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ARTICLE

Novel heterocyclic ring fused oleanolic acid derivatives as osteoclast inhibitors for osteoporosis ‡†

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Osteoporosis is a major public health problem in our aging society. In the present study, series of novel oleanolic acid (OA) derivatives were synthesized *via* modifications in A-ring and C-28 position of OA, and their anti-bone resorption activities were evaluated. Screening results revealed that most of the derivatives inhibited RANKL-induced osteoclast formation from RAW264.7 cells. Among the derivatives, **6g** exhibited a potent inhibitory activity with IC_{50} 24 nM. Furthermore, **6g** prevented ovariectomy-induced bone loss in rats. The preliminary mechanism investigation demonstrated that **6g** suppressed the protein and mRNA expressions of c-Fos and NFATc1, downregulated mRNA of other osteoclastogenic markers. The results suggested that the OA derivatives might serve as potential leads for the development of novel agents for the treatment of osteoporosis.

1. Introduction

Osteoporosis is characterized by low bone mass and structural deterioration of bone tissue, leading to enhanced bone fragility and consequent increase in fracture risk. It is a global public health problem currently affecting hundreds of millions of people worldwide, particularly postmenopausal women and older men.[1] Osteoporosis is the overall weakening of bone caused by an imbalance between bone building and bone destruction, in which new bone formation by osteoblasts is exceeded by the resorption of old bone by osteoclasts.[2] Therefore, in the case of osteoporosis, efforts have been primarily made on the development of drugs that block bone resorption by inhibiting osteoclasts differentiation or activity, or promote bone formation by increasing osteoblasts functions.[3] To date, various agents have been clinically available for the osteoporosis therapy, but few are ideal for the treatment of associated pathologies.[4] Hormone-replacement therapy (HRT) is effective in the prevention of bone loss in early menopause, but accompanied with several severe side effects such as uterine bleeding and carcinogenesis.[5] Although bisphosphonates (Alendronate) and an antibody to RANK-L (Denosumab) can increase bone mass by shutting down cells known as osteoclasts, they also hinder the creation of new bone.[6] Recombinant parathyroid hormone (PTH) is the only anabolic agent currently used for treatment of osteoporosis by stimulating bone formation. But PTH is an injectant and its clinical use is limited to a 2-year period due to its increased incidence of osteosarcoma.[7,8] Therefore, development of new alternative agents for the treatment of osteoporosis is still highly desirable.

Osteoclasts, the unique bone-resorbing polykaryons are responsible for the bone resorption. Hence, inhibitors of osteoclasts may present a potential to prevent the excessive bone resorption associated with osteoporosis.[9] Compounds originated from natural resources have exerted a major role as lead structures in drug discovery.[10] Oleanolic acid (OA) and its glycosides have a wide spectrum of important biological activities including anti-osteoporosis effect.[11,12,13] In our previous studies, we have already synthesized and evaluated anti-osteoporosis activity of several heterocyclic derivatives in A-ring of oleanolic acid and C28-amides using natural amino acids, and most of the compounds inhibited osteoclast formation without significant cytotoxicity.[14,15] The SAR proved that modifications in A-ring and C-28 position is required for anti-osteoporosis activity, therefore, the improved potency for anti-osteoporosis effects with modification of OA inspired us to continue our work for searching highly active lead compounds for the treatment of osteoporosis.

In the present study, as a continuing research of our project to further improve the inhibitory effect on osteoclast, series of new oleanolic acid derivatives *via* modifications in A-ring and C-28 position with heterocycles were designed and synthesized. Because sulfone-extended backbone analogs are known to be associated with a broad spectrum of bioactivity in many drugs,[16] various sulfonyl groups were further introduced into the heterocycle fused A-ring. The inhibitory activity of the synthesized derivatives on the osteoclast formation was evaluated *in vitro*. On the basis of the screening results, a structure-activity relationship of the derivatives was

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summarized. The osteoprotective effect of the most promising OA derivative was also evaluated in ovariectomized rats *in vivo*. Furthermore, the preliminary mechanism of the compound with the most potent inhibitory activity was examined.

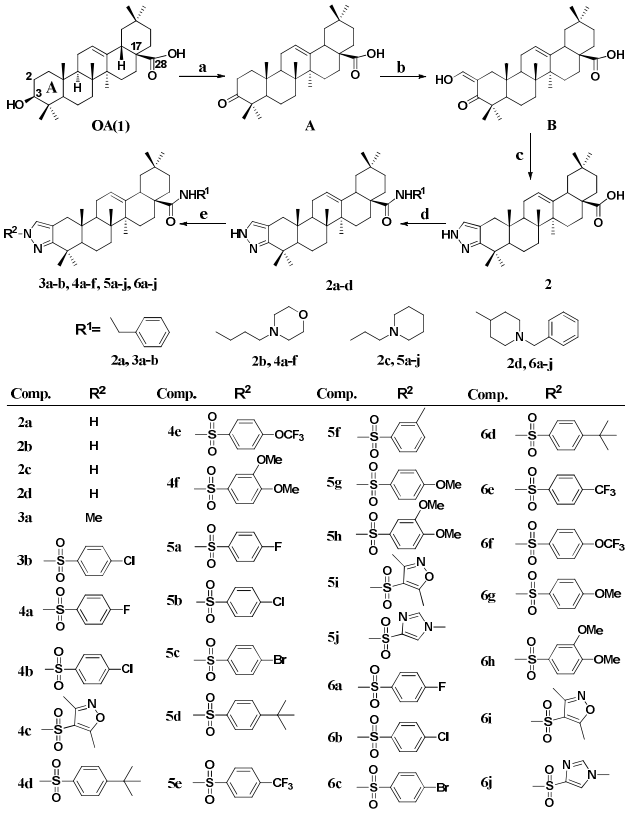
2. Results and discussion

2.1. Chemistry

Our previous work showed that modifications in A-ring and C-28 position of OA with heterocycles could improve potency and beneficial for anti-osteoclast activity.[14] Pyrazole, as an important class of nitrogen containing five membered heterocyclic rings has attracted much more attention in recent times due to its extensive range of pharmacological activities in the field of drug discovery. Some of drugs with pyrazole as basic moiety, such as celecoxib, deracoxib, etoricoxib and atorivodine are already available in the market.[17,18] Moreover, some natural products like betulinic acid derivatives which have similar structure to OA fused with pyrazole at C-2 and C-3 positions (A-ring) exhibited potent inhibitory activity on RANKL-induced osteoclast formation by TRAP assay.[19] Considering the importance of modified OA for its anti-osteoporosis effects and the pyrazole nucleus in medicinal chemistry, we first focused our work on the synthesis of the pyrazole derivatives of OA at A-ring. As shown in Scheme 1, 3-keto-oleanolic acid (A) was firstly prepared. For the oxidation of OA (**1**), Jones reagent was the most frequently used method,[20,21] however, because of the toxicity of chromium(VI) and a large quantity of metal waste, several other oxidation protocols were tested, and finally a Na_2WO_4-H_2O_2 system was applied.[22] With optimizing reaction conditions and minimizing generation of byproducts, the yield of target ketone A reached to 90%. After a simple ethyl acetate extraction, the product A was directly used for the next step without further purification. The key intermediate B was obtained in a high yield (96%) by Claisen condensation of A with ethyl formate in the presence of sodium methoxide in toluene, and the structure of compound B, especially an enol form at 2 position of A ring was elucidated based on the NMR data that were in agreement with those reported in literatures.[23,24] After extraction of the crude material, B was also directly applied to the next step without purification. Then, pyrazole OA was prepared *via* a reaction of B with hydrazine hydrochloride in 92% yield.[24] Next, preparations of several amides at C-28 position were tried. Firstly, attempts to directly couple **2** with amines under HOBt/EDCI catalytic condition were conducted. However, the target products were given in quite low yields, most probably due in part to the steric hindrance at C-28 position that had a great influence on the condensation reaction. Therefore, we first prepared 17-Acyl chlorides of OA derivatives by stirring compound **2** with thionyl chloride, then removed the solvent and treated with corresponding amines in the presence of triethylamine afforded amides **2a-d** in satisfied yields (> 60%), respectively.

It is frequently reported that molecules containing sulfonamides possessed a variety of biological activities, such as effects on bone remodeling, antibacterial, antitumoral, anti-inflammatory, and anti-cardiac disorders.[25,26] Because of the presence of hydrogen in N-2' of pyrazole ring, various sulfonyl groups together with methyl group were further introduced to the pyrazole ring to give target molecules **3a-b**, **4a-f**, **5a-j** and **6a-j** in high yields.

However, it is still ambiguous to which position the substituted groups was introduced in the pyrazole ring. Therefore, a detailed analysis of NOESY spectrum of compound **3a** was performed (see Supplementary Material). The structure of **3a** was optimized and analyzed with Material Studio 4.0 (Accelrys, USA). As shown in Fig. 1, a clear NOE



Scheme 1 Synthesis of pyrazole derivatives: (a) Na_2WO_4/H_2O_2, $NaH_2PO_4 \cdot 2H_2O/Na_2HPO_4 \cdot 12H_2O$, DMF; (b) HCOOEt, MeONa, toluene, r.t., overnight; (c) $NH_2NH_2 \cdot 2HCl$, EtOH, reflux, 4 h; (d) (i)$SOCl_2$, r.t., 1 h; (ii) amines, Et_3N, CH_2Cl_2, r.t., 0.5 h; (e) sulfonyl chloride, Et_3N, CH_2Cl_2, r.t., 0.5 h. 3a: CH_3I, K_2CO_3, DMF, r.t., 2 h.

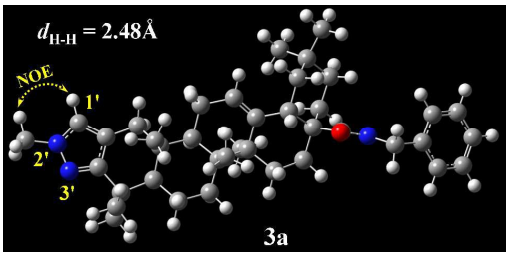
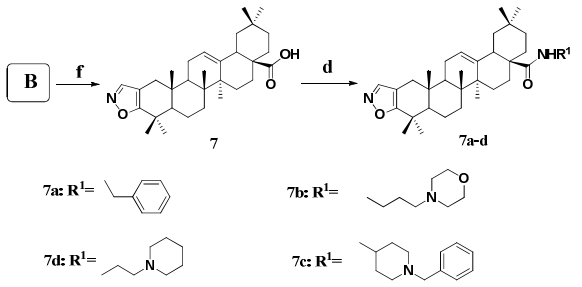


Fig. 1 NOE correlation (dashed arrow) in NOESY spectrum of compound **3a**



Scheme 2 Synthesis of isoxazole derivatives: (f) $NH_2OH·HCl$, EtOH, reflux, 4 h. (g) (i) $SOCl_2$, r.t., 1 h; (ii) amines, Et_3N, CH_2Cl_2, r.t., 0.5 h.

correlation between proton signals of NCH_3 [δ 3.83 (s, 3H)] and H-1' [δ 6.94 (s, 1H)] with a distance of 2.48Å in NOESY spectrum of **3a** was observed, indicating that the methyl group located at N-2' position. This result also implied that other sulfonyl groups should be assigned at the same position, N-2'.

Isoxazole derivatives are also an important class of five membered nitrogen- and oxygen-containing heterocyclic molecules that exhibit various bioactivities.[27] Especially, some of compounds bearing an isoxazole moiety provided a possible lead for therapeutic intervention in osteopenia and osteoporosis.[28] Encouraged by the reported data and screening results of pyrazole derivatives, four isoxazole derivatives of OA with C-28 substitutions were synthesized. The synthetic route is outlined in Scheme 2. Isoxazole OA (**7**) was prepared by condensation of B with hydroxylamine hydrochloride in EtOH.[24,29] Final compounds **7a**-**d** (yields > 70%) were obtained by the same protocol as described for the synthesis of compounds **2a**-**d**.

2.2. Biological activity and SAR

Osteoclasts are multinucleated bone-resorbing giant cells derived from hematopoietic cells with a monocyte/macrophage lineage. Terminal differentiation in this lineage is characterized by tartrate-resistant acid phosphatase (TRAP) staining and expressions of osteoclast markers. Receptor activator of nuclear factor-κB Ligand (RANKL) is preferentially expressed on committed preosteoblastic cells, whereas its specific receptor RANK is expressed in hematopoietic osteoclast progenitors.[2] RANKL is the key osteoclast differentiation factor and absolutely required for osteoclast development and bone remodeling *in vivo*, and also acts as a survival factor for osteoclast precursors.[30] In the present bioevaluation, multinucleated osteoclasts generation was achieved using RAW264.7 cells in the presence of RANKL, which has been used as a standard osteoclast formation model. The mature osteoclasts containing three or more nuclei from RAW264.7 cells at the differentiation day 5 were identified by the TRAP staining and morphological observation (Fig. 2). The inhibitory activity of all the heterocyclic ring-fused OA derivatives together with OA at the concentrations of 2 and 20 μM were assayed against RANKL-induced osteoclast formation.

Table 1 Inhibitiory activity of synthesized compounds against RANKL-induced osteoclast differentiation from RAW264.7 cells

Compd	Inhibition (%) OC		IC50 (μM)	Compd	Inhibition (%) OC		IC50 (μM)
	20 μM	2 μM			20 μM	2 μM	
Ctr.	0.0 ± 12.7	0.0 ± 8.2		5f	100.0**	33.6 ± 10.8*	
OA	82.1 ± 5.3**	11.4 ± 3.3		5g	100.0**	100.0**	0.088
2	99.0 ± 0.9**	32.1 ± 11.5*		5h	100.0**	100.0**	0.130
2a	100.0**	67.7 ± 7.5**		5i	100.0**	22.3 ± 1.0*	
2b	100.0**	49.8 ± 10.4**		5j	100.0**	89.5 ± 0.6**	
2c	100.0**	95.1 ± 2.2**	0.099	6a	82.9 ± 15.4*	73.0 ± 2.4**	
2d	100.0**	88.6 ± 0.1**		6b	41.3 ± 7.7**	18.2 ± 7.0*	
3a	99.7 ± 0.5**	76.0 ± 10.8**		6c	19.6 ± 13.9*	20.3 ± 26.9*	
3b	0.0 ± 5.8	0.0 ± 10.9		6d	65.6 ± 17.3**	39.2 ± 8.6**	
4a	97.7 ± 2.2**	56.1 ± 2.4**		6e	82.1 ± 6.4**	83.6 ± 3.3**	
4b	56.1 ± 5.2**	20.6 ± 6.6*		6f	71.9 ± 25.6**	2.2 ± 9.7	
4c	100.0**	75.2 ± 7.1**		6g	100.0**	90.0 ± 6.3**	0.024
4d	97.7 ± 2.2**	56.1 ± 2.4**		6h	100.0**	93.2 ± 4.1**	0.035
4e	86.3 ± 5.8**	31.8 ± 1.4*		6i	69.5 ± 11.8**	64.0 ± 11.2**	
4f	50.9 ± 9.1**	34.4 ± 2.7*		6j	100.0**	80.7 ± 3.4**	
5a	100.0**	100.0**	0.043	7	100.0**	27.4 ± 9.1*	
5b	100.0**	0.9 ± 9.8		7a	69.3 ± 11.7**	27.3 ± 11.0*	
5c	100.0**	100.0**	0.078	7b	45.9 ± 7.9**	23.2 ± 7.1*	
5d	89.8 ± 4.6**	20.8 ± 5.7*		7c	100.0**	78.5 ± 9.3**	
5e	100.0**	100.0**	0.068	7d	99.6 ± 0.8**	17.6 ± 12.1*	
AS		93.2 ± 4.7**	0.084				

TRAP+ osteoclasts were generated from RAW264.7 after 5 days of culture in the presence of RANKL (40 ng/ml) and the synthesized compounds at different concentrations. TRAP+ osteoclast number at control group (Ctr.) was 128. AS (alendronate

sodium) was used as a positive control. The data of control group was pegged as 100%, while inhibition (%) = 100% − osteoclast formation (%) of the compounds. Each data was expressed as mean ± S.D., n = 3. * $p < 0.05$ and ** $p < 0.01$ vs Control (Ctr).

As shown in Table 1, the derivatives containing pyrazole (**2**) or isoxazole (**7**) moieties both showed a better inhibitory activity compared with OA (**1**) did. Very interestingly, when the C-28 position was further modified by various heterocycles, all the compounds containing pyrazole ring **(2a**, **2b**, **2c** and **2d)** showed a much stronger activity than the unmodified one (**2)**. While, for those containing isoxazole ring (**7a**, **7b** and **7d**), except **7c**, modification of C28 carboxyl group did not cause any improvement in the inhibitory activity compared with the parent compound (**7**). These data demonstrated that the pyrazole derivatives led to a better inhibitory activity than isoxazole derivatives did (**2a** vs **7a**, **2b** vs **7b**, **2c** vs **7c** and **2d** vs **7d**). In addition, the pyrazole derivative (**2c**, inhibitory rate 95.1%) with piperidine at the position of C-28 showed higher inhibitory values than other amines (**2c** vs **2a**, **2b**, **2d**) at 2 μM.

Moreover, when various phenylsulfonyl substituent groups were introduced to the pyrazole ring, the inhibitory effects of the derivatives especially with moieties containing piperidine ring at C-28 position displayed almost 100% inhibition at 2 μM (**5a**, **5c**, **5g**, **5h**, **5e**, **6g** and **6h**). Substitution of $–OCH_3$ at phenylsulfonyl groups (**4f**, **5h**, **5g**, **6g** and **6h**) resulted in a much more potent inhibitory effect, while substituent of –Cl exhibited a negative impact on the activity (**3b**, **4b**, **5b** and **6b**), which was even lower than OA. Other substituents showed no distinct regularity.

Because compounds **2c**, **5a**, **5c**, **5e**, **5g**, **5h**, **6g** and **6h** showed almost full inhibition at 2 μM, further detailed tests on their half inhibitory concentration (IC_{50}) were performed. To our delight, these compounds still possessed potent inhibitory effects even at quite low concentrations. As shown in Table 1, all of the derivatives inhibited RANKL-induced osteoclast formation at nM concentration level, especially compounds **6g** (IC_{50} = 24 nM) and **6h** (IC_{50} = 35 nM). Fig. 2 displayed that compound **6g** significantly reduced the number of multinucleated osteoclasts.

To ascertain that the inhibitory activity of OA derivatives on osteoclastogenesis was not due to the decrease in viability of the precursor cells, we investigated the cytotoxicity of these compounds upon RAW264.7 cells by standard MTT assay. The results from Table 2 revealed that at concentrations up to 20 μM, **2c** and **6h** did not show any cytotoxicity on RAW264.7 cells. Compound **6g** showed a slight toxicity at 10 μM, and other compounds showed high cytotoxicity at the same concentration, while, those compounds did not show any cytotoxicity at their IC_{50} of osteoclastogenesis.

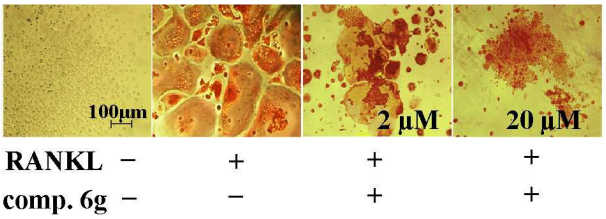


Fig. 2 Inhibitory effect of **6g** on RANKL-induced osteoclast formation

Table 2 Cytotoxicity of Compounds on Precursor of Osteoclasts (RAW264.7)

Compd	Inhibition(%) RAW 264.7			
	20 μM	10 μM	5 μM	2 μM
2c	0.0 ± 2.1	0.0 ± 1.4	0.0 ± 1.7	0.0 ± 3.3
5a	70.8 ± 1.9**	74.0 ± 1.3**	0.0 ± 4.8	0.0 ± 3.5
5c	70.8 ± 2.4**	67.2 ± 3.2**	0.0 ± 2.3	0.0 ± 7.1
5g	72.9 ± 1.6**	67.5 ± 4.1**	0.0 ± 6.1	0.0 ± 3.6
5h	71.1 ± 6.8**	52.6 ± 7.2**	0.0 ± 2.9	0.0 ± 5.4
5i	72.0 ± 2.2**	70.7 ± 9.3**	0.0 ± 3.4	0.0 ± 5.6
6g	58.2 ± 4.0**	6.6 ± 8.5	0.0 ± 8.7	0.0 ± 4.8
6h	0.0 ± 5.7	0.0 ± 8.0	0.0 ± 9.9	0.0 ± 6.2

MTT assay was used for the cytotoxicity evaluation. Data are expressed as mean ± S.D., *n* = 3. * $p < 0.05$ and ** $p < 0.01$ vs Control (0.0% inhibition).

Taking the above results together, a preliminary structure activity relationship (SAR) for synthesized derivatives could be outlined as follows: (1) Introduction of pyrazole or isoxazole moieties into OA could enhance the activity, while the pyrazole-bearing derivatives showed a much better performance than isoxazole-bearing ones. (2) Although isoxazole moiety improved the inhibitory activity in some extent, further modification at C-28 position did not give favorable results. (3) Modification at C-28 position of pyrazole-bearing derivatives with piperidine-containing moieties seems to be relevant to a strong activity. (4) Substitutions at pyrazole ring, such as phenylsulfonyl groups could ameliorate the activity. (5) Substituted group at phenylsulfonyl groups impacted the activity that the methoxyl group could improve the activity, while chloro group should be detrimental to the activity.

2.3. Prevention of OVX-induced bone loss in vivo

Because osteoporosis occurs most frequently in postmenopausal women due to the dramatic estrogen withdrawal, and ovariectomized (OVX) rat model is most commonly used in research on postmenopausal

osteoporosis.[31] As compound **6g** exhibited the most potent activity on osteoclast formation, OVX rat model was used to further test the preventive effect of **6g** on this low-bone-mass disease. The female rats (8 weeks old) were sham-operated or ovariectomized, and then orally treated immediately after ovariectomy with either vehicle, alendronate sodium as a positive control (AS, 1 mg/kg/day, orally) or increasing amounts of **6g** (0.1, 1 or 10 mg/kg/day). The dosing period was 4 weeks.

The efficiency of OVX was assessed through the significant weight gain, the body weight of OVX rats increased about 8% compared with that of Sham rats (Supplementary material Fig.

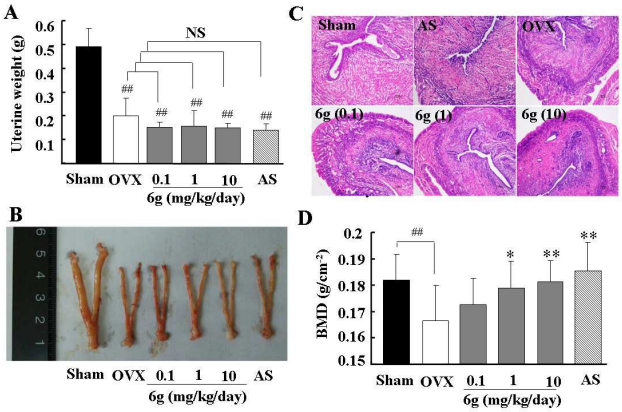


Fig. 3 6g protects against ovariectomy-induced bone loss: (A), (B) Uterine wet weight of OVX groups and sham group after euthanized (mean ± s.d., n = 10. $^{\#\#}P < 0.01$ versus sham group, Student's t-test). (C) The hematoxylin and eosin/Orange-G-stained uterine sections from sham and OVX groups. (D) The change of bone mineral density (BMD) of lumbar spine (L3-L5) between OVX groups and sham group, measured by Dual-energy X-ray absorptiometry (DXA) after treated with 6g or AS (mean ± s.d., n = 10. * $P < 0.05$, ** $P < 0.01$ versus OVX group, $^{\#\#}P < 0.01$ versus sham group, Student's t-test).

S1). The uterine weight of OVX rats also showed a marked reduction (Fig. 3A) due to uterine atrophy. These data suggested the success of the ovariectomy operation. Four weeks treatment of **6g** at all doses displayed no effect on the uterine weight (Fig. 3A), further histopathological observations of uterine displayed undeveloped endometrial luminal epithelium and endometrial glands (Fig. 3B, 3C). These data demonstrated that **6g** had no hormone-like side effects that are a big concern for female osteoporosis patients.[32]

Measurement of bone mineral density (BMD) was conducted with dual x-ray absorptiometry (DXA). As expected, OVX rats treated with vehicle showed significant bone loss at the lumbar spine, which is one of the most severely affected sites by osteoporosis. Alendronate sodium (AS), a clinically available bisphosphonate-type oral drug, significantly increased the BMD. On the other hand, although OVX rats treated with **6g** at 0.1 mg/kg/day did not show significant improvement of BMD, while the doses of 1 and 10 mg/kg/day displayed a significant effect to restore the deterioration of bone mass, similar to sham-operated animals (Fig. 3D). The results suggest that **6g** protected OVX-induced bone loss.

2.4. Possible mechanism

RANKL, as a key factor for osteoclast formation and activation, activates the expression of c-Fos and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), two of the most important osteoclast specific transcription factors.[33] c-Fos, as a member of the Fos family of genes, acts as an essential switch between osteoclast and macrophage differentiation, and even osteoclasts do not form in its absence.[34] NFATc1, a

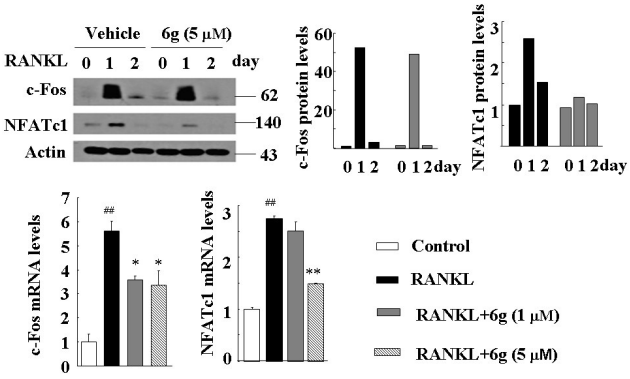


Fig. 4 The effects of **6g** on the protein and mRNA expressions of c-Fos and NFATc1: RAW264.7 cell cultures incubated on vehicle (DMSO) or with 6g in the presence of RANKL (40 ng/ml) for 2 days. Western blot analysis of c-Fos and NFATc1 proteins, normalized versus actin. The mRNA expression of the indicated genes was analyzed by real-time qPCR, normalized with actin expression (mean ± S.D., n = 3. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus control, * $p < 0.05$ and ** $p < 0.01$versus vehicle (RANKL only), Student's t-test).

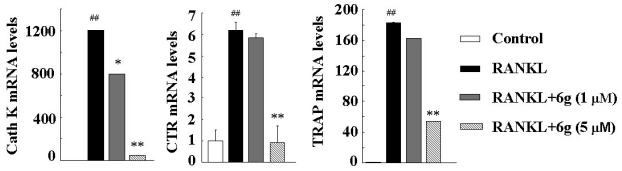


Fig. 5 The effects of **6g** on the mRNA expressions of osteoclastogenic markers: RAW264.7 cell cultures incubated on vehicle (DMSO) or with 6g in the presence of RANK-L (40 ng/ml) for 4 days. The mRNA expression of the indicated genes was analyzed by real-time qPCR, normalized with actin expression (mean ± S.D., n = 3. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus control, * $p < 0.05$ and ** $p < 0.01$versus vehicle (RANKL only), Student's t-test).

member of NFAT proteins, involves in all aspects of osteoclast formation and activation, and also seems like a prime target for antiosteoclast therapy.[35] In deed, the

calcineurin/NFATc1 inhibitors, tacrolimus (FK506) and cyclosporine A, prevent bone loss in inflammatory arthritis because they reduce the inflammation and inhibit osteoclast formation and its bone resorptive activity.[36,37] To examine the effects of **6g** on the mRNA and protein expressions of c-Fos and NFATc1, RAW264.7 cells were cultured in osteoclastogenic media with or without **6g** for 2 days. As shown in Fig. 4, RANKL stimulation increased the protein expressions of c-Fos and NFATc1 in vehicle-treated RAW264.7 cells, **6g** significantly inhibited the protein levels of c-Fos and NFATc1 in response to RANKL. The effects of **6g** on mRNA expressions of the two transcription factors were analyzed further by real time qPCR (RT-qPCR). The data exhibited that **6g** also suppressed mRNA expressions of c-Fos at both 1 and 5 μM, and NFATc1 at 5 μM, respectively.

As NFATc1 further directly induces osteoclast-specific genes, such as cathepsin K (CathK), tartrate resistant acid phosphatase (TRAP), calcitonin receptor (CTR), which are also important osteoclastogenesis-related biomarkers.[38] Therefore, the effects of **6g** on the mRNA expressions of those genes were further assayed with RT-qPCR. As can be seen in Fig. 5, RANKL markedly enhanced the mRNA levels of CathK, TRAP and CTR in vehicle-treated cells, and those mRNA increases were significantly attenuated by **6g** treatment.

Above data clearly indicated that the inhibitory effects of **6g** on osteoclast formation was *via* suppressing key transcriptional factors, c-Fos, NFATc1, and several down-stream signals, such as CathK, TRAP and CTR.

Conclusions

In summary, novel heterocyclic oleanolic acid derivatives were synthesized, and their inhibitory activity on RANKL-induced osteoclast formation generated from RAW264.7 cells was evaluated. Most of the compounds showed a better inhibitory activity than OA, and the pyrazole derivatives led to a better inhibitory activity than isoxazole derivatives. When various phenylsulfonyl groups were introduced to the pyrazole ring, some derivatives displayed much better inhibitory activity, especially, compounds **6g** exhibited IC_{50} values of 24 nM, while without cytotoxicity. Furthermore, **6g** prevented ovariectomy-induced bone loss *in vivo.* Preliminary mechanism study revealed that compound **6g** exerted its inhibitory activity *via* attenuating several key osteoclastogenic markers, such as c-Fos, NFATc1, CathK, TRAP and CTR. We deem the oleanolic acid derivatives as a promising new class of anti-osteoporosis leads deserving of further studies.

Acknowledgements

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Graphical abstract

Series of novel oleanolic acid (OA) derivatives were synthesized via modifications of A-ring and C28-amides of OA, and their anti-bone resorption activities were evaluated *in vitro* and *in vivo*.