR8 (0.5 mg/kg)	DR8 (2.5 mg/kg)	DR8 (12.5 mg/kg)	DR8 (62.5 mg/kg)
0	Female	25.33 ± 1.72	24.72 ± 1.79	24.96 ± 1.21	25.10 ± 1.10	25.39 ± 1.11
	Male	27.64 ± 1.90	26.80 ± 1.52	27.20 ± 1.12	27.52 ± 1.24	27.82 ± 1.57
1	Female	28.29 ± 1.97	26.51 ± 1.96	26.49 ± 1.95	28.10 ± 2.17	28.28 ± 1.07
	Male	31.69 ± 1.97	31.03 ± 1.60	30.73 ± 1.07	31.33 ± 1.18	31.33 ± 2.22
3	Female	30.96 ± 2.15	29.21 ± 2.52	29.03 ± 1.58	31.19 ± 1.89	30.96 ± 0.71
	Male	34.98 ± 2.18	33.46 ± 2.48	33.58 ± 1.46	33.60 ± 1.67	34.22 ± 2.55
5	Female	33.37 ± 2.48	31.51 ± 2.55	30.78 ± 1.97	34.10 ± 1.82	33.75 ± 2.44
	Male	40.09 ± 2.79	38.79 ± 3.07	37.63 ± 1.79	37.40 ± 1.98	38.12 ± 3.58
7	Female	33.53 ± 2.14	30.97 ± 3.07	31.52 ± 1.72	33.68 ± 1.53	33.99 ± 1.74
	Male	38.77 ± 2.82	36.06 ± 3.05	37.24 ± 1.87	36.87 ± 1.83	38.03 ± 2.97
9	Female	33.38 ± 2.13	32.22 ± 2.84	31.45 ± 1.78	34.27 ± 1.14	33.93 ± 2.58
	Male	39.17 ± 2.67	39.65 ± 2.84	37.95 ± 1.50	38.70 ± 2.10	39.24 ± 3.40
11	Female	34.04 ± 2.53	33.08 ± 3.13	31.98 ± 1.90	35.51 ± 1.40	34.63 ± 2.02
	Male	41.43 ± 2.52	40.83 ± 2.87	39.19 ± 2.01	39.51 ± 2.53	41.30 ± 3.68
13	Female	35.78 ± 2.84	34.22 ± 2.48	33.45 ± 2.30	37.45 ± 1.96	35.89 ± 3.06
	Male	42.79 ± 2.78	43.66 ± 2.81	41.12 ± 2.40	41.82 ± 2.83	42.53 ± 4.34

Table S3 Antibodies used for western blotting, immunohistochemistry and immunofluorescence.

Antibodies	Species	WB	IHC	IF	Source
α-SMA	Rabbit	1:500			Abcam
E-cadherin	Rabbit	1:1000			Servicebio
E-cadherin	Mouse			1:200	Abcam
Vimentin	Rabbit	1:1000		1:200	Cell Signaling Technology
Collagen I	Rabbit	1:1000			Cell Signaling Technology
Collagen I	Rabbit	1:1000	1:500	1:100	Servicebio
Fibronectin	Rabbit	1:1000	1:200		Abcam
MMP2	Rabbit	1:500			Servicebio
TGF-β1	Rabbit	1:1000			Abcam
AQP5	Rabbit	1:1000			Proteintech
pERK	Rabbit	1:1000			Cell Signaling Technology
p-p38	Rabbit	1:1000			Cell Signaling Technology
pSmad2/3	Rabbit	1:1000			Cell Signaling Technology
pAKT	Rabbit	1:1000			Cell Signaling Technology
ERK	Rabbit	1:1000			Beyotime
p38	Rabbit	1:1000			Cell Signaling Technology
Smad2/3	Rabbit	1:1000			Cell Signaling Technology
AKT	Rabbit	1:1000			Cell Signaling Technology
GAPDH	Rabbit	1:5000			Proteintech
HRP-conjugated IgG	Goat	1:10,000			ImmunoWay
CoraLite594-conjugated Goat Anti-Rabbit IgG	Goat			1:200	Proteintech
CoraLite488-conjugated Goat Anti-Mouse IgG	Goat			1:200	Proteintech

Table S4 Primer pairs used for quantitative real-time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Human		
ACTA2	ACTGCCTTGGTGTGTGACAA	CACCATCACCCCCTGATGTC
VIM	AGGCAAAGCAGGAGTCCACTGA	ATCTGGCGTTCCAGGGACTCAT
CDH1	CTTCTGCTGATCCTGTCTGATG	TGCTGTGAAGGGAGATGTATTG
FN1	ACAACACCGAGGTGACTGAGAC	GGACACAACGATGCTTCCTGAG
COL1A1	GTGCGATGACGTGATCTGTGA	CGGTGGTTTCTTGGTCGGT
MMP2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC
ABCA1	AGCTGTTCACCGACAATAAG	CCACCAAGTCCCAAGATAA
NKD1	AGACACGCTCAGCGAGGAA	TGCCGTTGTTGTCAAAGTCATA
CMTM4	TTCAATCGTACTGGCTGCTTT	CCAGGAATGTGTTCACTGCATA
GPX3	CATTCGGTCTGGTCATTCTGG	GGTCGGACATACTTGAGGGTA
MTMR4	GGACTCCAAAGTGGTGAGGTG	ACCAGGCATGGTAGGCAAAG
AQP5	GCCATCCTTTACTTCTACCTGC	CTCCTCCCAGTCCTCGTCA
STK38	TCAGCACATGCTCGGAAGG	ACAAGCCGTACCTCACCAAAT
SIRT5	GTCCACACGAAACCAGATTTGCC	TCCTCTGAAGGTCGGAACACCA
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
Mouse		
Acta2	ACTGGGACGACATGGAAAAG	CATCTCCAGAGTCCAGCACA
Col1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Vim	CGTCCACACGCACCTACAG	GGGGGATGAGGAATAGAGGCT
Cdh1	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
Mmp2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC
Fn1	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG
Sod1	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
Sod2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
Cat	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG
Nox1	GGTTGGGGCTGAACATTTTTC	TCGACACACAGGAATCAGGAT
Nox4	GAAGGGGTTAAACACCTCTGC	ATGCTCTGCTTAAACACAATCCT
Gapdh	AGGAGTAAGAAACCCTGGAC	CTGGGATGGAATTGTGAG

Table S5 Globally harmonized classification system.

Acute toxicity level	LD50（mg/kg BW）
I	0.5–5
II	5–50
III	50–300
IV	300–2000
V	2000–5000
Unclassified	＞ 5000

Supplemental Materials and Methods

Cell cytotoxicity assay

MTT assays were used to assess the cytotoxicity of NIH3T3 and A549 cells. NIH3T3 cells plated in 96-well plates were treated with DR8 analogs at different concentrations (0, 10, 20, 40, 80 and 160 μmol/L) for 24 h when cells reached a confluence of 80%. A549 cells were treated with DR3penA at the indicated concentrations for 48 h. Then, 10 μL of MTT (5 mg/mL, Sigma–Aldrich) was incubated with cells for 4 h, and 150 μL of DMSO was used to dissolve the formazan crystals after the supernatant in the wells was discarded. The absorbance at 570 nm was detected with a multimode microplate reader (MD, CA, USA). The formula used to calculate cell viability was as follows: cell viability (%) = $(A_{treated} - A_{blank}) / (A_{control} - A_{blank}) \times 100$.

DR8 analogs were diluted in serum-free medium at concentrations of 10, 20, 40, 80 and 160 μmol/L to measure toxic effects. NIH3T3 cells were incubated for 24 h, and A549 cells were incubated for 48 h.

Lung injury in mice after DR3penA treatment

Female C57BL/6 mice were randomly divided into 2 groups (n = 8 per group): (1) normal control group (normal); (2) DR3penA (2.5 mg/kg) group. Mice in the normal group and DR3penA group were subcutaneously injected with PBS or DR3penA (2.5 mg/kg) every day for 21 days, respectively. Mice were sacrificed on the 22nd day, and blood and lung tissue samples were collected for follow-up studies.

Dual-luciferase reporter assay

293T cells were cultured in 24-well plates at 1×10^5 cells/well, and cotransfected with miR-23b and AQP5-3'UTR wild-type (WT) or AQP5-3'UTR mutant (Mut) plasmids for 48 h. The cells were lysed with Passive Lysis Buffer at 4 ℃ for 20 min. Luciferase activity was measured with a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA). After quantification of the firefly luciferase fluorescence intensity of, 20 μL of Stop & Glo® Reagent was added to each well to terminate the reaction, and then, the Renilla luciferase activity was detected using a microplate reader.

Quantitative detection of intracellular ROS using DCFH-DA

The intracellular ROS levels in NIH3T3 cells and A549 cells were detected using the fluorescent probe DCFH-DA (Sigma–Aldrich). Cells were plated in the black 96 well plates for 24 h. After starvation with serum-free medium overnight, the cells were treated with TGF-β1 alone or with DR8 or DR3penA for 12 h. Then, 10 μmol/L DCFH-DA dissolved in DMSO and diluted with serum-free medium was added to the cells for 30 min at 37 °C. The fluorescence intensity of DCFH-DA was detected with a multimode microplate reader after washing away the DCFH-DA that failed to enter the cells with PBS. The fluorescence excitation and emission wavelengths were 488 nm and 525 nm, respectively.