Identification of a binding site on soluble RANKL that can be targeted to inhibit soluble RANK-RANKL interactions and treat osteoporosis

Dane Huang1,2,3, Chao Zhao1,3, Ruyue Li2, Bingyi Chen1, Yuting Zhang1, Zhejun Sun1, Junkang Wei1, Huihao Zhou1, Qiong Gu1 & Jun Xu1

One of the major challenges for discovering protein-protein interaction inhibitors is identifying selective and druggable binding sites at the protein surface. Here, we report an approach to identify a small molecular binding site to selectively inhibit the interaction of soluble RANKL and RANK for designing anti-osteoporosis drugs without undesirable immunosuppressive effects.

Through molecular dynamic simulations, we discovered a binding site that allows a small molecule to selectively interrupt soluble RANKL-RANK interaction and without interfering with the membrane RANKL-RANK interaction. We describe a highly potent inhibitor, S3-15, and demonstrate its specificity to inhibit the soluble RANKL-RANK interaction with in vitro and in vivo studies. S3-15 exhibits anti-osteoporotic effects without causing immunosuppression. Through in silico and in vitro experiments we further confirm the binding model of S3-15 and soluble RANKL. This work might inspire structure-based drug discovery for targeting protein-protein interactions.

Protein-protein interactions (PPIs) inhibitors gain increasing attentions1,2 in pharmaceutical sciences. Because most proteins must accomplish their functions through PPIs. One of the challenges, however, is to identify a druggable binding site for a molecule to selectively inhibit the PPIs3,4. Compared to the classic binding site of drug targets like enzymes or kinases, the PPIs interface is large, flat, and lacks a suitable size site for the small molecules binding with. Therefore, the small molecular PPIs inhibitors own large molecular weight and consist with many hydrophobic functional groups to form more interactions with their targets. These usually lead to poor pharmacokinetic properties and off-target effects. Tumor necrosis factor superfamily (TNFSF) is a typical example that has such challenge. TNFSF superfamily plays important roles in the rheumatoid arthritis (RA), Crohn’s disease (CD), inflammatory bowel disease (IBD), psoriasis, atherosclerosis, bone loss, and Alzheimer’s disease (AD)5-7.

Many TNFSF antibodies such as adalimumab, infliximab, and denosumab have been employed for treating rheumatoid, osteoporosis, and osteosarcoma. However, the severe immune-related side effects (such as increased risk of infection, autoimmune diseases, cancer, and reactivation of tuberculosis) caused by these pan-PPIs inhibitory biologics limit their clinic values8-10. TNFSF proteins own two forms, membrane-bound form (mTNFSF) and soluble form (sTNFSF) that is cleaved from mTNFSF11,12. The main reason for those side effects is mTNFSF expressed as type II transmembrane proteins (receptors) in immune cells, such as antigen presenting cells and T cells. mTNFSF proteins function as immune enhancers through reverse signaling pathways11,12. On the other hand, sTNFSF proteins are...
over expressed in disease status. Hence, the therapeutic agents targeting sTNFSF PPIs must be selective to sTNFSF without interfering mTNFSF PPIs. Antibodies cannot distinguish mTNFSF and sTNFSF per se because they blankety wrap up the same PPIs binding domain of mTNFSF or sTNFSF. Therefore, scientists including FDA agents suggest to discover small molecular TNFSF PPIs inhibitors. Suramin is a small molecular inhibitor that inhibits TNF activity by disrupting the TNF trimer. Another small molecular TNF PPIs inhibitor is SPD304, which binds to the interface of a TNF dimer, disrupts the TNF trimer, and inhibits the cytokine function. Unfortunately, selective small molecular PPIs inhibitors are not reported so far.

Receptor Activator of Nuclear Factor-κB Ligand (RANKL) belongs to TNFSF family. Therefore, RANKL also exists soluble and membrane forms. Normally, the functions of soluble RANKL (sRANKL) and membrane RANKL (mRANKL) are similar in osteoclast differentiation. However, over-expressed sRANKL can cause excessive bone resorption that induces osteoporosis. The osteoporosis patients with therapy exhibited decreased level of sRANKL. Interfering RANKL–RANK interaction can inhibit osteoporosis or bone metastasis. Denosumab, a human monoclonal antibody, is the only approved therapeutic agent targeting RANKL for treating postmenopausal osteoporosis, giant cell tumor of bone, and bone cancer metastasis. For the same reason, denosumab can reduce severe immune side effects due to its blankety wrapping the same PPI binding domain of sRANKL and mRANKL. The mRANKL is also expressed in the surface of lymphocytes, like T cell and B cell. When T-cells with antigen, mRANKL becomes a receptor of RANK-RANKL reverse signaling pathway and enhances immune functions such as, T-cell proliferation, T-cell–dendritic cell (DCs) interactions, DCs survival, thymus and lymph node development. Blocking mRANKL RANK reverse interaction results in osteopetrosis due to the lack of osteoclast; defective T-cell and B-cell differentiation, a failure of mammary gland lobuloalveolar development during pregnancy, decrease monocytes, DCs survival and their effective function. This mRANKL can be cleaved by TNF-α converting enzyme (TACE) to form sRANKL. Many researchers report that sRANKL from B cell and T cell promotes osteoclastogenesis, subsequently leads to osteoporosis and cancer metastasis. In addition, the serum level of sRANKL was shown to be a significant risk predictor of type 2 diabetes mellitus (T2DM) in a large prospective study. Therefore, the selective sRANKL inhibitor may avoid immune side effects.

A small molecule that can selectively inhibit sRANKL–RANK interactions is a therapeutic solution for treating osteoporosis avoiding immune side-effects. Until now, small molecular sRANKL-selective PPIs inhibitor is not discovered yet. The major premise of discovering such small molecular inhibitors is to identify the binding site which is druggable and can discriminate sRANKL from mRANKL.

Here, we hypothesize that protein thermal fluctuation can lead us to discover this binding site. One traditional method of PPIs inhibitors discovery usually based on the PPIs interface from x-ray structures. As known, the x-ray structure usually presents the most stable conformation. However, the protein conformation is not constant, but fluctuant. During this thermal motivation process, it may form a druggable binding site. On the other hand, the mRANKL is rigidified by the cell membrane while sRANKL has no C-terminal extracellular connecting stalk domain and, is free from the cell membrane. Hence, the fluctuation may also distinguish the sRANKL and mRANKL. Therefore, we applied a molecular dynamic (MD) study to mimic thermal fluctuation and identified a selective binding site on sRANKL based on our hypothesis. This site was further proved to be druggable by biostudies. Then, a series of sRANKL–RANK small-molecular inhibitors was discovered based on this site, confirming the inhibitor’s selectivity toward sRANKL and, validating the efficacy of anti-osteoporosis activity of the sRANKL without causing immunosuppression side effects in vivo and in vitro. This work provides a selective sRANKL small molecule inhibitor as a promising anti-osteoporosis agent without immune side effects. It also might inspire the structure-based drug discovery targeting PPIs for other drug target families.

Results

Identifying small molecule binding site that can be used to distinguish sRANKL from mRANKL

sRANKL is a non-covalently formed homo trimer, and cleaved from mRANKL. mRANKL has C-terminal extracellular connecting stalk binding to the membrane. Hence, a binding site of the homo trimer is induced easily for a small molecule to bind sRANKL rather than mRANKL. Therefore, our initial effort attempts to identify selective binding sites for disturbing sRANKL–RANK interaction by molecular dynamics (MD) simulations. Firstly, a homology model of mouse mRANKL was created based on a mouse sRANKL crystal structure (PDB ID: 1SSS) by freezing the stalk amino acids close to the membrane because the crystal structure of mRANKL was not available. Then, 100 ns MD simulations on the models of sRANKL and mRANKL based on crystal a structure were conducted. The MD simulations results are documented in supplementary material (Supplementary Fig. 1).

In sRANKL or mRANKL, three key residues (K180, I246, and Q236 in mRANKL) are regarded as key residues for RANK binding. Thus, we hypothesize that a druggable binding site that can be used to distinguish sRANKL from mRANKL should be close to these key residues. Therefore, we focus on the variation of Y234, Q236, K180, Q291, H252, I246, W192, Q302, D301, D299, R222, and H224 (Fig. 1a) during MD simulation. The MD results indicated four key residue pairs (Y234–H224, Y234–D301, K180–H224, and Q236–D301) demonstrated different behaviors in sRANKL and mRANKL. The distances of these residue pairs stand for the flexibility and size of the binding site.

To examine the flexibility of the binding site, the frequencies of the four residue-pair staying in long-distance status (> 2 Å) during the 100 ns MD simulations were studied in Fig. 1b, which indicated these residue-pairs significantly stayed at longer distance statuses in mouse sRANKL (red bars) rather than the ones in mouse mRANKL (blue bars).

To further examine the size of the binding site, we also defined the sum of the distances of the four residue-pairs (SDRP). Longer SDRP means higher flexibility and larger size and ligand-inducibility. The distance distribution of SDRP during the 100 ns MD simulations is depicted in Fig. 1c, which demonstrates that the SDRP in mRANKL (blue bars) tended staying shorter distances rather than the one in sRANKL (red bars). The common conformation of sRANKL (SDRP is 76–78 Å) and mRANKL (SDRP is 70–72 Å) were selected (Fig. 1d, e). The size of site was measured by distance of Y234–D303 and K180–R222 that present the length and width. The results revealed that the site size in sRANKL is much larger than mRANKL.

To experimentally confirm in silico results, the single-point mutation experiments on rat sRANKL were conducted (The residue ID of rat RANKL is 2 more than that of mouse RANKL, like rat sRANKL K182 is equivalent to mouse sRANKL K180). We mutated K182A, R224A, H226A, E227A, Q238A, Y242A, E270, D301A, and D303A. Since these residues were possible to form the binding site and play important roles for binding with RANK, Osteoclastogenic assays were conducted on these mutated and the wild type (WT) RANKL proteins. As shown in Fig. 1f–h, these mutated sRANKL proteins were not able to promote osteoclasts except Q238A. Thus, K182, R224, H226, E227, Y242, E270, D301, and D303 are experimentally confirmed to be important for RANK–RANKL binding and inhibiting osteoclastogenesis. In short, a binding site of RANKL for developing anti-osteoporosis drug is identified.

Discovery small-molecule inhibitors of sRANKL

An in-house library of 10,016 compounds were virtually screened by docking the compounds into the above-mentioned binding site in Nature Communications | (2022) 13:5338
mRANKL (Fig. 1c) and sRANKL (Fig. 1d). This docking campaign consists of two steps:

1. The library was screened with the rigid-body docking packages of MOE 2018 (Chemical Computing Group, Montreal, Canada) and Glide 2018-01 (Schrödinger, New York, USA). 2,061 hits were predicted by both programs as stronger binders to sRANKL rather than mRANKL;

2. The hits from the step (1) were further screened with the induced-fit docking package of MOE 2018. Thus, 51 refined hits were predicted as even stronger binders to sRANKL rather than to mRANKL.

The 51 refined hits were then validated with osteoclastogenesis assay and surface plasmon resonance (SPR) assays. These result in a sRANKL inhibitor S3 with most potent osteoclast inhibition effect (IC50 = 0.096 μM, KD = 34.80 μM, Fig. 2a, b and Supplementary Table 1).

To further improve the potency and selectivity of S3, 21 derivatives were synthesized and evaluated (Supplementary Tables 1, 2).
To further study the binding selectivity of S3-15 to sRANKL, we linked two soluble RANKLs (termed as binary-RANKL or L-RANKL) to mimic mRANKL behavior. The binary-RANKL motif is rigidified while still maintains osteoclast differentiation ability (Supplementary Fig. 3a). Then, S3-15 binding affinities with sRANKL and binary-RANKL were measured using isothermal titration calorimetry (ITC). The results (Fig. 3a) show that the binding affinity of S3-15 with sRANKL (KD = 5.78 μM) is significantly stronger than the one of S3-15 with binary-RANKL (KD = 124 μM) indicating S3-15 selectively binds sRANKL (SI = 21). The affinities of S3-05 binding sRANKL and binary-RANKL were also measured in ITC experiments. The results show that S3-05 also selectively binds to sRANKL (Supplementary Fig. 3a, Table 2).

To cross-validate the above experimental results, the 1H saturation transfer difference (STD)-nuclear magnetic resonance (NMR) assays were used to measure the binding selectivity of S3-15 to sRANKL and binary-RANKL. The results demonstrate that S3-15 binds to sRANKL (Fig. 3d, red color) rather than mRANKL (Fig. 3d, green color).

As shown in Fig. 3c, the osteoclasts are significantly inhibited by the S3-15-sRANKL binding in the dose-dependent manner. However, the osteoclasts are weakly inhibited (IC50 = 4.14 μM) due to the weak binding of S3-15 with mRANKL (Fig. 3d). Interestingly, S3-15 binds to sRANKL with a modest binding affinity, however, the potency against osteoclastogenesis is high. In this case, the simultaneously binding of sRANKL trimer with three RANK receptors are necessary for the formation of a functional sRANKL-RANK signal transduction complex to induce the osteoclastogenesis. It means that binding of a molecule of S3-15 to anyone of the three surface clefts of sRANKL functionally blocks all the three sRANKL-RANK binding sites on sRANKL trimer, thus S3-15-sRANKL interaction cause a greater effect on sRANKL-RANK interactions as well as a greater biological consequence.

To further study S3-15 inhibits osteoclastogenesis through specially binds to sRANKL, we synthesized the probes by labeling S3 derivatives with biotin (Supplementary Fig. 3c) for activity-based protein profiling (ABPP) assay. This resulted in S3B and S3-15B (Supplementary Fig. 3c, d) for fishing protein targets. S3B was used as negative control because it significantly lost osteoclast inhibition (IC50 = 49.29 μM); and S3-15B was used as positive control due to its similar activity as S3-15 (IC50 = 0.26 μM, S3-15 IC50 = 0.37 μM). A pull-down assay reveals that S3-15B can bind with sRANKL, however S3B cannot bind with sRANKL (Fig. 3e). In osteoclasts, both extracellular cytokines and intracellular proteins regulate osteoclastogenesis. Therefore, three protein samples were applied to ABPP assay: (1) in vitro osteoclastogenesis culture supernatants (Fig. 3f), (2) total protein lysate of osteoclasts at the differentiation stage (Supplementary Fig. 3e), and (3) rat serum (Supplementary Fig. 3f). Several bands that specially bound to S3-15B were analyzed by Mass spectra, and sRANKL is observed in all three samples (Supplementary Tables 3–5). The results indicate that S3-15 binds to sRANKL more preferred than other osteoclastogenesis targets.

Two in vivo rescue experiments were conducted to further prove the osteoclastogenesis inhibition effect of S3-15 is caused by inhibiting sRANKL. Q238A sRANKL (equivalent to hRANKL Q237A) can stimulate osteoclast differentiation (Fig. 1f, h) but without binding affinity with S3-15 (Fig. 4a, b). Our experiments demonstrated S3-15 can block osteoclastogenesis when the osteoclasts are incubated with WT RANKL; however, it cannot block osteoclastogenesis when incubated with Q238A sRANKL (Fig. 3g). Another H226A-mutated-RANKL (equivalent to hRANKL H225A) that cannot stimulate osteoclast differentiation (Fig. 1f, g) but with binding affinity with S3-15 (Fig. 4a, c) was then applied. As shown in Fig. 3h, WT RANKL induced osteoclastogenesis was decreased by S3-15; however, it was rescued in a dose-dependent manner by adding H226A sRANKL. Using W194A sRANKL (similar as H226A sRANKL, no osteoclastogenesis activity but binds...
Mechanism of selectivity studied at molecular level
To prove the binding site and its specificity, we elucidated the mechanism of S3-15 selectively blocking sRANKL and inhibiting osteoclastogenesis through in silico and in vivo experiments. Biotin-mediated pull-down experiments with S3-15 were conducted with S3-15 in the rescue experiments also have the same results (Supplementary Fig. 4a, b).

Mechanism of selectivity studied at molecular level
To prove the binding site and its specificity, we elucidated the mechanism of S3-15 selectively blocking sRANKL and inhibiting osteoclastogenesis through in silico and in vivo experiments. Biotin-mediated pull-down experiments with S3-15 were conducted with mutated rat sRANKL proteins. The results demonstrate that S3-15 loses binding affinity when the key residues (such as K182A, Q238A, E270A, D301A, and D305A) are mutated (Fig. 4a); and S3-15 still binds to the site when other residues of sRANKL (such as R192A, W194A, H254A, N255A and K283A) are mutated (Fig. 4a). STD-NMR experiments further confirm that the key residues K182, Q238, E270, D301 and D305 (Fig. 4b) are essential for S3-15 binding, and residues R224, H226, E227, and Y242 have no contributions to S3-15 binding to sRANKL (Fig. 4c).
A docking study was applied to other inhibitors, such as S3-15, and rat sRANKL. The pull-down proteins by S3-15 were observed by Coomassie blue staining. A dimmed band means the interaction of S3-15 and some mutants are weakened.

**Fig. 4 | Binding models identification of selective sRANKL inhibition.** a The pull-down assay results showing the binding affinities of S3-15 and rat sRANKL mutants. The pull-down proteins by S3-15 were observed by Coomassie blue staining. b The binding surface of sRANKL (PDB ID: 1S55). c, d The binding surface (yellow) of sRANKL (PDB ID: 1S55) and mRANKL (purple) and sRANKL (green). The purple area is shrunken (compared with the yellow area) due to the residues near by the membrane are fixed. The green area is expanded (compared with the yellow area) because sRANKL has no residues restricted by membrane.

to investigate the binding mode of S3-15 and sRANKL, molecular docking and MD studies have been conducted. As our described, the small-molecular binding region (Fig. 4d, yellow color) is shrunken in mRANKL (Fig. 4e, purple color) and enlarged in sRANKL (Fig. 4f, green color). Thus, S3-15 can bind to sRANKL at the enlarged stable region (binding site), and unable to bind to mRANKL at the shrunken stable region (Fig. 4g, h). Actually, S3-15 was dissociated from mRANKL after the 54th ns MD simulation (Fig. 4g and Supplementary Fig. 4c). However, S3-15 was observed to generate induced-fit conformations that let S3-15 interacts with K180 and Q236 (mouse RANKL K180 and Q236 are equivalent to rat RANKL K182 and Q238) and form a H-bond between S3-15 and D303 (mouse RANKL D303 is equivalent to rat RANKL D305) (Fig. 4h, cyan color). Therefore, S3-15 can selectively form a stable complex with sRANKL at the interface of a sRANKL dimer. This interface consists of positively charged portion (Fig. 5a, blue color) and negatively charged portion (Fig. 5a, colored in red). S3-15 resides at the positively charged portion (Fig. 5a) because the surface of S3-15 is mainly positively charged.

To further confirm key residues for the binding of S3-15 and sRANKL, a docking study was applied to other S3 derivatives. The results indicate that residues K180, Q236 and K256 in mouse RANKL are essential for inhibiting osteoclastogenesis (these residues are equivalent to rat sRANKL K182, Q238 and K258; Supplementary Fig. 4a). When compounds interact with all key residues, such as S3, S3-05, S3-08, S3-01, and S3-03, their IC50 values are less than 0.1 μM (Fig. 5b, Supplementary Fig. 4d-g). While the compounds (S3-10, S3-00, S3-11 and S3-02) interact with only one or two of the residues (mouse sRANKL K180, Q236 and K256), the RANKL binding affinities and osteoclastogenesis inhibitory activities are reduced (IC50 >1 μM) (Fig. 4c, Supplementary Fig. 5h-j). When compounds (such as S3-07 and S3-04) do not interact with sRANKL or interact with only one of the key residues (mouse sRANKL K180, Q236 or K256), they lose osteoclastogenesis inhibitory activities (Fig. 5d and Supplementary Fig. 4k).

There are three key residues provide the most important interactions with H125 and E126 in RANK for RANK-RANKL binding. Our inhibitors, such as S3-15, occupied the binding region of H125 and E126 in RANK, and destroyed RANKL-RANK binding. This is consistent with the previous observation. The GST-pull down assay was then applied with a GST tag recombinant rat sRANKL (GST-sRANKL) and its receptor RANK. As shown in Fig. 4f, the binding affinities of the S3, S3-05, S3-07, S3-08, and S3-15 were consistent with their inhibitory activities on sRANKL-induced osteoclast formation. Further cross-link assay results indicate that S3-15 cannot affect the trimerization of sRANKL (Supplementary Fig. 5i). These results confirmed that our sRANKL selective
signaling (Fig. 6c, d). Correspondingly, the osteoclastogenesis marker sRANKL, ever, but not lower concentration (0.3 μM) dose-dependently blocked both downstream transcription factor NF-κB and AKT (Supplementary Fig. 5a). Moreover, p38 (Fig. 6b), reduced PI3K-AKT signaling by decreasing the phosphorylation of ERK, JNK, and MAPK signaling by decreasing the phosphorylation of the IκB kinase-α, NF-κB inhibitor-α (IκBα) and p65 (Fig. 6a). It also abrogated MAPK signaling by decreasing the phosphorylation of ERK, JNK, and p38 (Fig. 6b), reduced PI3K-AKT signaling by decreasing the phosphorylation of P38 and AKT (Supplementary Fig. 5a). Moreover, S3-15 dose-dependently blocked both downstream transcription factor NF-κB and NFATC luciferase reporter–gene expression of RANKL-RANK signaling (Fig. 6c, d). Correspondingly, the osteoclastogenesis marker genes, DC-stamp, Ctsk, MMP9, Tracp, Oscar, and Calcr mRNA level of were also significantly suppressed (Fig. 6e).

Nonetheless, we found that S3-15 cannot affect TNF-α, IL-β and LPS activating NF-κB and NFATC osteoclastogenesis signaling pathway (Supplementary Fig. 3g, h). Moreover, S3-15 cannot affect the osteoclastogenesis related gene expression without RANKL (Supplementary Fig. 3g). All the data demonstrate that S3-15 specifically binds to sRANKL and plays a key role in osteoclast differentiation inhibition. NF-κB and MAPK signaling pathways are related to cell survival and functions. Therefore, we examined S3-15 in osteoclast survival and bone resorption. Apoptosis of mature osteoclasts were significantly increased after S3-15 treatment with EC50 of 0.55 μM (Fig. 6f and Supplementary Fig. 5b). Moreover, S3-15 attenuated bone resorption in a dose-dependent manner (Fig. 6g). Similar effects were observed in other S3 derivatives, such as S3, S3-05, S3-07, S3-08, and S3-15 (Supplementary Fig. 5c, d).

Recent studies reveal that RANKL-RANK signaling regulates osteoblastogenesis by RANK reverse signaling. Therefore, the influence of S3 derivatives on osteoblasts were examined. sRANKL was observed to significantly suppress the mineralization of osteoblast. However, S3-15 exhibits potent activity of improving osteoblastic mineralization on human primary osteoblasts in presence of sRANKL (Fig. 6h). Another study indicates that S3-15, S3 and S3-05 increase cell proliferation and mineralization in mouse embryonic mesenchymal
Selective sRANKL inhibitor (S3-15) inhibits the RANKL biological functions of osteoclasts and osteoblasts. a, b Western blot experimental results show that S3-15 suppresses RANKL mediated NF-κB and MAPK signaling pathway. Images represent three independent experiments. c, d Luciferase reporter assay results show that NF-κB and NFATC-luciferase expression are reduced in doge manage when treated with S3-15. e RT-qPCR experimental results show that S3-15 suppresses osteoclast markers (DC-stamp, Ctsk, MMP9, Tracp, Oscar, and Calcr) expression in BMMs cells stimulated with 100 ng/mL sRANKL. f Cell apoptosis assay results show that S3-15 induces osteoclast apoptosis. The apoptosis (Annexin V stained showing green fluorescence), death (PI stained showing green fluorescence), and nucleic acid breaks (DAPI stained showing blue fluorescence) of osteoclasts were observed. TRAP stained also observed osteoclast death. Representative images (n = 3 images taken in total with technical triplicate repeat) (left) and dead osteoclast (yellow arrowheads) quantification (right). g Bone resorption experimental results show that S3-15 suppresses RANKL-induced resorption pits in dose-dependent manner. Representative resorption pits images (n = 3 images taken in total with technical triplicate repeat) (left) and resorption pits area quantification (right). h Human primary osteoblasts cell culture experiments show that S3-15 promotes osteoblast mineralization. Image was taken after 12 days cell culture with S3-15 and Alizarin Red S staining. Data are presented as the mean ± s.d. from three biological replicate per group. Statistical difference was determined by unpaired two-tailed Student’s t test. Significant difference p value is < 0.05.
stem cell line C3H10T1/2 or human primary osteoblasts with osteogenic differentiation medium (Supplementary Fig. 5e, f).

**S3-15 selectively inhibits sRANKL without changing T lymphocyte differentiation**

mRANKL dominates T cell differentiation and maintains dendritic cells survival and regulates T-cell/dendritic cell communication in the immune system. Both mouse CD4+ and CD8+ T cells express mRANKL, and their proliferation are inhibited by anti-CD3Ab, anti-CD137Ab and OPG-Fc (the decoy receptor of RANKL). Hence, we used lymphocyte proliferation assay to evaluate the effect of selective inhibitor on immunity.

The results demonstrated that S3-15 did not change lymphocyte proliferation (Supplementary Fig. 6a), and cell ratio of CD4+ T cell and CD8+ T cell (Fig. 7a-c and Supplementary Fig. 6b). However, the nonselective RANKL monoclonal antibody - Denosumab notably inhibits lymphocyte proliferation at high concentration (Supplementary Fig. 6a) together with a decreased of CD4+ T cell differentiation (Fig. 7a-c). The immunosuppression effect of Denosumab could be due to the decreased CD4+ ratio. In three healthy human T lymphocyte samples, we found that the proportion of CD4+ T cells in two of them were decreased. For human 1, later stages of cell differentiation (CD4+CFSE- cells) are decreased after treated with Denosumab for 3 days (Fig. 7c, left) and 12 days (Fig. 7a, right). For human 2, after treated with Denosumab for 9 days, total CD4+ and CD4+CFSE+ T cell dose-dependently decreased, it dropped by nearly half at the concentration of 100 μg/ml (Fig. 7b). In cancer patient, the suppression effect in CD4+ T cell seems to be more significant. After Denosumab treatment, total T cells isolated from cancer patient were reduced from 14.51 ± 3.71% to 3.88 ± 1.77% and CD4+CFSE+ cell reduced from 5.32 ± 0.76% to 1.33 ± 2.31% (Fig. 7c). However, all these cells are not affected when treating sRANKL selective inhibitor S3-15 (Fig. 7a-c and Supplementary Fig. 6a-c). Those results indicated that selectively blocking sRANKL had less effect on T cell differentiation than nonselective RNKL blockers. The immunosuppression effect of Denosumab could be mainly caused by the decreased of CD4+ differentiation.

**Anti-osteoporosis effects for sRANKL inhibitors in vivo**

Previous studies have demonstrated that the formation of osteoclasts was mainly driven by mRANKL on osteoblast, through cell-cell contact. However, recent studies showed that sRANKL is sufficient to increase osteoclasts and bone resorption in vivo. To prove that S3 series inhibits osteoporosis by selectively disturbing the binding of sRANKL-RANKL, an in vivo experiment was applied. The bone volume / total volume (BV/TV) of ovariectomy (OVX) rats was increased when administrated with S3, S3-05, and S3-15 orally (Fig. 8a and Supplementary Fig. 7a-d). S3-15 can significantly improve bone trabecular parameters, and the values of BS/BV, Tb.N, and Tb.Th recovered to the sham-operated rats (Fig. 8a). In addition, in OVX mice, the trabecular parameters were also improved after treating with S3-15 (Fig. 8c). To further evaluate the biomechanical strength of the bones, a three-point bending test was applied. The result indicated that the bone strength was increased after S3-15 treatment (Fig. 8c). Moreover, the data of a histology experiment demonstrated that S3-15 significantly decreased the numbers of osteoclast as well as its potent in vivo activity (Supplementary Fig. 7f). The serum markers of bone resorption such as cross-linked carboxy-terminal telopeptide of type I collagen (CTX-I), osteocalcin, and propeptide of type I procollagen (PINP), OCN (Osteocalcin), and Glu-Ocn (Glu-osteocalcin) were significantly decreased after S3-15 administration (Fig. 5b).

Routine blood examination indicated that rats treated with S3-15 exhibited similar results to those of normal rats (sham group) and had no organ toxicity in vivo (Supplementary Fig. 7e). Similarly, S3 and S3-05 can also increase the BMD although their efficacies are not as good as S3-15 (Supplementary Fig. 7a-d).

To prove S3-15 does not disturb the immune system in OVX-induced osteoporosis mice, the biomarkers in peripheral blood lymphocyte were examined. It was observed that the declined ratios of CD3+ T cell and CD3+CD8+ T cell, the increased ratio in CD4+CD8+ (Fig. 8d) in OVX operation mice. In CD3+ T cell subsets, the ratio of CD4+ T cell and CD4+/CD8+ were significantly increased (Fig. 8e) in the OVX models. These results are consistent with the previous report. S3-15 improved the immune condition (Fig. 8d, e), reduced the swollen spleen (Fig. 8f) in vivo. Lymphocyte transformation tests were applied to evaluate the immune function of spleen lymphocytes. The lymphocyte transform rate in OVX mice were lower. OVX mice with S3-15 administration have improved transform rate that is comparable to the normal mice (Fig. 8g). In summary, the selective sRANKL inhibitor can inhibit osteoporosis without immunosuppression.

**Discussion**

Discovery of small molecule PPIs inhibitors have been recognized as a major challenge due to off-target and undruggable of these small molecular inhibitors. The main reason is that the PPIs interface is a much larger and flat surface area (~800 Å²) than a conventional substrate-binding cavity and one with a less well-defined shape. Therefore, it requires the inhibitors disobey the Lipinski’s rules and own large molecular weight for occupying the large region and inhibiting the PPIs. Another reason is that the classic strategy of discovering these compounds usually based on ‘hot-spot’ for two proteins interacting. It is reported that the hot-spot residues are usually arginine (21%) and tyrosine (12.3%). The residues surround these hot spots are hydrophobic because they can provide a shield to protect the hot spot from waters. Hence, the small molecular PPIs inhibitors usually own similar structure and large molecular weight. For these reasons, the small molecular PPIs inhibitors usually are unspecific and undruggable.

Therefore, discovering druggable site on the PPIs area is preferred and it can help us to overcome the disadvantage of known PPIs inhibitors. The x-ray structure of protein, which is usually applied for drug discovery, usually presents the most stable state. However, the structure of protein is not constant, but exhibit pronounced fluctuations. This gives an opportunity to find out a traditional small molecule binding site in the surface area. Here, we perform a MD study to mimic the protein fluctuation, and thereby discovery a series druggable and selective small molecular PPIs inhibitors. These compounds not only target RANKL mainly, but also distinguish sRANKL and mRANKL which share the exact same sequence. However, our work cannot exclude that these compounds exhibit the anti-osteoporosis activity through additional mechanisms.

During the simulation, we observed that the amino acids in the surface area (rat sRANKL H226, Y236, K182 and D303) will move and the fluctuation. The fluctuations of sRANKL also provide a deeper and larger site for small molecule binding compared to x-ray structure. Further, the fluctuation difference between sRANKL and mRANKL provides us a possibility to discover S3-15 selective inhibitors. All these revealed that performing MD to mimic protein fluctuation can help us to find out the specific small molecule binding site on PPIs surface.

Here, we also show the benefit of selective sRANKL inhibitors in vitro and in vivo, which is no T-cell proliferation suppression. This hints us selective sTNFSF inhibitors overcome the side effects in clinical.

In summary, the results described herein suggest that a small molecular binding site for PPIs inhibitor discovery can be discovered by a MD study. This site is benefit for discovering more druggable inhibitors with specificity. Therefore, the future of small molecular PPIs inhibitors development will base on the protein fluctuation.
Methods

Materials

The information of reagents, antibodies, cell, animal, bacterial, chemicals and recombinant proteins, oligonucleotides, and software were displayed in supplemental information file name “KEY RESOURCES TABLE”.

Cell culture

All mammalian cells were culture in complete medium (containing 10% fetal bovine serum and 50 IU mL⁻¹ penicillin/streptomycin) and maintained in a humidified at 37 °C with 5% CO₂ atmosphere. RAW264.7 cells (ATCC, Cat# TIB-71) were cultured in MEM-alpha complete medium. Bone marrow cells (BMMs) were isolated from 8-week-old

---

### Table: CFSE-CD4+ cells in total cells (%)

|                  | Denosumab (μg/mL) | S3-15 (μM) |
|------------------|-------------------|------------|
| **Healthy donors** |                   |            |
| 3 day            |                   |            |
| Control          | 2 10 50 100 0.2 1 5 10 |            |
| p=0.7730         |                   |            |
| p=0.3697         |                   |            |
| p=0.0831         |                   |            |
| p=0.5390         |                   |            |
| p=0.0059         |                   |            |
| p=0.0499         |                   |            |
| p=0.0080         |                   |            |
| p=0.0419         |                   |            |
| 12 day           |                   |            |
| Control          | 2 10 50 100 0.2 1 5 10 |            |
| p=0.5212         |                   |            |
| p=0.2387         |                   |            |
| p=0.9938         |                   |            |
| p=0.0413         |                   |            |
| p=0.0116         |                   |            |
| p=0.0439         |                   |            |
| p=0.0133         |                   |            |

|                  | Denosumab (μg/mL) | S3-15 (μM) |
|------------------|-------------------|------------|
| **Healthy donors** |                   |            |
| 9 day            |                   |            |
| Control          | 2 10 50 100 0.2 1 5 10 |            |
| p=0.2738         |                   |            |
| p=0.8748         |                   |            |
| p=0.5322         |                   |            |
| p=0.2969         |                   |            |
| p=0.0113         |                   |            |
| p=0.0743         |                   |            |
| p=0.0283         |                   |            |
| p=0.0085         |                   |            |
| 9 day            |                   |            |
| Control          | 2 10 50 100 0.2 1 5 10 |            |
| p=0.3668         |                   |            |
| p=0.4845         |                   |            |
| p=0.8615         |                   |            |
| p=0.0030         |                   |            |
| p=0.0083         |                   |            |
| p=0.0599         |                   |            |
| p=0.0278         |                   |            |

|                  | Denosumab (μg/mL) | S3-15 (μM) |
|------------------|-------------------|------------|
| **Cancer patient donors** |               |            |
| 3 day            |                   |            |
| Control          | 2 10 50 100 0.2 1 5 10 |            |
| p=0.2985         |                   |            |
| p=0.6969         |                   |            |
| p=0.7295         |                   |            |
| p=0.4657         |                   |            |
| p=0.0198         |                   |            |
| p=0.0040         |                   |            |
| p=0.0011         |                   |            |
| p=0.0020         |                   |            |
| 3 day            |                   |            |
| Control          | 2 10 50 100 0.2 1 5 10 |            |
| p=0.6153         |                   |            |
| p=0.3346         |                   |            |
| p=0.9613         |                   |            |
| p=0.4446         |                   |            |
| p=0.0100         |                   |            |
| p=0.0489         |                   |            |
| p=0.3246         |                   |            |
| p=0.0015         |                   |            |

---

**Article**

https://doi.org/10.1038/s41467-022-33006-4

Nature Communications | (2022) 13:5338

10
Fig. 7 | S3-15 has no adverse effects on T lymphocyte differentiation. a CD4+ T cell differentiation ratio in gate of CFSE− cells in T cell sample of healthy donors 1. T cells of healthy donors 1 were induced by anti-CD3 antibody (5 μg/mL) and anti-CD28 antibody (5 μg/mL) for 3 days or 12 days with or without treatment. b CD4+ T cell differentiation ratio in gate of total cells and gate of CFSE− cells in T cell sample of healthy donors 2. T cells of healthy donors 2 were induced by anti-CD3 antibody (5 μg/mL) and anti-CD28 antibody (5 μg/mL) for 9 days with or without treatment. c CD4+ T cell differentiation ratio in gate of total cells and gate of CFSE− cells in T cell sample of cancer patient donors 1. T cells of cancer patient donors 1 were induced by anti-CD3 antibody (5 μg/mL) and anti-CD28 antibody (5 μg/mL) for 3 days with or without treatment. In experiments of a–c, CFSE− cells, CD4+ cells, CD8+ cells were assayed by flow cytometry and analyzed their percentage in the corresponding gate after treatment. Data are presented as the mean ± s.d. from three or four biological replicates per group. Statistical difference was determined by unpaired two-tailed Student’s t-test. Significant difference p-value is < 0.05.

Docking and virtual screening

The structure of RANKL was prepared from MD results analysis. A protein pose on 70 ns MD simulation was extract as sRANKL structure, and a protein pose on 26 ns MD simulation was extract as mRANKL structure. The waters and ions on the structure were removed and then prepared by QuickPrep function on MOE. The small-molecular structures were minimized (within RMS gradient of 0.1 kcal/mol/Å²) in MMFF94x force field. Further, Triangle Matcher method (30 s allowed for each ligand placement) with rigid-body approach was applied to small-molecular compounds. Ten key residues were selected as docking site (K180, Y234, Q236, M238 and K256 in 1st monomer of RANKL; R222, H224, E268, D299 and D303 in 2nd monomer of RANKL). London dG scoring method was performed to evaluate the results. The compounds with S-score lower than -6 for sRANKL and mRANKL were selected independently. Among them, compounds that only docked with sRANKL with GLIDE docking were selected. After analysis of results, the compounds with score less than -3.5. Next, these compounds were applied to induced-fit docking (IFD) by Triangle Matcher method (225 s allowed for each ligand placement).

3D structure of sRANKL were prepared by Schrödinger package (v2018-1): a) adding hydrogen atoms; b) adjusting the ionization and tautomerization state of the protein using PROPKA; c) interactive optimization of the hydrogen bonding network; and d) restrained minimization of the structure (within RMSD of 0.3 Å of the initial structure) with the OPLS3 force field. A grid box of 20.0 × 20.0 × 20.0 Å³ were formed based on same residues as MOE docking. Then a rigid-body docking was performed using GLIDE for the selected compounds. After analysis of results, the compounds with score less than -3.5. Next, these compounds were applied to induced-fit docking (IFD) by Triangle Matcher method (225 s allowed for each ligand placement).

In vitro cytotoxicity assay

MTT assay was used to determine cell viability. RAW264.7 cells in the logarithmic growth phase were cultured in 96-well plates with 6 × 10³ cells in each well and incubated for 24 h. The surviving cells were then determined by MTT method. Absorbance was measured at 570 nm using a microplate reader and displayed with OD value. Cell growth inhibition ratio was calculated as follows:

\[
\text{Cell growth inhibition ratio} = \frac{\text{OD vehicle} - \text{OD treated}}{\text{OD vehicle}} \times 100\%
\]

Cell cytotoxicity CC₅₀ value were conducted by using Nonlinear regression (curve fit) analytic procedure in GraphPad Prism 8.

In vitro anti-osteoclastogenesis activity determination

Mouse osteoclasts were cultured according to the previously reported protocols. BMMs were plated in 96-well plates at a density of 6 × 10³ cells/well with complete medium containing 10 ng/mL M-CSF. The following day, cells were then stimulated with 100 ng/mL sRANKL in the presence or absence of compounds with different concentrations every 2 days until osteoclasts formed. In order to calculate IC₅₀, we set 9 concentrations for each compound and untreated and non-sRANKL unstimulating group as controls, each with triplicated repeated wells. After 5 days, cells were fixed with 4% paraformaldehyde and then applied TRAP-staining to identify osteoclasts by using the TRAP-staining kit. Osteoclasts number (OC No.) in each well was counted and
Fig. 8 | In vivo anti-osteoporosis activity and immunological effects of S3-15.

a micro-CT images of the trabecular part of distal femur of rat treated with S3-15 shows higher bone density than rat not treated (OVX). Histological analysis of trabecular bone parameters including BV/TV, Tb.Th, Tb.N and Tb.Sp demonstrate that S3-15 improve significantly the morphometric characteristics of trabecular bone.

b Serum PINP, CTX-I, OCN, and Glu-OCN osteoporosis related marker levels were decreased in rats treated with S3-15. c micro-CT images of the trabecular part of distal femur of mouse treated with S3-15 shows higher bone density than mouse not treated (OVX). Histological analysis of trabecular bone parameters including BV/TV, Tb.Th, Tb.N, and Tb.Sp demonstrate that S3-15 improve significantly the morphometric characteristics of trabecular bone. Biomechanical testing shows S3-15 treated mice improving ultimate load. d, e S3-15 can improve the imbalance of immune cell ratio caused by osteoporosis. In peripheral blood total CD3+, CD8+ and CD3+CD8+ T cells are decreased in osteoporosis mice, (left). In CD3+ T cells subset, osteoporosis mice show an increased percentage of CD3+CD4+ T cells and a decreased percentage of CD3 + CD8 + T cells, that results in the increased the value of CD3+CD4+/CD3+CD8+(right). S3-15 treatment can reverse those abnormal indicators. f S3-15 alleviating spleens swelling in mice with osteoporosis. g Spleens of sham, OVX and S3-15 treated mice are got and applied splenic lymphocyte transformation experiment. Spleen lymphocyte transforming index is lower in osteoporosis mice than sham mice and can improve to normal level after S3-15 treatment for 4 weeks. Rat number of sham and OVX + S3-15 group, n = 9. Rat number of OVX group, n = 10. In mouse study, n = 8 mice per group. Data are expressed as means ± s.d. Statistical difference for ultimate load was determined by one-way ANOVA followed by Uncorrected Fisher’s LSD, and the others were followed by Tukey’s multiple comparisons test. Significant difference p-value is <0.05. Scalar bar, 500 μm.

The dosages and their correspondent osteoclastogenesis inhibitory activity curve and IC50 value were conducted by using Nonlinear regression (curve fit) analytic procedure in GraphPad Prism 8. Photographs of individual wells were taken using Leica microscope.

the percentage of inhibition ratio was calculated as follows:

\[
\text{OC inhibitory ratio(%) = } 100 - \frac{\text{OC No treated} - \text{OC No unstimulating}}{\text{OC No untreated} - \text{OC No unstimulating}} \times 100\%
\] (1)
Osteoclasts apoptotic activity assay

Mature osteoclasts were generated according to “Primary cultures of BMMs and osteoclastogenesis assay”. Mature osteoclasts were treated with or without compounds for 24 h. At the end of incubation, cells were fixed with 4% paraformaldehyde and applied TRAP-staining. Apoptosis and cell death was measured by in situ fluorescence staining with Annexin-V-FITC (Beyotime Biotechnology) and propidium iodide or DAPI staining flowing by their instructions. TRAP-positive cells, Annexin V, PI, and DAPI were visualized by Leica fluorescence microscopic.

Hydroxyapatite resorption assay

BMMs (1 × 10^6 cells/well) were cultured onto 6-well collagen-coated plates and stimulated with 100 ng/mL sRANKL and 10 ng/mL MCSF for 3 days to generate early stage of osteoclasts began differentiation. Then early-stage osteoclasts were gently harvested using cell dissociation solution, and seeded into OsteoAssay Surface (hydroxyapatite-coated) 24-well plates. Early-stage osteoclasts were continue incubated in a medium containing sRANKL and MCSF with or without treatment. After 48 h, wells were bleached to remove cells, followed by image acquisition for the measurement of resorbed areas using a Leica microscope. The percentage of surface resorbed was analyzed using ImageJ software.

Cloning, expression, and purification of recombinant wild-type and mutant sRANKL

GST-sRANKL was expressed and purified following a previously reported protocol. Rat sRANKL (aa160–318) cDNA was cloned into the bacterial expression vectors pGEX-3X and pGEX-2T with purification GST tag and resultant plasmid named p3rRANKL. Label-free sRANKL, mutational sRANKL, and L-RANKL were purified using the Superdex 200 Increase 10/300 GL (GE Healthcare). DNA sequence encoding mouse extra-cellular RANK fragment (residues 26–210) was cloned into pET28a using NdeI and XhoI restriction enzyme sites. The recombinant RANK was produced as inclusion body in E. coli BL21(DE3) cells. The transformed cells were grown in LB in LB media till OD600 about 0.6, 0.5 mM IPTG was added to induce protein overexpression in 37 °C for 7 h. The cell pellet harvest after centrifugation was resuspended in washing buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100. The cells were disrupted by sonication for 20 min (2 s on, 2 s off). After repeating the sonication and centrifugation, the precipitate was dissolved in 6 M guanidine hydrochloride, 50 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 10 mM DTT to a protein concentration of ~30 mg/mL with stirring at room temperature for 2 h. The soluble RANK was diluted to ~10 mg/mL using buffer containing 20 mM Na2HPO4 pH 7.3, 1 M L-arginine, 20% glycerol, 10 mM reduced glutathione and 1 mM oxidized glutathione. Then, sequential dialysis was followed to refold protein, against 20 mM Na2HPO4 pH 7.3, 0.5 M L-arginine, and 10% glycerol for 12 h, 20 mM Na2HPO4 pH 7.3, 0.2 M L-arginine, and 5% glycerol for 12 h, and finally twice against 20 mM Na2HPO4 pH 7.3 for 12 h. After centrifugation, the refold RANK was concentrated and further purified by Superdex 200 Increase 10/300 GL (GE Healthcare).

Surface plasmon resonance (SPR) assay

A ProteOn XPR36TM SPR instrument (BioRad Hercules, CA) was used for carrying out SPR assays. sRANKL protein in PBS buffer, pH 7.4, was diluted to 20 nM in 10 mM sodium acetate buffer, pH 5.5 and immobilized in parallel flow channels of a the EDAC/Sulfo-NHS activated surface of a GLH biosensor chip (Bio-Rad) with an amine coupling kit (Bio-Rad). The surface was blocked with 1 M ethanolamine. The final immobilization level for sRANKL was approximately 14,000 RU. A blank immobilization channel in the same GLH biosensor chip was also used with activated EDAC/Sulfo-NHS and blocked with 1 M ethanolamine to use as a reference.

Biosensor binding experiments were conducted in filtered and degassed running buffer (PBS containing 0.005% Tween-20, pH 7.4) at 25 °C. Compounds were dissolved in 100% DMSO in appropriate serial concentrations of stock solutions. Before detecting, compounds were diluted in six or five concentrations with running buffer and ensured that the content of DMSO contained in each detected concentration was consistent. Compounds in different concentrations were injected simultaneously at a flow rate at 20 μL/min for 180 s and 120 s for the association phase, followed by 180 s for the disassociation. Following the compound injection, the chip surface was regenerated with 40 s pulses of 0.85% H3PO4 and running buffer.

ProtedOn Manager 2.0 was used for data analysis. Each set of sensorgrams was globally analyzed using the 1:1 Langmuir binding model to obtain the kinetic rate constants (KOn and Koff). Global kinetic rate constants (ka and kd) were derived for each reaction, and the equilibrium dissociation constant, KD, was calculated using the equation KD = kd/ka.

STD NMR binding assay

For NMR studies, purified sRANKL or L-RANKL protein samples were buffer exchanged into PBS (Gibco, C100105080BT, pH7.4) using a Millipore spin column (1500 × g for 15 min at 4 °C, repeated six times, each time adding fresh PBS and discarding the flow-through). Protein

Nature Communications | (2022)13:5338 https://doi.org/10.1038/s41467-022-33006-4
concentration was determined using BCA method according to the procedure of Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Compound S3-15 powder was dissolved in H2O as a concentration of 10 mM.

Protein saturation transfer difference (STD) experiments were performed on Bruker Avance III 500 (500 MHz) spectrometer (Columbia University) at 300 K using triple-resonance cryogenic probes optimized for proton detection. All experiments were performed using an independent sample for each experimental measurement as a 500 μL sample with 100 μM protein and 5 mM S3-15 in final concentration. Uniformly, for STD assay all samples were contained 50 μL protein, 200 μL S3-15 (10 mM in H2O), and 250 μL D2O; for S3-15 two-dimensional spectra reference, samples were 50 μL PBS, 200 μL S3-15 (10 mM in H2O) and 250 μL D2O.

Isothermal titration calorimetry (ITC) assay

ITC titrations were performed on the MicroCal iTC200 system at 25 °C. Working stocks of compound S3-15 were prepared in 100% DMSO at 50 mM and then diluted to 500 μM at PBS with final DMSO concentration at 0.8% (v/v). PBS with 0.8% DMSO was used as ITC buffer. Purified sRANKL or L-RANKL protein was buffer exchanged into same ITC buffer using a Millipore spin column. S3-15 at 500 μM was loaded into a syringe and protein was loaded into an isothermal calorimeter cell. During titration, S3-15 in the cell was injected (2 μL per injection, 25 injections in total, and 180 s between each injection) regularly from a rotating syringe into protein solution in the isothermal calorimeter cell. The sample cell was stirred at 385 rpm. Reference cell power was set to 5 μcal/s. Control experiments were conducted under the same conditions using buffer solution instead of S3-15. Data were analyzed using a one-site binding model in Origin 7.1 software for ITC version 7.0 (MicroCal). The dissociation constant, KD, was calculated according to equation KD = 1/K. All other parameters, K (varying the stoichiometry), ΔH (entropy of the reaction), and ΔΔG (entropy of the reaction) were determined from the titration data.

GST pull-down assay

The RANKL-RANK interaction destructive activity of sRANKL selective inhibitors were detected by GST pull-down assay. 120 μg purified GST-sRANKL in 1 mL PBS containing 1% BSA buffer was loaded into the resin and incubated at room temperature for 45 min. After that, compounds S3, S3-05, S3-07, S3-08 and S3-15 with final concentration of 50 μM were added and incubated for 30 min. And then put 25 μg RANK into each tube, gently mix, incubated for another 20 min. 1% BSA PBS buffer with 0.8% (v/v) DMSO solution was used as control tube and performed with the same procedure. The resins were subsequently centrifugal settling and washed with 1 mL PBS 3 times and resuspended in 1×SDS gel loading buffer (20 μL PBS, 5 μL 5× Loading Buffer) and denatured at 100 °C for 5 min. The denatured samples were analyzed by SDS-PAGE electrophoresed and Coomassie blue staining. Finally, the band were observed, photographed and analysis of grayscale by Image J.

WT sRANKL or mutation sRANKL enrichment by pull-down assay

500 μM S3-15-biotin conjugate (S3-15B, positive control), biotin (B, blank control), and/or S3-biotin conjugate (S3B, negative control) in 400 μL PBS buffer were incubated with 40 μL streptavidin bead suspension for overnight at 4 °C. After conjugated, the beads were collected and washed with PBS buffer thrice, and then resuspended in 400 μL PBS buffer with 100 μg WT sRANKL or 15 mutation sRANKL proteins in each tube. All the tubes were incubated with stirring at 4 °C for 1 h. The beads were gently washed with 500 μL of PBS buffer for three times. The beads were resuspended in 20 μL PBS buffer with 5 μL 5× Loading Buffer and denatured at 100 °C for 5 min. The denatured samples were analyzed by SDS-PAGE electrophoresed and Coomassie blue staining. Finally, the band were observed, photographed and analysis of grayscale by Image J.

In vitro pull-down assay of sRANKL or mRANKL binding to OPG

To evaluate whether S3-15 affects the binding of sRANKL or mRANKL to OPG, we did the pull-down assay. sRANKL-OPG: 200 ng human sRANKL with his-sumo tag was incubated with Ni gel at 4 °C for 10 min. Then the Ni gel was washed with PBS for 3 times. 500 μL PBS that contains 100 ng Fe tag human OPG. Then S3-15 was treated in different concentration (0, 10, 1 and 0.3 μM). The mixtures were incubating at 4 °C for 30 min. Washed with PBS for 3 times. 500 μL 1% Triton X-100 in PBS was then added at 4 °C. The concentration of OPG was tested by OPG ELISA kit (USCN, China). mRANKL-OPG: MC3T3-E1 cells were differentiated by osteoblast differentiation medium (Pythomibio, China) for 2 days. Then cells were exposed to 1M NaCl for 5 minutes to remove endogenous OPG, washed once in PBS, and fixed with 70% ethanol for 5 minutes at 4 °C. Fixed cells were wash once by PBS and then exposed to PBS that containing 100 ng mouse OPG (Sino, China) or 100 ng mouse OPG with 1 μM S3-15 and incubated for an additional 30 min at 4 °C. Cell layers were washed 2 times with PBS and lysed with 1% triton in PBS. The concentration of OPG in cell lysates were measured with an OPG ELISA kit (USCN, China). Similar proceed were performed using HEK 293 T cells that transfer full length mouse RANKL or human RANKL.

Affinity-based target validation with cell lysates, culture supernatant or rat serum

Target enrichment was performed by incubating early-stage OC cell lysates, culture supernatant or serum samples with streptavidin beads pre-loaded with biotinylated compound S3-15B (positive control), B (blank control), and S3B (negative control). To obtain cell lysates and culture supernatant, BMUs were cultured and stimulated osteoclastogenesis for 3 days. Cell culture supernatant was collected, and cells were by PBS and lysate by 1× RIPA with phosphatase inhibitors and EDTA-free protease inhibitors. Then we collected the cell lysates and centrifuged at 18,386 × g for 10 min at 4 °C. To obtain rat serum sample, a female rat serum was extracted form aortaventrals after anesthesia, follow by centrifuged at 18,386 × g for 10 min at 4 °C. All the protein samples were adjusted to 1 mg/mL with PBS buffer and incubated with streptavidin beads pre-loaded with biotinylated compounds at 4 °C for 1 h with shaking. Then the beads were washed, denatured and separated proteins by SDS-PAGE electrophoresis. Finally, the band in gels were revealed by Coomassie blue staining or silver staining. In-gel protein digestion and desalting of differential bands in the gels were performed as described with some modification. Briefly, the gel slices were hydrated with 100 μL of 50 mM NH4HCO3:30%CH3CN (v/v) and incubated at room temperature for 6 h. This hydrated step was only performed to gel with Coomassie blue staining but not silver staining. Hydrated solvent was eliminated and all the gel slices form both Coomassie blue staining and silver staining were dried with 300 μL 100%ACN Gel slices followed by shaking 5 min and then freeze-drying 3 min. Dried slice were then rehydrated with 300 μL of 10 mM DTT/ 50 mM NH4HCO3 and incubated at 56 °C for 60 min. Remove solvent and repeated the dried gel procedure once more. All samples were added 300 μL of 60 mM IAA/ 50 mM NH4HCO3 and left in dark at room temperature for 30 min. Removed IAA solution and dried the gel with 300 μL 100%ACN by 5 min shaking and 3 min freeze-drying. Then added 80 μL 50 mM NH4HCO3 and 8 μL 0.25 μL trypsin solution (dissolved in 10 mM acetic acid). The digestion was carried out at 37 °C overnight. Tryptic peptide extracts with 200 μL 0.1% FA acetonitrile by shaking for 5 min and speed vac to dry. The dry digested samples were desalted using C18 Stage Tip (Thermo Fisher) according to the manufacturer’s
instructions. The desalted samples were sent to School of Pharmaceutical Sciences Central Laboratory in Sun Yat-sen University and performed the LC-MS/MS protein identification by nanoRPLC-Q Exactive Orbitrap (Thermo Fisher).

**Reporter-gene assay for NF-κB and NFAT**

In order to investigate the NF-κB and NFAT transcriptional inhibition activity of selective sRANKL inhibitor, luciferase reporter gene assays were used. RAW 264.7 cells stably transfected with an NF-κB luciferase reporter gene (3κB-Luc-SV40) or NFAT luciferase reporter gene were seeded into 96-well plates at a density of $1.5 \times 10^4$ or $1.0 \times 10^5$ cell per well respectively for 24 h. Cells were pretreated with 50 ng/mL of 100 ng/mL sRANKL and stimulated with 100 ng/mL sRANKL combined W194A, H226A sRANKL in different concentrations respectively. For the osteoclast formation induced by WT and Q238A sRANKL experiments, cells were stimulated with 50 ng/mL of PTG-κB transcriptional or 24 h for NFAT transcriptional. After treatment both cells were lysed and measured luciferase activity using a Promega Luciferase Assay system (Promega Corporation) by a Variscan flash Multimode Microplate Reader (Thermo Scientific).

**RT-qPCR and Western Blotting**

The mRNA expression level of S3-15 was measured by RT-qPCR as primary described. Total cellular RNA sample was prepared using RNAiso Plus, followed by reverse transcription with PreIMESTM MiXRT Master Mix reverse transcriptase kit. Then quantitative real-time PCR assays were performed with TB Green™ MixRT Master Mix reverse transcriptase kit. Primary antibody binding was detected using rat secondary antibodies (1:5000) coupled with enhanced chemiluminescence (ECL) reagents (Thermo Scientific) and visualized on Tanon 5200. Primers used for qPCR and antibodies used for western blotting were seen supplementary materials for details.

**Rescue experiments**

For the osteoclast formation induced by WT and Q238A sRANKL experiments, BMMs were seeded in 96-well plate in density of $6 \times 10^3$ cell/well with α-MEM completed medium containing 10 ng/mL M-CSF for 24 h. Then the cells were treated with 0.5 μM S3-15 with or without sRANKL (100 ng/mL) for 5, 10, 30 and 60 min after starved for 1 h. Then the cells were washed with cold PBS and lysed by RIPA Lysis Buffer. Cell lysates were then processed for Western Blotting analyses. Primary antibody binding was detected using rat secondary antibodies (1:5000) coupled with enhanced chemiluminescence (ECL) reagents (Thermo Scientific) and visualized on Tanon 5200. Primers used for qPCR and antibodies used for western blotting were seen supplementary materials for details.

**Drug concentration in plasma**

Plasma samples were stored at −80°C. Additionally, ten rats were used as a control and not treated with compound. Plasma from control mice was used to calibrate LC-MS and correct for matrix effects. Drug concentration in plasma was quantified by LC-MS-based methods. Pharmacokinetic parameters were estimated using Phoenix WinNonlin (version 6.3) (Certara USA, Princeton, New Jersey) from mean plasma concentration-time profiles.

**Osteoporosis therapeutic study in rats and mice**

Female SD rats or C57BL/6 mice were cared until they grew up to 6 months old or 10 weeks old, respectively. Then we subjected them to either bilateral ovarietomy or sham operation after anesthesia with 10% chloral hydrate (0.3 mL/100 g). Then all the rats or mice were intramuscular injected of penicillin to prevent infection in the first week of surgery. For rat experiments, after 12 weeks of operation, 3 rats from sham-operated or ovarietomized groups were random selected to determine bone mass by micro-CT imaging biopsy to verify successful produced bone loss(data unshown). Then the OVX rats were individually administrated with S3-15, S3, and S3-05 orally with (10 mg/kg/day) for another 12 weeks. For mouse experiments, after 4 weeks of surgery, the OVX mice were treated with 10 mg/kg/day S3-15 for another 4 weeks.

The weight of rats or mice were recorded every week in the treatment period. After treatment, the rats were harvested, the serum, heart, liver, spleen, lung, kidney, thymus, and femurs were all collected and subjected to subsequent analysis. The right femurs of rats or mice were cleaned excess soft tissue, fixed in 10% formalin, and processed them for microcomputed tomography (micro-CT) analysis. Left mice femurs were collected and excarnate for biomechanical strength

---

**Splenocyte transformation test**

Spleens were aseptically isolated from sham group, OVX group and OVX treatment group after treating S3-15 for 4 weeks in a clean bench (II Class biosafety Cabintes, Escolifesciences, Singapore). Splenocytes were used as a control and not treated with compound. Plasma from sham group was used to calibrate LC-MS and correct for matrix effects. Drug concentration in plasma was quantified by LC-MS-based methods. Pharmacokinetic parameters were estimated using Phoenix WinNonlin (version 6.3) from mean plasma concentration-time profiles.
testing. Mice peripheral blood were as well as collected for flow cytometry analyzing.

**Micro-CT analyses**

The right femurs of rats or mice were further analyzed with micro-CT on Inveon PET/CT (Siemens, Germany). We used micro-CT to scan the femur from the femoral head to the femoral condyle, using 19 μm resolution, 80 kV, 500 μA, 360 projections, 3000-6000 image threshold, full rotation cone beam. Three-dimensional reconstruction and histomorphometric analyses of trabecular bone were conducted with the IAW (Inveon analysis workstation) analysis software. The trabecular extended 1 mm (rat) or 0.5 mm (mouse) proximally to the end of the distal growth plate over 2 mm (rats) or 1 mm (mice) toward the diaphysis was selected as the region of interest (ROI) for the analysis. The resulting two-dimensional images of trabecular bone in relative cross sections were shown in grayscale. Trabecular bone parameters were measured including bone volume/tissue volume (BV/TV), bone surface area/bone volume (BS/BV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular pattern factor (Tb.P.F).

**Histological examination**

The mice left femur was fixed in paraformaldehyde for 72 h, demineralized using 10% Ethylenediamine tetra-acetic acid (EDTA-2Na) for 3 weeks, and then dehydrated with ethanol, clarified with xylene, and embedded with paraffin. Paraffin embedded tissue was sectioned on a rotary microtome. The sectioned tissues were stained with TRAP. The histologic changes of the femur caused by the ovariectomy were observed with a light microscope.

**Osteoporosis biomarkers determination**

Change of rat osteoporosis biomarkers after treating with S3-15 were determined by enzyme-linked immunosorbent assay (ELISA). Bone resorption serum biomarkers including CTx-I (C-terminal telopeptide of type I collagen), OC(Osteocalcin), PINP (Procollagen I N-Terminal propeptide), and RANKL(Receptor activator of nuclear factor-kB ligand). Rat blood was collected from aortaventralis after anesthesia, and centrifuged in 14000 × g at 4 °C to get the serum samples. The protein contents of those biomarkers in serum samples were detected according to manufacturer’s instruction (CTX-I and OC, Systems IDS, UK; RANKL, R&D Systems, USA; PINP, USCN Life Science, Inc., China.).

**Serum biochemical markers determination**

After treating with S3-15 for 12 weeks, rat serum was harvested and analyzed routine blood biochemical markers, including calcium (Ca), phosphorous (P), total cholesterol (TC), triglyceride (TG), glucose (Glu), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bile acid (TBIL), total bilirubin (TBA), creatinine (CREA), urea nitrogen (BUN). Ca, P, AST, ALT, ALP, CREA, and BUN were purchased from Wako Pure Chemical Japan; Glu,TG, TBIL, and TBA were purchased from Kehua Bio-engineering Co.LTD, China. Routine blood biochemical markers were examined with a Hitachi chemistry automated analyzer (Hitachi 7020, Japan) according to manufacturer’s instruction.

**Rats or mice organ coefficients determination**

We dissected all rats after 12 weeks of treatment, then took the main organs like heart, liver, spleen, lung, kidney, and thymus. All the organs were removed the attached adipose and weighted with an electronic balance (Sartorius, Germany). Mice spleen and cervical lymph node were also taken and weighted. Organ index (mg/g) was calculated as the following formula:

\[
\text{Organ index (mg/g)} = \frac{\text{Organ weight (mg)}}{\text{Rat or mouse total weight (g)}}
\]

**Splenic lymphocyte transformation test**

Spleens were aseptically isolated from sham group, OVX group and OVX treatment group after treating S3-15 for 4 weeks in a clean bench (II class biosafety Cabinets, Esolificiences, Singapore). Splenic single cell suspensions were obtained by gentle squeezing spleen tissue by injecting 2 mL cold Hank’s solution. Splenic cells were filtered through 40 μm nylon filters (BD Biosciences, Darmstadt, Germany), and centrifuged at 1400 × g for 10 min. Then cells were resuspended in 1640 completed medium (containing 10%FBS, 1% penicillin/streptomycin, 1% glutamine (200 mmol/L) and 5 × 10^{-3} mol/L 2-mercaptoethanol) with a concentration of 5 × 10^{6} cells/mL and seeded 100 μL into 96-well plates. Two wells for each mouse, and one well was stimulated with 50 μL CoA (Sigma-Aldrich, Germany) solution (working concentration 7.5 μg/mL in 1640 completed medium), another well added 50 μL 1640 complete medium as a control. After that, the plates were incubated at 37 °C in a CO2 incubator at 5% CO2 for 72 h. After stimulation, cells were measured by MTT method. Stimulation of cell proliferation ability was determined as the absorbance (OD) value of stimulated minus unstimulated cells for each cell sample.

**Mice peripheral blood T lymphocyte subsets analysis**

In order to study the effect of S3-15 on the immune system, after 4 weeks of S3-15 treatment, orbital venous blood with EDTA-anticoagulated was collected for T lymphocyte subsets analysis in osteoporosis mice. We used flow cytometry to determine T lymphocyte subsets according to the guidelines for Flow Cytometric of Beckman Coulter, FC500. Cells were stained according to manufacturer’s instruction of PE-Cyanine7 anti-Human CD8a and PE anti-Human CD4 antibodies (Tonbo Biosciences). Data analyzing was performed using CXP software (Beckman Coulter, Supplementary Fig. 9).

**General procedure for compound synthesis**

(1 mmol, 2 eq.). Water (3 mL) was also added, and stirred at 0 °C for 30 min to form mixture A. Compounds 2 (0.5 mmol, 1 eq.) was dissolved in MeOH (5 mL), followed by adding NaOAc (1.1 mmol, 2.2 eq.) and H2O (5 mL) to form mixture B. The mixture A was added into mixture B by drop wise, stirred at room temperature for 8 h. The precipitate was filtered, and washed with a mixture of MeOH:H2O = 3 : 1 to give S3 series. Detail of synthetic methods were seen in “Compounds synthesis” of Supplementary Information.

**Statistical analysis**

All statistical analysis was performed using Graphpad Prism Version 8. All the in vitro or in vivo data were shown as mean ± s.d. In vitro studies n represents biological replicates, in the animal studies n represents sample size (the number of mice or rats). For SPR and ITC graphs,
individual data points are plotted and calculated binding parameters by using the analytical software of the instrument according to the points value. Dose-dependent inhibition studies were statistically analyzed using two-tailed, paired t-test. For the comparisons of multiple groups, statistical analysis method was ANOVA. A p-value of < 0.05 was considered to be statistically significant.

Data availability
The synthesis routes and analytical spectra of the chemical compounds presented in this study are provided in the Supplementary Information document. The crystal structure of RANKL used in this study are available in Protein Data Bank database under accession code 1S55 and 3Q8Q. The protein mass spectrometry mass spectrometry results of pull-down assay in excel form. Sequence data from this article can be found in supplementary information. Source data are provided with this paper. All data supporting the findings of this study are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

References
1. Scott, D. E., Bayly, A. R., Abell, C. & Skidmore, J. Small molecules, big targets: drug discovery faces the protein-protein interaction challenge. Nat. Rev. Drug Discov. 15, 541–550 (2016).
2. Corbi-Verge, C., Garton, M., Nim, S. & Kim, P. M. Strategies to develop inhibitors of motif-mediated protein-protein interactions as drug leads. Annu Rev. Pharmacol. Toxicol. 57, 39–60 (2017).
3. Touti, F., Gates, Z. P., Bandyopadhyay, A., Lautrette, G. & Pentelute, B. L. In-solution enrichment identifies peptide inhibitors of protein-protein interactions. Nat. Chem. Biol. 15, 410–418 (2019).
4. Modell, A. E., Blosser, S. L. & Arora, P. S. SystematicTargeting of Protein-Protein Interactions. Trends Pharmacol. Sci. 37, 702–713 (2016).
5. Steeland, S., Libert, C. & Vandenbroucke, R. E. A New Venue of TNF Targeting. Int. J. Mol. Sci. 19, 1442 (2018).
6. Croft, M. et al. TNF superfamily in inflammatory disease: Translating basic insights. Trends Immunol. 33, 144–152 (2012).
7. Locksley, R. M., Killeen, N. & Lenardo, M. J. The TNF and TNF receptor superfamilies: Integrating mammalian biology. Cell 104, 487–501 (2001).
8. Nunez Martinez, O., Ripoll Noisexe, C., Carneros Martin, J. A., Gonzalez Lara, V. & Gregorio Maranon, H. G. Reactivation tuberculosis in a patient with anti-TNF-alpha treatment. Am. J. Gastroenterol. 96, 1665–1666 (2001).
9. Xie, X., Li, F., Chen, J. W. & Wang, J. Risk of tuberculosis infection in anti-TNF-alpha biological therapy: from bench to bedside. J. Microbiol. Immunol. Infect. 47, 268–274 (2014).
10. Aggarwal, B. B. Signalling pathways of the TNF superfamily: A double-edged sword. Nat. Rev. Immunol. 3, 745–756 (2003).
11. Croft, M. & Siegel, R. M. Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases. Nat. Rev. Rheumatol. 13, 217–233 (2017).
12. Eissner, G., Kolch, W. & Scheurich, P. Ligands working as receptors: Reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. Cytokine Growth Factor Rev. 15, 353–366 (2004).
13. Palladino, M. A., Bahjat, F. R., Theodorakis, E. A. & Moldawer, L. L. Anti-TNF-alpha therapies: The next generation. Nat. Rev. Drug Discov. 2, 736–746 (2003).
14. Mancini, F. et al. Inhibition of tumor necrosis factor-alpha (TNF-alpha)/TNF-alpha receptor binding by structural analogues of suramin. Biochem Pharmacol. 58, 851–859 (1999).
15. He, M. M. et al. Small-molecule inhibition of TNF-alpha. Science 310, 1022–1025 (2005).
16. Lacey, D. L. et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93, 165–176 (1998).
17. Lacey, D. L. et al. Bench to bedside: Elucidation of the OPG–RANK–RANKL pathway and the development of denosumab. Nat. Rev. Drug Discov. 11, 401–419 (2012).
18. Jones, D. H. et al. Regulation of cancer cell migration and bone metastasis by RANKL. Nature 440, 692–696 (2006).
19. Black, D. M. et al. Effects of continuing or stopping alendronate after 5 years of treatment - The Fracture Intervention Trial long-term extension (FLEX). A randomized trial. JAMA 296, 2927–2938 (2006).
20. Reid, I. R. Short-term and long-term effects of osteoporosis therapeutics. Nat. Rev. Endocrinol. 11, 418–428 (2015).
21. Raje, N. S., Bhatta, S. & Terpos, E. Role of the RANK/RANKL Pathway in Multiple Myeloma. Clin. Cancer Res. 25, 12–20 (2019).
22. Elango, J., Hao, B. & Wu, W. The hidden secrets of soluble RANKL in bone biology. Cytokine 144, 155559 (2021).
23. Ono, T., Hayashi, M., Sasaki, F. & Nakashima, T. RANKL biology: Bone metabolism, the immune system, and beyond. Inflamm. Regen. 40, 2 (2020).
24. Fata, J. E. et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell 103, 41–50 (2000).
25. Fouque-Aubert, A. & Chopural, R. Influence of RANKL inhibition on immune system in the treatment of bone diseases. J. Bone Spine 75, 5–10 (2008).
26. Walsh, M. C. & Choi, Y. Regulation of T cell-associated tissues and T cell activation by RANKL–RANK–OPG. J. Bone Miner. Metab. 39, 54–63 (2021).
27. Kanazaki, H. et al. Soluble RANKL Cleaved from Activated Lymphocytes by TNF-alpha-Converting Enzyme Contributes to Osteoclastogenesis in Periodontitis. J. Immunol. 197, 3871–3883 (2016).
28. Kitaura, H. et al. Osteocyte-related cytokines regulate osteoclast formation and bone resorption. Int. J. Mol. Sci. 21, 5169 (2020).
29. Celea-Terrassa, T. & Kang, Y. Metastatic niche functions and therapeutic opportunities. Nat. Cell Biol. 20, 868–877 (2018).
30. Kiechl, S. et al. Blockade of receptor activator of nuclear factor-kappaB (RANKL) signaling improves hepatic insulin resistance and prevents development of diabetes mellitus. Nat. Med. 19, 358–363 (2013).
31. Phelps, D. K. & Post, C. B. Molecular dynamics investigation of the effect of an antiviral compound on human rhinovirus. Protein Sci. 8, 2281–2289 (1999).
32. Mashiah, E., Nussinov, R. & Wolfson, H. J. FiberDock: A web server for flexible induced-fit backbone refinement in molecular docking. Nucleic Acids Res 38, W457–W461 (2010).
33. Lam, J., Nelson, C. A., Ross, F. P., Teitelbaum, S. L. & Freemont, D. H. Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. J. Clin. Invest. 108, 971–979 (2001).
34. Liu, C. et al. Structural and Functional Insights of RANKL–RANK Interaction and Signaling. J. Immunol. 184, 6910–6919 (2010).
35. Ito, S. et al. Crystal structure of the extracellular domain of mouse RANK ligand at 2.2-A resolution. J. Bone Miner. Metab. 20, 6631–6636 (2002).
36. Xu, J. et al. Cloning, sequencing, and functional characterization of the rat homologue of receptor activator of NF-kappaB ligand. J. Bone Miner. Res. 15, 2178–2186 (2000).
37. Nelson, C. A., Warren, J. T., Wang, M. W., Teitelbaum, S. L. & Freemont, D. H. RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor. Structure 20, 1971–1982 (2012).
38. Ta, H. M. et al. Structure-based development of a receptor activator of nuclear factor-kappaB ligand (RANKL) inhibitor peptide and molecular basis for osteopetrosis. Proc. Natl Acad. Sci. USA 107, 20281–20286 (2010).
39. Roodman, G. D. Treatment strategies for bone disease. Bone Marrow Transpl. 40, 1139–1146 (2007).
40. Kawai, M., Modder, U. I., Khosla, S. & Rosen, C. J. Emerging therapeutic opportunities for skeletal restoration. Nat. Rev. Drug Discov. 10, 141–156 (2011).
41. Chen, X., Zhi, X., Wang, J. & Su, J. RANKL signaling in bone marrow mesenchymal stem cells negatively regulates osteoblastic bone formation. Bone Res 6, 34 (2018).
42. Ikebuchi, Y. et al. Coupling of bone resorption and formation by RANKL reverse signalling. Nature 561, 195–200 (2018).
43. Ferrari-Lacraz, S. & Ferrari, S. Do RANKL inhibitors (denosumab) affect inflammation and immunity? Osteoporos. Int. 22, 435–446 (2011).
44. Sanchez-Paulete, A. R. et al. Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy. Eur. J. Immunol. 46, 513–522 (2016).
45. Arthur, K. K. et al. In vitro stoichiometry of complexes between the soluble RANK ligand and the monoclonal antibody denosumab. Biochemistry 51, 795–806 (2012).
46. Xiong, J. et al. Matrix-embedded cells control osteoclast formation. Nat. Med. 17, 1235–1241 (2011).
47. Cappariello, A. et al. Biotechnological approach for systemic delivery of membrane Receptor Activator of NF-kappaB Ligand (RANKL) active domain into the circulation. Biomaterials 46, 58–69 (2015).
48. Xiong, J. et al. Soluble RANKL contributes to osteoclast formation in adult mice but not ovariectomy-induced bone loss. Nat. Commun. 9, 2909 (2018).
49. Delgado-Calle, J. et al. MMP14 is a novel target of PTH signaling in T-cell costimulation for translation into successful cancer immunotherapy. Eur. J. Immunol. 46, 513–522 (2016).
50. Breuil, V. et al. Immune changes in post-menopausal osteoporosis: the Immunos study. Osteoporos. Int. 21, 805–814 (2010).
51. Nyfeler, B. & Pichler, W. J. The lymphocyte transformation test for the diagnosis of drug allergy: Sensitivity and specificity. Clin. Exp. Allergy 27, 175–181 (1997).
52. Zhao, C. et al. Identifying novel anti-osteoporosis leads with a chemotype-assembly approach. J. Med. Chem. 62, 5885–5900 (2019).
53. Yan, L., Lu, L., Hu, F., Shetti, D. & Wei, K. Piceatannol attenuates RANKL-induced osteoclast differentiation and bone resorption by suppressing MAPK, NF-kappaB and AKT signalling pathways and promotes Caspase-3-mediated apoptosis of mature osteoclasts. R. Soc. Open Sci. 6, 190360 (2019).
54. Walter, T. S. et al. Crystallization and preliminary X-ray analysis of mouse RANK and its complex with RANKL. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 65, 597–600 (2009).
55. Freyer, M. W. & Lewis, E. A. Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions. Methods Cell Biol. 84, 79–113 (2008).
56. Magni, F. et al. Expanding the proteome two-dimensional gel electrophoresis reference map of human renal cortex by peptide mass fingerprinting. Proteomics 5, 816–825 (2005).
57. Hong, G. et al. Asiatic Acid Inhibits OVX-Induced Osteoporosis and Osteoclastogenesis Via Regulating RANKL-Mediated NF-kappab and Nfatc1 Signaling Pathways. Front. Pharm. 11, 331 (2020).
58. Riddell, S. R. & Greenberg, P. D. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J. Immunol. Methods 128, 189–201 (1990).

Acknowledgements
This work was supported by the Natural Science Foundation of China (81573210, 8204473, 22717140), Guangdong Basic and Applied Basic Research Foundation (2019A1515110484) and the Science and Technology Planning Project of Guangdong Province (2016A020217005). We thank Prof. J.X. (School of Biomedical Sciences, University of Western Australia) for kindly providing full length mouse and human mRANKL plasmids and other kindly assistance.

Author contributions
D.H. and C.Z. contribute equally to this work. D.H. carried out most of the pharmacological and biological experiments, analyzed data, and wrote the paper. C.Z. carried out the experiments of the molecular modeling and chemical synthesis, wrote the paper. R.L. contributed to the in vitro and in vivo activity evaluation experiments. B.C. contributed to protein cloning, expression, and purification experiments. Y.Z. carried out plasmid transfection and functional evaluation experiments. Z.S. and J.K. performed the pharmacokinetic studies. Q.G. H.Z., and J.X. designed and supervised this project, as well as revised the manuscript.

Competing interests
The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-33006-4.

Correspondence and requests for materials should be addressed to Huihao Zhou, Qiong Gu or Jun Xu.

Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022