A novel RANKL/RANK inhibitor IMB-R38 inhibits osteoporosis through regulating bone metabolism

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Supplemental Materials and Methods:

Supplementary Methods.

Supplementary Table S1-S2;

Supplementary Figure S1-S3.

1. Supplementary Methods

1.1 Expression and purification of RANKL and RANK protein

The BL21 (DE3) harboring the pGEX-4T-RANKL plasmid was cultured in LB medium containing 100 μg/ml ampicillin at 37 ℃ with agitation (200 rpm) until optical density at 600 nm (OD_{600}) reached 0.6~0.8. Following mid-log phase growth, recombinant protein expression was induced by adding 0.05 mM isopropyl β-D-thiogalactopyranoside (IPTG) (I8070, Solarbio, Beijing, China) and incubating at 22 ℃ overnight. Cells were then collected by centrifugation and subsequently lysed in lysis buffer 1 (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM lysozyme, and 0.5% (v/v) Triton X-100, pH = 8.0). After centrifugation, the lysate supernatant was collected and loaded onto a GST column (GE Healthcare, USA) with an AKTA Explorer System (GE Healthcare, USA). After washing the

column-bound glutathione S-transferase (GST)-RANKL fusion protein, the RANKL was cleaved and eluted from the beads by on-column treatment with PreScission Protease (GE Healthcare, USA) for 4 hours at 4℃ (Figure S2A).

BL21 (DE3) containing the pET28a (+)-RANK plasmid was cultured in LB medium supplemented with 100 μg/ml kanamycin at 37 ℃ with agitation at 250 rpm until the OD_{600} reached 0.6~0.8. Then 1 mM IPTG was added and the culture was incubated for 4 h. Following incubation, BL21 cells were harvested via centrifugation, and the precipitate which contained the inclusion bodies was re-suspended in 100 ml of ice-cold washing buffer 2 (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% v/v Triton X-100, pH = 8.0). Finally, the cells were disrupted and homogenized using sonication, followed by thorough washing of the inclusion bodies.

To refold the RANK protein, the inclusion bodies firstly were dissolved in buffer 3 (6 M Guanidine–HCl, 50 mM Tris, 1 mM EDTA, 150 mM NaCl, 0.5 M L-arginine, 20% v/v glycerol, 10 mM DTT, pH = 8.0) at RT. After centrifugation, supernatant was collected and transferred into a dialysis bag and subsequently dialyzed by buffer 4 (2 M Guanidine–HCl, 0.5 M L-arginine, 50 mM Tris, 1 mM EDTA, 150 mM NaCl and 10% v/v glycerol, pH = 8.0) for 12 h. Then, a second dialysis was performed using buffer 5 (0.5 M Guanidine–HCl, 0.2 M L-arginine, 50 mM Tris, 1 mM EDTA, 150 mM NaCl and 5% v/v glycerol, pH = 8.0) for 12 h. Finally, the solution was dialyzed by PBS (containing 1 mM DDT, pH = 7.3). After that, the solution in the dialysis bag which containing RANK refolding protein was centrifuged and collected for further experiment (Figure S2B).

1.2 Cellular thermal shift assay (CESTA)

RAW264.7 cells were treated with IMB-R38 (10 μM) for 6 h and then washed three times with ice-cold PBS. The cell pellets were equally aliquoted into eight PCR tubes (50 μL/tube) and subjected to thermal challenge in a PCR thermocycler (AL050123, Bio-Rad, USA) under the following conditions: incubation at specified temperatures (range: 37-48.9°C) for 3 min, followed by equilibration at 25 °C for 3 min. To ensure complete cell lysis, samples underwent three freeze-thaw cycles alternating between liquid nitrogen immersion and 37 °C water bath. After centrifugation at 13,500 × g for 30 min at 4 °C, 40 μL of supernatant was mixed with

10 μL 5× loading buffer (P1040, Solarbio, Beijing, China,) and denatured at 100 °C for 10 min. The relative abundance of RANK and RANKL was measured by Western blot.

To further assess the concentration-dependent stabilizing effects of IMB-R38 on RANK and RANKL protein, RAW264.7 cells were pretreated with serially diluted IMB-R38 (0.1-10 μM) or equivalent DMSO for 6 h. The cell lysates were then subjected to thermal stress through sequential incubation at 45 °C for 3 min followed by 25 °C for 3 min. After high-speed centrifugation (13,500 × g, 30 min, 4 °C), the supernatant fractions were analyzed by Western blot.

Supplementary Table S1. Antibodies used in Western blot.

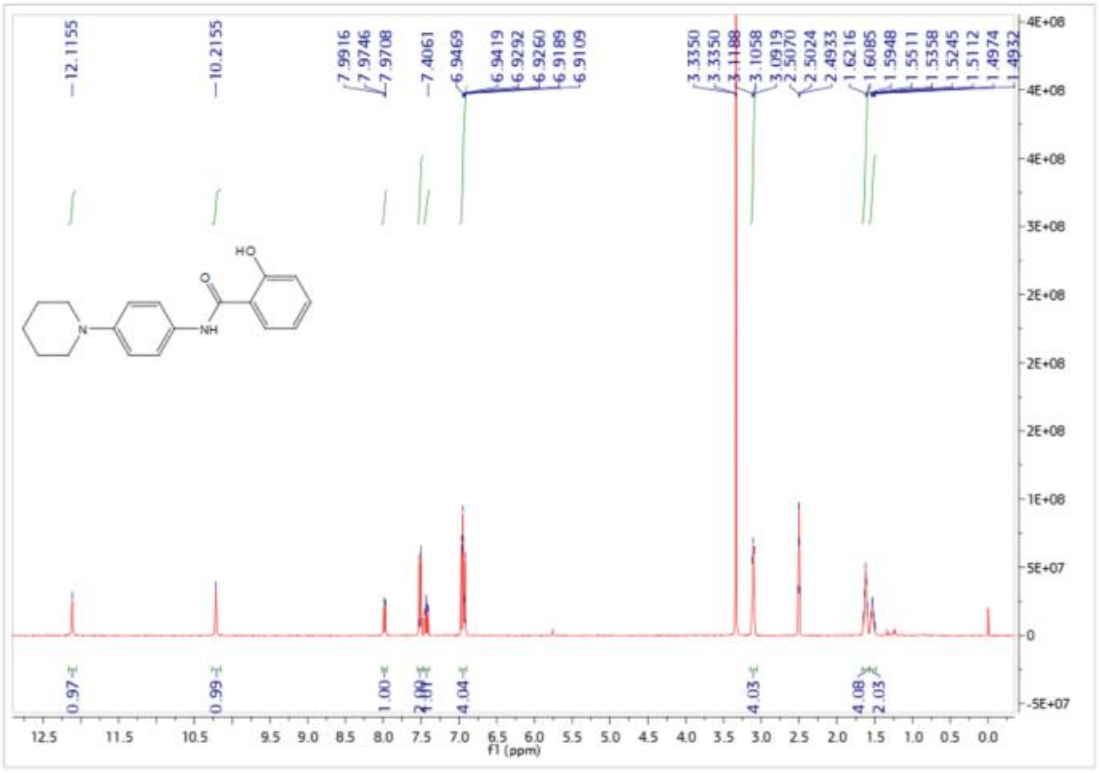
Targetantigen	Vendor orSource	Catalog #	Workingconcentration	Persistent ID / URL
OPG	abcam	ab183910	1:500	https://www.abcam.cn/products/primary-antibodies/osteoprotegerin-antibody-ab183910.html
BMP2	abcam	ab284387	1:1000	https://www.abcam.cn/products/primary-antibodies/bmp2-antibody-epr24209-61-ab284387.html
RUNX2	abcam	ab236639	1:1000	ab236639https://www.abcam.cn/products/primary-antibodies/runx2-antibody-epr22858-106-chip-grade-ab236639.html
β-catenin	abcam	ab32572	1:5000	https://www.abcam.cn/products/primary-antibodies/beta-catenin-antibody-e247-chip-grade-ab32572
Phospho-Smad1/5/9	selleck	F2875	1:1000	https://www.selleck.cn/antibodies/phospho-smad1-5-9-s463-465-467-antibody-k12g16.html
RANK	abcam	ab305233	1:1000	https://www.abcam.cn/products/primary-antibodies/rank-antibody-epr26196-15-ab305233.html
RANKL	abcam	ab62516	1:1000	https://www.abcam.cn/products/primary-antibodies/srankl-antibody-ab62516.html
MMP9	abcam	ab38898	1:1000	https://www.abcam.cn/products/primary-antibodies/mmp9-antibody-ab38898.html
NFATc1	abcam	ab2976	1:1000	https://www.abcam.cn/products/primary-antibodies/nfat2-antibody-7a6-ab2796.html
PI3K	CST	4257	1:1000	https://www.cellsignal.cn/products/primary-antibodies/pi3-kinase-p85-19h8-rabbit-monoclonal-antibody/4257
p-PI3K	CST	4228	1:1000	https://www.cellsignal.cn/products/primary-antibodies/phospho-pi3-kinase-p85-tyr458-p55-tyr199-antibody/4228
AKT	CST	4691	1:1000	https://www.cellsignal.cn/products/primary-antibodies/akt-pan-c67e7-rabbit-monoclonal-antibody/4691
P-AKT	CST	4060	1:2000	https://www.cellsignal.cn/products/primary-antibodies/phospho-akt-ser473-d9e-rabbit-monoclonal-antibody/4060
ERK	CST	4695	1:1000	https://www.cellsignal.cn/products/primary-antibodies/p44-42-mapk-erk1-2-137f5-rabbit-monoclonal-antibody/4695
P-ERK	Biodragon	BD-PB5113	1:1000	https://www.biodragon.cn/cn/goods/goodsView?GoodsId=79598&Catalog=
JNK	Biodragon	RM1210	1:1000	https://www.biodragon.cn/cn/goods/goodsView?GoodsId=53634&Cat

Targetantigen	Vendor orSource	Catalog #	Workingconcentration	Persistent ID / URL
				alog=
p-JNK	CST	4668	1:1000	https://www.cellsignal.cn/products/primary-antibodies/phospho-sapk-jnk-thr183-tyr185-81e11-rabbit-monoclonal-antibody/4668
P38	Bioworld	AP0424	1:1000	https://www.bioworlde.com/Primary-Antibodies/139978.html
p-P38	abcam	ab195049	1:1000	https://www.abcam.cn/products/primary-antibodies/p38-phospho-t180-y182-antibody-epr18120-ab195049
P65	abcam	Ab16502	1：1000	https://www.abcam.cn/products/primary-antibodies/nf-kb-p65-antibody-ab16502
P-P65	Bioss	bs-0982R	1:1000	https://www.biosschina.com/productDetail?goods_id=7439
lkB	Biodragon	RM0145	1:1000	https://www.biodragon.cn/cn/goods/goodsView?GoodsId=52581&Catalog=
p-lkB	abcam	9246	1:1000	https://www.cellsignal.cn/products/primary-antibodies/phospho-ikappab-alpha-ser32-36-5a5-mouse-monoclonal-antibody/9246
α-tubulin	Proteintech	66031-1-Ig	1:50000	https://www.ptgcn.com/products/tubulin-Alpha-Antibody-66031-1-Ig.htm
GAPDH	Proteintech	10494–1-AP	1:50000	https://ptgcn.com/products/GAPDH-Antibody-10494-1-AP.htm

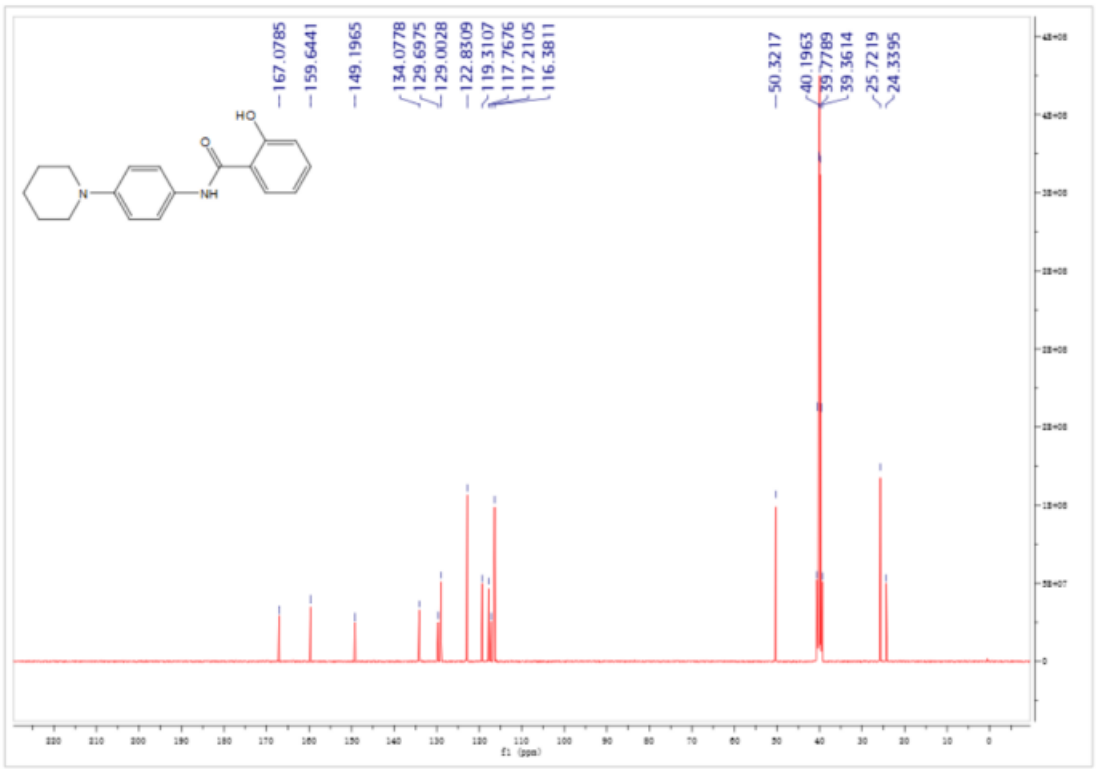
Supplementary Table S2. Primers Sequences of the RT-qPCR.

Gene	Forward Primer	Reverse Primer
Bmp2b	GAAGTATCCGAGGAGGCTGA	CCTCCACCACCATGTCCT
Runx2a	AGACTCCGACCTCACGACAACC	GGCAGCACCGAGCACAGAAAG
Runx2b	GAAGCGGAAGAGGGAAGAGC	AATGGCTCTGTGGTAAGTGGC
Sp7	AAGAAACCTGTCCACAGCTG	GAGGCTTTACCGTACACCTT
Alp	CTGGTGGAAGGTGGACGCATTG	CGTGTCATACTCGCTTGTCAGGAG
Oc	TGGCCTCTATCATCATGAGACAGA	CTCTCGAGCTGAAATGGAGTCA
Mmp9	CAGAGAGGAAAAGGCAAGGTG	AGGAATACATCATGTGAATCAATG
Mmp2	CTGGGAACAAGTTCTGGAGATAC	GTGAGTCTTTGAAGAAGTAGCTG
Mmp13	AGACCAGGACACACTCGCAGAG	CTGCTGCATCTCCTTGAGCCT
Il-6	GCGTCCTGACGTGGTATAAAG	GTCGTTTGGTGCTGTGTTTG
Aggrecan	ACAGGTGTGGTCTTCCATTAC	GACTGCGCCGATACTCTTAC

A

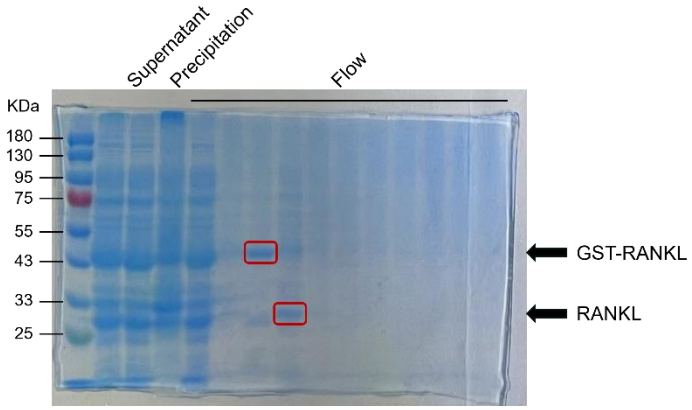


B

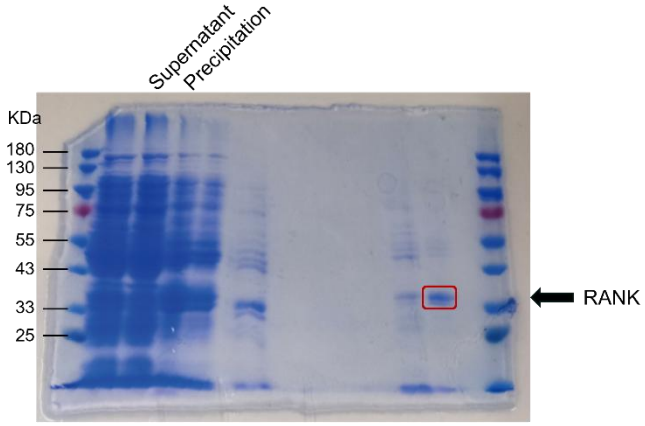


Supplementary Figure S1. (A, B) ^{1}H NMR, ^{13}C NMR for compound IMB-R38.

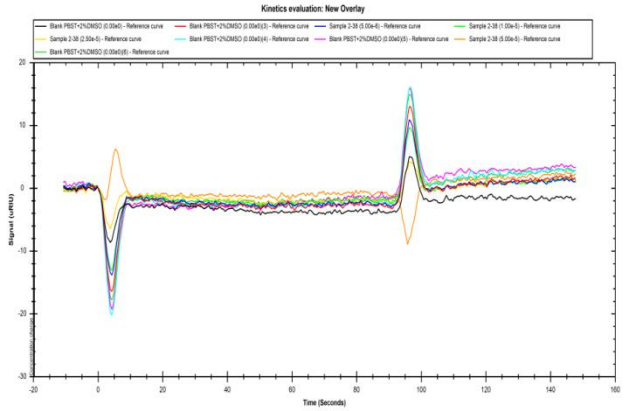
A



B



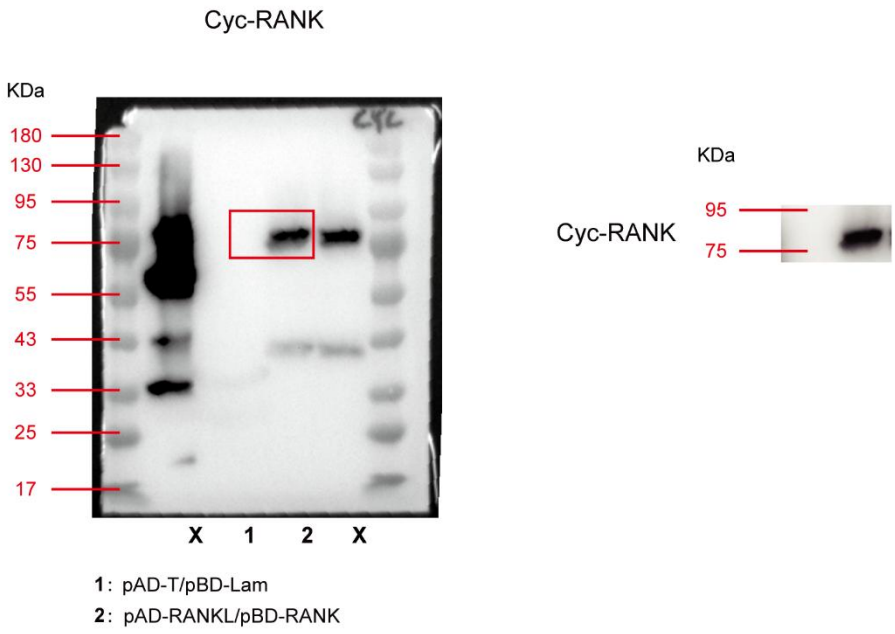
C



Supplementary Figure S2. (A-B) The SDS-PAGE of the recombinant GST-tagged RANKL and his-tagged RANK. The red boxes are labeled with purified RANKL or RANK. (C) SPR sensorgram for the interaction between IMB-R38 and RANKL protein.

Supplementary Figure S3. All uncropped membranes

Figure 1B



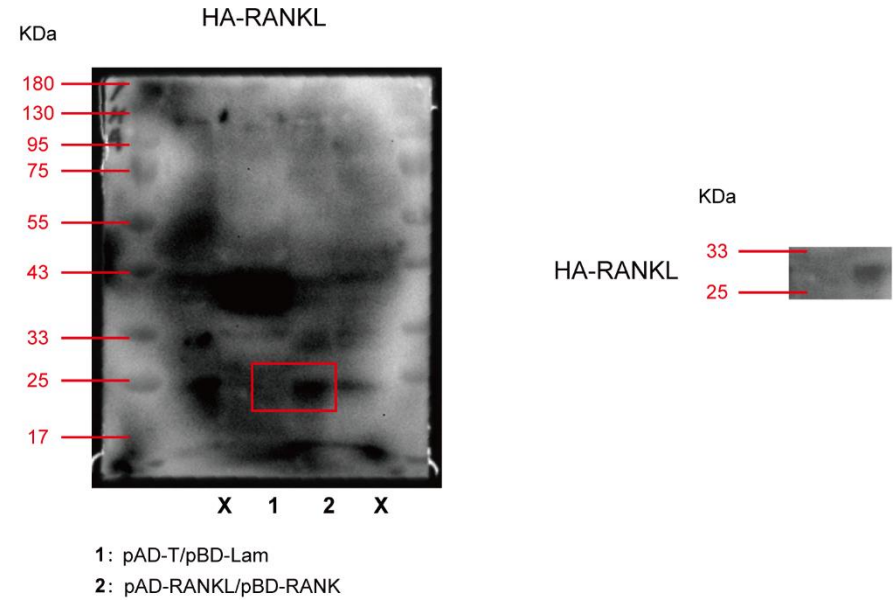


Figure 2E

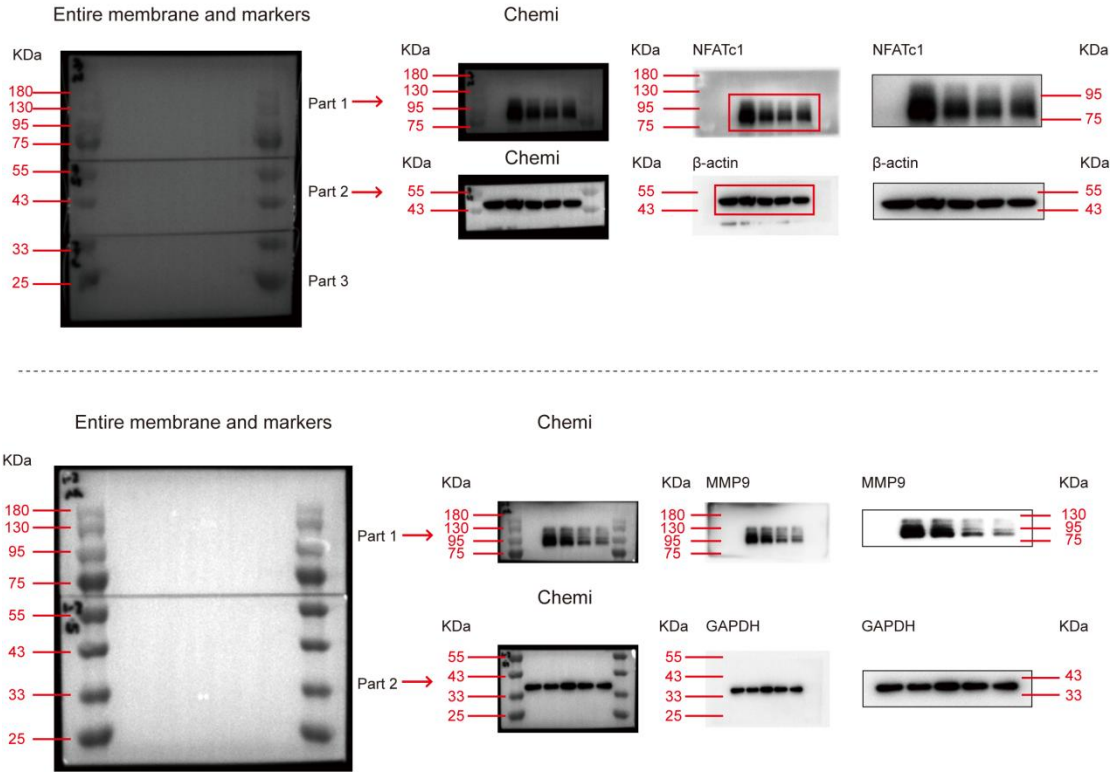

Entire membrane and markers
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Figure 3C

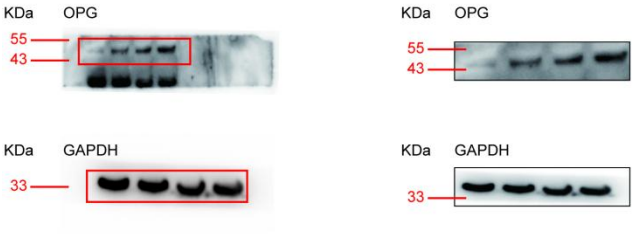


Figure 3F

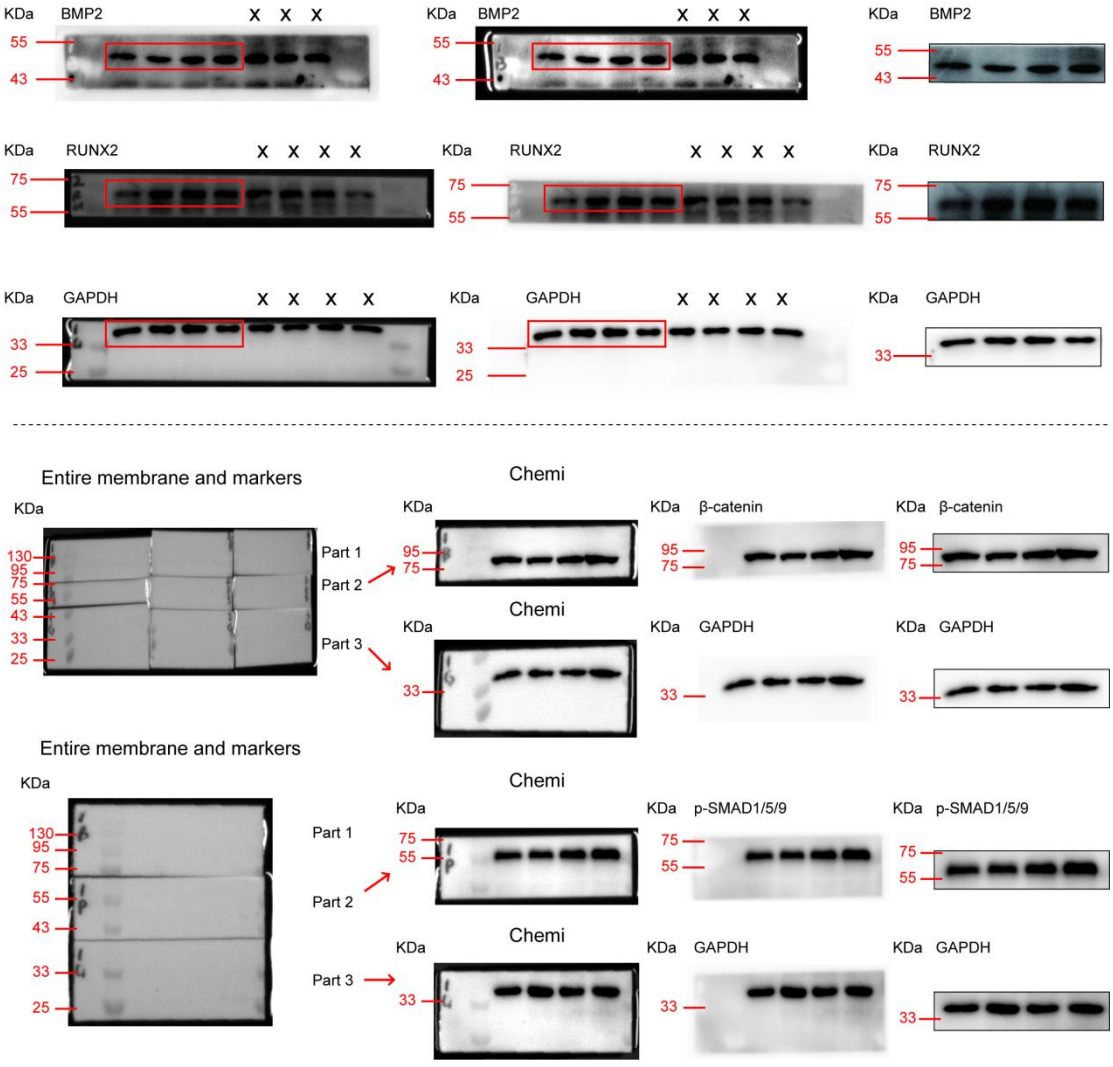


Figure 5A

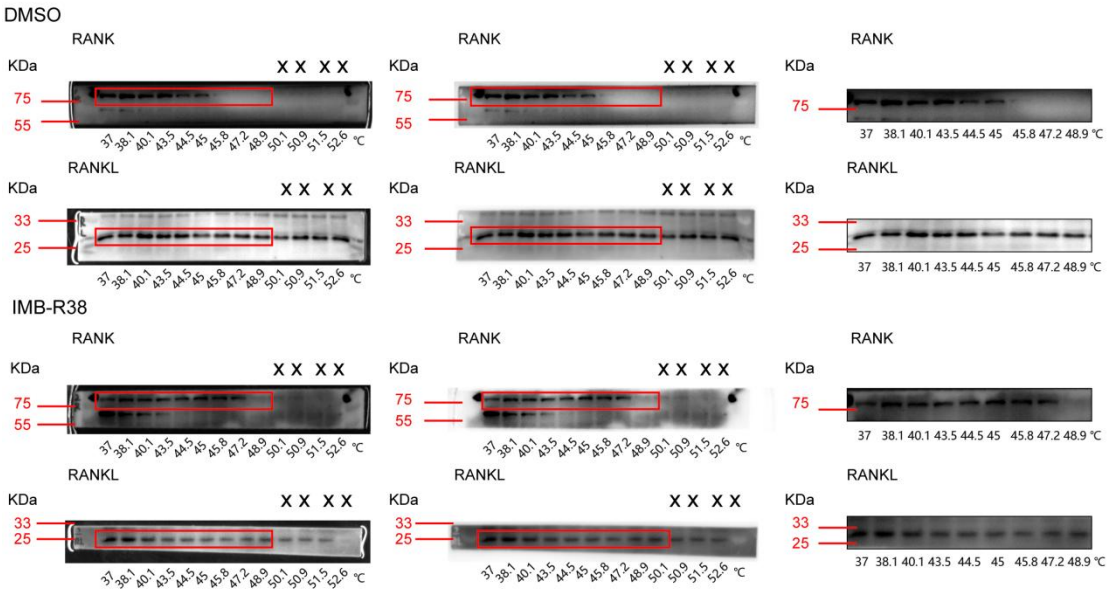


Figure 5D

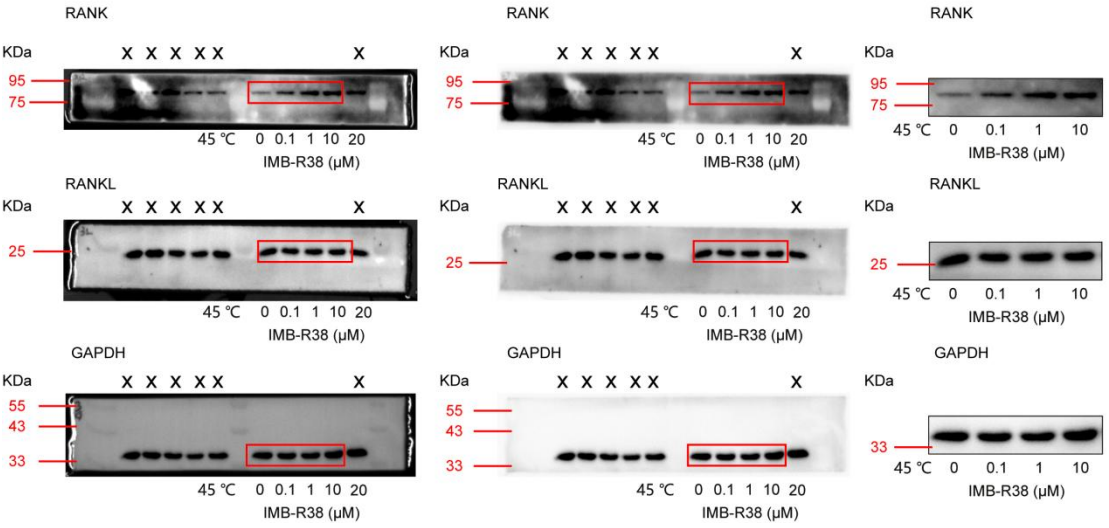


Figure 6A

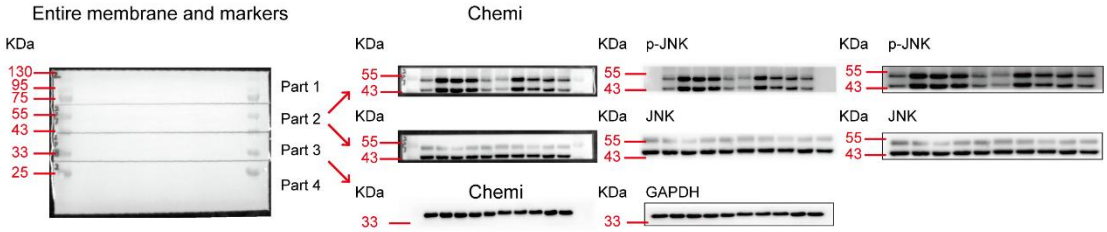


Figure 6C

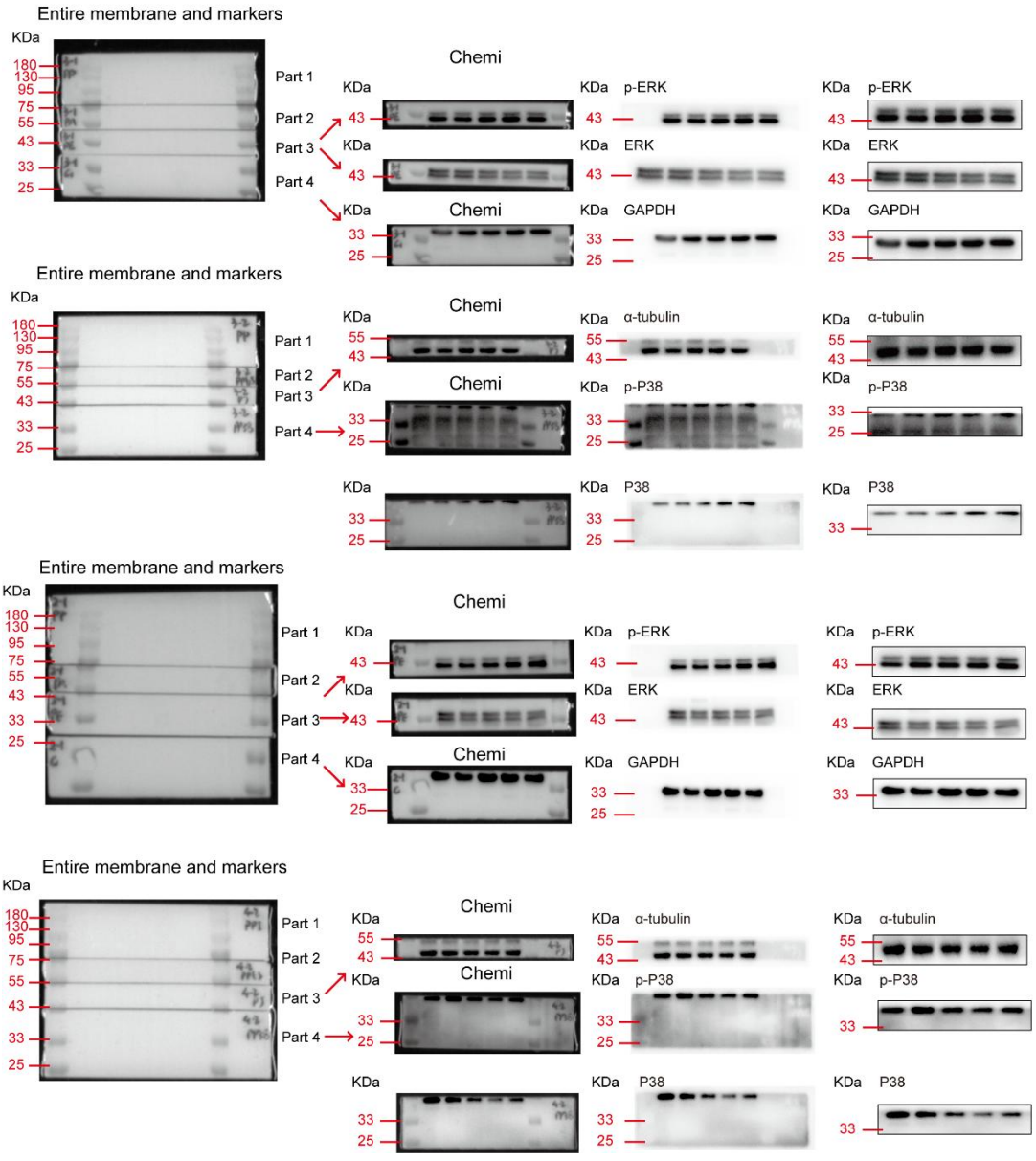


Figure 6D

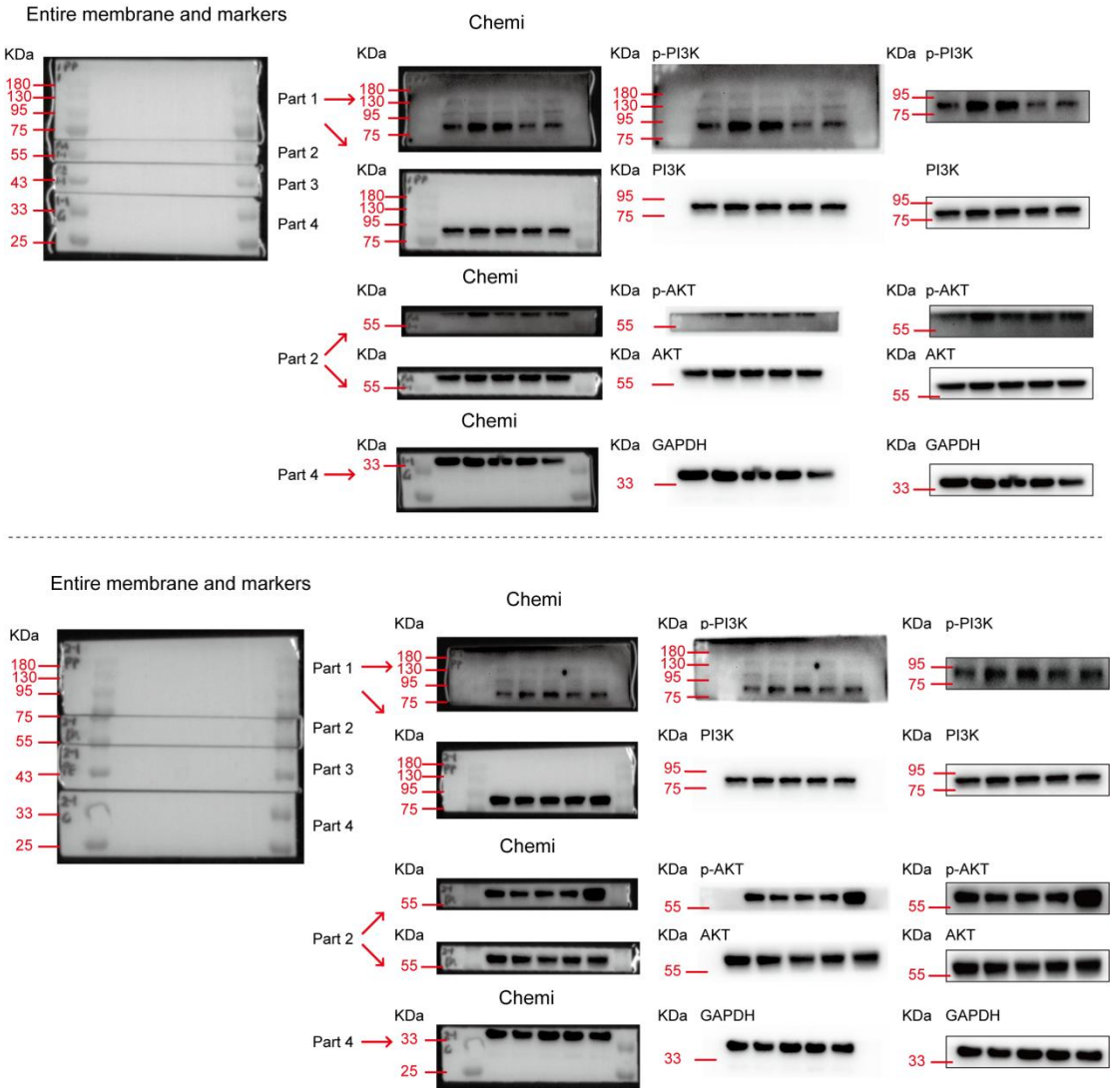
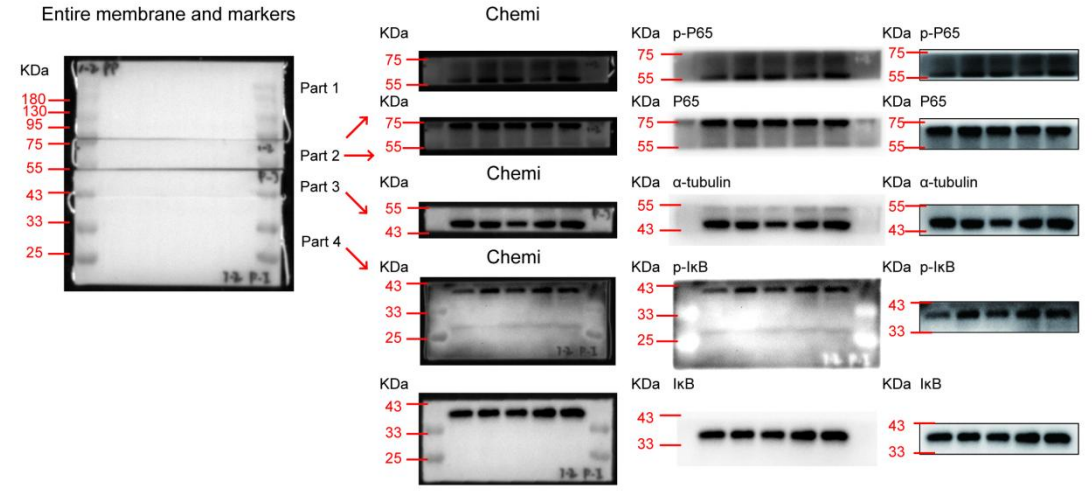
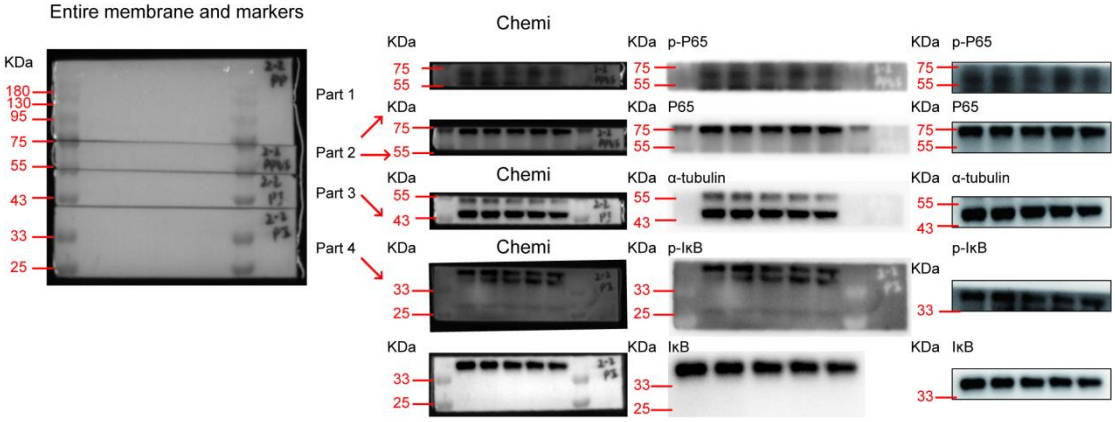

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Figure 6E


Entire membrane and markers
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