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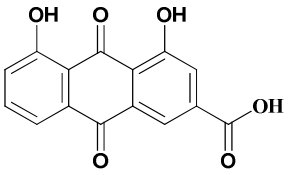

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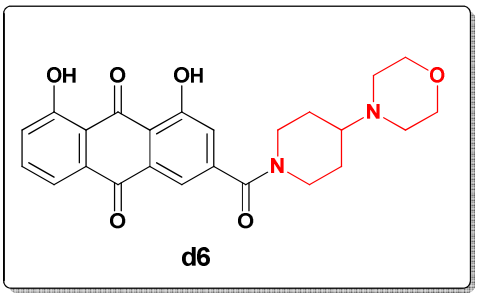

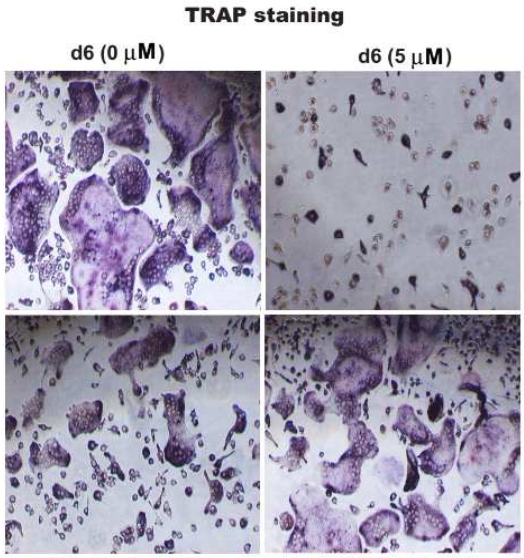

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Synthesis and biological evaluation of rhein amides as inhibitors of osteoclast differentiation and bone resorption

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Abstract:

Approaches of targeting excessive activation and differentiation of osteoclasts were considered as an effective treatment option for osteoporosis or osteopenia. In the present work, a series of rhein derivatives were synthesized and employed for their cytotoxicity screening against bone marrow-derived macrophages cells (BMMs) and their inhibition effects on osteoclasts activation and differentiation *in vitro* using an MTT assay and a TRAP activity assay respectively. Two rhein derivatives **d6** and **d11** inhibited BMMs activation and differentiation with 98% and 85% inhibitory activity respectively, without showing any cytotoxicity on BMMs. Subsequently, the most potent compound **d6** was further validated for its inhibitory effects on the formation of TRAP-positive multinucleated cells and bone resorption as evaluated by TRAP staining and bone resorption assay. The regulation by **d6** of osteoclast marker genes assay revealed that treatment of BMMs with M-CSF and RANKL resulted in the

stimulation of mRNA expressions of NFATc1, c-fos, TRAP, MMP-9 and cathepsin K which were highly related with osteoclast activation and differentiation, while **d6** decreased mRNA expressions of these genes. It was indicated that **d6** might regulate osteoclasts activity through RANKL/RANK/NFATc1 pathway. Thus our current work is expected to provide a highly promising approach for the development of a new type of anti-osteoporosis agent.

Key words:

Osteoclast, rhein derivatives, bone resorption, inhibitor, RANKL/RANK/NFATc1 pathway

1. Introduction

Bone is a highly dynamic tissue that undergoes significant modifications. Bone metabolism consists of a life-long remodeling process which is mediated by the balance between bone resorption by osteoclasts and bone formation by osteoblasts [1-5]. An imbalanced bone remodeling is involved in many diseases such as osteoporosis [6-7], rheumatoid arthritis [8-9], multiple myeloma [10] and Paget's disease [11].

The functional role of osteoclasts generated from bone marrow-derived macrophages cells (BMMs) is to resorb bone matrix through secreting acid and proteases to dissolve the organic and mineral components of bone [12]. Osteoblasts derived from mesenchymal stem cells (MSCs) mediate the main part in bone formation [13]. Osteoblasts, stromal cells and osteocytes express receptor activators of the nuclear factor-κb (NF-κB) ligand (RANKL) and macrophage colony-stimulating

factor (M-CSF) which are essential factors for the osteoclast differentiation [14-15]. RANKL is a member of the tumor necrosis factor (TNF) family and binds to the RANK receptor expressed in osteoclast precursor cells [12]. After the activation of RANKL–RANK signaling transduction cascades, various downstream signaling are triggered including nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) / c-fos pathway. Furthermore, genes such as tartrate-resistant acid phosphatase (TRAP), matrix metalloprotein-9 (MMP-9) and cathepsin K are highly expressed in the mature osteoclast [16] which are directly regulated by NFATc1. Currently, searching for osteoclast activation and differentiation inhibitors via RANKL signaling has turned to be a promising strategy for the development of antiresorptive agents.

Currently, bisphosphonates have been the most commonly used medications in the treatment of osteoporosis and prevention of fracture in osteoporotic patients. However, it is also reported that bisphosphonates have serious adverse effects such as osteonecrosis of the jaw and subtrochanteric fractures which are partly attributed to its promoting effects on osteoclasts apoptosis [17]. Another first-line therapy-ERT (estrogen-replacement therapy) increases the risk of breast cancer, stroke, clots and heart attack [18]. Hence, we need to develop more small molecules that regulate osetoclast activity and differentiation to improve the treatment of bone loss.

Natural products have made a significant contribution to drug discovery, and nearly half of the new drugs introduced into the market are natural products or their derivatives [19]. To develop novel inhibitors on RANKL induced osteoclast formation with more favorable pharmacological properties, we began to focus on natural

products because they were evolved to interfere and interact with their biological targets *in vivo*. Numerous natural products have been reported as potential osteoclastgenesis inhibitors such as paeonol[20], magnolol[21], halenaquinone[22] and emodin[23] (**Fig. 1**). As a privileged structure of medicinal chemistry, polyol anthraquinones have drawn high attention because of their extensive application in the biological areas [24, 25, 26, 27]. Particularly, emodin was reported to regulate bone remodeling by inhibiting osteoclastogenesis and stimulating osteoblast formation both *in vitro* and *in vivo* [23]. Moreover, diacerein inhibited the expressions of bone resorption enzymes and reduced osteoclastic differentiation in osteoarthritic subchondral bone [28]. Diacerein has been used for the treatment of osteoarthritis in Egypt and the Middle East as a prodrug of rhein. In addition, the bone affinity of rhein had been confirmed by hydroxyapatite affinity experiment and rhein hybrid compounds had been developed as bone-targeting agents [29, 30].

(Fig 1)

In the present work, we developed a series of novel rhein amide derivatives (**Fig. 2**). All compounds were also tested for their cytotoxicity on BMMs by MTT assay. The inhibitory activities of synthesized compounds with no cytotoxicity toward osteoclast were evaluated by TRAP activity assay on RANKL-induced osteoclastic activation and differentiation from BMMs. Based on this screen, we identified 1,8-dihydroxy-3-(4-morpholinopiperidine-1-carbonyl)anthracene-9,10-dione (**d6**) can effectively inhibit RANKL-induced osteoclastogenesis. Furthermore, **d6** was further validated to inhibit RANKL-induced osteoclast formation and bone resorption by

suppressing osteoclast marker genes through RANK/RANKL/NFATc1 pathway without any cytotoxicity on MC3T3-E1 osteoblast cells and MSCs. Detailed illustration of the synthetic procedure, biological experiments is presented as follows.

(Fig 2)

2. Chemistry

The compounds were synthesized as shown in Scheme 1. Commercially available rhein (**a**) was subjected to o-acetylation with acetic anhydride.[31] The diacerein (**b**) was transformed into the corresponding acid chloride using excess oxalyl chloride, and the acid chloride was reacted with aromatic or aliphatic amine to give the corresponding amides (**c**) with moderate to good yields. The compounds **d** were generated by hydrolysis of amides (**c**).

(Scheme 1)

3. Biological results and discussion

3.1 MTT assay for cytotoxicity

In order to confirm that the anti-osteoclastogenic effects of compounds are not attributable to cellular toxicity, twenty rhein derivatives, along with rhein and its analogue emodin were assessed their cytotoxic effects against BMMs *in vitro* at a concentration of 5 μM by performing MTT assay firstly. As shown in **Table 1**, the viability of untreated cells was set as 100%, the lead compound rhein and emodin displayed neither proliferation nor cytotoxicity on BMMs. Among these derivatives, the results showed that five compounds such as **c1**, **c2**, **d1**, **d3**, **d5** dramatically decreased the viability of BMMs, while six compounds, such as **c7**, **d2**, **d4**, **d6**, **d11**,

d12, had slight or no effect on cell viability at a concentration of 5 μM which indicated these compounds had no cytotoxicity toward osteoclast precursor cells. The effects of all derivatives on BMMs viability were summarized in **Table 1**.

(Table 1)

3.2 Effects of the rhein amide derivatives on RANKL-induced osteoclastogenesis

Our study was designed to identify a new class of safe and effective small molecules that blocked bone resorption through inhibiting the activity and differentiation of osteoclast, not promoting its apoptosis. Therefore our subsequent evaluation was focused on compounds **c7**, **d2**, **d4**, **d6**, **d11**, **d12** which showed ineffective activities on the viability of osteoclast. TRAP activity assay was conducted to investigate the activity of TRAP in BMMs osteoclastgenesis induced by M-CSF and RANKL. TRAP is an established marker for osteoclast and its activity is highly related with osteoclast differentiation and bone resorption. As shown in **Table 2**, compounds **c7**, **d2** and **d4** did not exhibit any inhibitory effects, whereas compounds **d6**, **d11** and **d12** dramatically inhibited TRAP activity with 98%, 85% and 65% inhibitory activity respectively. It was suggested that compounds **d6**, **d11** and **d12** were potent in inhibiting osteoclast activation and differentiation, which were more effective than rhein and emodin.

(Table 2)

Compounds **d6** and **d11** were the most potent compounds among 20 derivatives. In view of the relatively high activity and low cytotoxicity, we were determined to further investigate the effects of **d6** on osteoclast activation and differentiation. As

shown in **Fig 3A**, compound **d6** strongly inhibited TRAP activity in a dose dependent manner especially at the concentrations of 5 μM and 7.5 μM. This compound showed no cytotoxicity even at 7.5 μM (**Fig 3B)**.

(Fig 3)

Once BMMs were differentiated into mature osteoclasts, monocytes integrated into TRAP-positive multinucleated cells induced by M-CSF and RNAKL. We thus examined the effects of compound **d6** on the formation of TRAP-positive multinucleated cells. As shown in **Fig. 3C-D**, M-CSF and RANKL induced the formation of TRAP-positive multinucleated cells, while treatment of compound **d6** (5μM) significantly reduced osteoclast formation. Emodin exhibited relatively low inhibitory effects compared with compound **d6**, while rhein did not show any effects on the formation of TRAP-positive osteoclast. These results indicated that compound **d6** could significantly inhibit the differentiation of osteoclast and **d6** was more effective than its lead compound rhein.

3.3 Effects of **d6** on RANKL-induced bone-resorbing activity of osteoclasts

Bone resorption assay was further used to investigate the inhibitory effects of compound **d6** on osteoclast activity of bone resorption. BMMs were plated on to bone slices and compound **d6** was subjected to interfere bone resorption of mature osteclasts induced by M-CSF and RANKL. As shown in **Fig 4A**, osteoclastic bone resorption was almost completely inhibited after treatment with 5μM compound **d6** as **d6** treatment substantially reduced bone resorption area. The areas were also calculated in **Fig 4B**. Collectively, all above-mentioned results supported that compound **d6** could negatively regulate osteolclasts formation and osteoclastic bone

resorption activity *in vitro*.

(Fig 4)

3.4 Effects of **d6** on osteoclastogenesis-specific marker genes during the RANKL-induced osteoclast development

Osteoclasts activation and differentiation are associated with the up-regulation of specific genes such as the master regulators of osteoclast NFATc1 and c-fos, as well as bone resorption-related genes TRAP, MMP-9 and Cathepsin K responding to RANKL stimulation. As compound **d6** had been determined as an inhibitor of osteoclasts activation and differentiation, we next assessed the inhibitory effects of compound **d6** on the mRNA expressions of osteoclastogenesis-related genes to elucidate the mechanism underlying the inhibition of compound **d6** on osteoclastogenesis. In the assay, BMMs were treated with M-CSF and/or RANKL alone or combination with 5μM compound **d6**. As shown in **Fig. 5**, RANKL substantially induced the mRNA expressions of osteogenesis-related genes including NFATc1, c-fos, TRAP, MMP-9 and Cathepsin K as evaluated by qRT-PCR. In contrast, compound **d6** strongly suppressed the expressions of all studied genes. Here, we suggested that compound **d6** might regulate the RANKL induced osteoclast differentiation and bone resorption activity *in vitro* through regulating the expressions of NFATc1, c-fos, TRAP, MMP-9 and Cathepsin K.

(Figure5)

4. Conclusions

In the current study, we focused on a natural product rhein aiming to develop

novel inhibitors on osteoclasts activation and differentiation with more favorable pharmacological properties. Therefore, a series of rhein derivatives were designed and synthesized. Screening by MTT assay, it was found that there were four compounds showing none cytotoxicity while other compounds showing moderate or significant cytotoxicity against BMMs among these twenty derivatives. The identified novel osteoclast inhibitor **d6** through TRAP activity assay was determined to be highly potent compared to the lead compound rhein. Consequently, compound **d6** was further investigated for its effects on osteoclasts activation and differentiation using TRAP staining and bone resorption assay.

Chemokines such as M-CSF and RANKL induced osteoclast activation and differentiation by regulating the activity of nuclear receptor NFATc1 and c-fos and then controlled the expressions of bone resorption enzymes such as TRAP, MMP-9 and cathepsin K. Based on the current finding that **d6** repressed gene expressions of NFATc1, c-fos, TRAP, MMP-9 and cathepsin K induced by M-CSF and RANKL, we determined that compound **d6** might participate in osteoclastgenesis through regulation of RANKL/RANK/NFATc1 pathway.

During the bone remodeling, bone continuously undergoes coupled resorption and formation. Osteoclasts produced cytokines, chemokines and growth factors for coupling with osteoblasts to maintain normal bone formation. Some antiresorptive agents such as bisphosphonates may carry long-term risks, not only because of the effects of inducing osteoclast apoptosis, but also inhibiting persistent osteoblasts viability and cellular activity. Specifically, as shown in supplementary (**S1**),

compound **d6** at a concentration of 5 μM showed none cytotoxicity on osteoblast cells MC3T3-E1 and MSC survival as evaluated by MTT assay. The results above suggested a potentially important difference between compound **d6** and other antiresorptive agents.

In summary, we designed and synthesized a series of rhein derivatives and identified a novel compound **d6** as an inhibitor of osteoclastgenesis. It effectively inhibited the formation of TRAP-positive multinucleated cells and bone resorption induced by RANKL. We also elucidated its mechanism on inhibiting osteoclast function. Though SARs were not discernible in the present work, we are exploring new rhein derivatives based on this study and SARs research is ongoing.Our current work is expected to provide a highly promising approach for the development of a new type of anti-osteoporosis agent.

5. Materials and methods

5.1 Chemistry

5.1.1 General information

Column chromatography was performed on silica gel (200~300 mesh). NMR spectra were recorded on Brucker AVANCE 300 NMR spectrometer or Brucker AVANCE III 400 NMR spectrometer or Brucker AVANCE III 500 NMR spectrometer or Brucker AVANCE III 600 NMR spectrometer. Chemical shifts were reported in parts per million (ppm, δ). Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), heptet (hept), multipet (m) and broad (br). Low-resolution mass spectra (ESI) was obtained using Agilent HPLC-MS (1260-6120B). UPLC spectra were recorded on WATERS ACQUITY UPLC H-Class.

5.1.2. The procedure for preparation of **c1-c8**

The compound rhein (284 mg, 1 mmol) in acetic anhydride (612 mg, 6 mmol) was added with $Zn(CF_3SO_3)_2$ (2 mg, 0.005 mmol) and stirred at 130 ℃ for 2 h. Then the resulting yellow solution was poured into ice water, filtered and dried under reduced pressure, and recrystallized from ethyl acetate to obtain pure 4,5-diacetoxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid **b**. Oxalyl chloride (3 mmol) was added to a solution of diacerein **b** (1 mmol) in DCM (20 mL) dropwise with stirring and the mixture was added a catalytic amount of DMF. After 1 h, the mixture was distilled off under reduced pressure and the produced acid chloride was collected. The acid chloride residue directly reacted with aromatic or aliphatic amine (1.5 mmol) and $N(Et)_3$ (2 mmol) in DCM (20 mL) and stirred at room temperature (RT) for 3 h. The mixture was poured into water (30 mL), extracted with DCM (20 mL×2), washed with brine (10 mL), dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo to give the crude product, which was further purified by silica gel chromatography to afford the desired products **c**.

5.2 Biology

5.2.1 Biological materials

α-Modified eagle's medium (α-MEM), fetal bovine serum (FBS), penicillin and streptomycin (PS) were from Gibco. Dimethyl sulfoxide (DMSO), MTT, glucorticoid dexamethasone, β-glycerophosphate, ascorbic acid and TRAP staining kit were purchased from Sigma. M-CSF and RANKL were obtained from Peprotech. Reverse transcript reagents and SYBR Green PCR Master Mix were from Takara. TRAP activity assay kit and BCA protein assay kit were from Beyotime Biotechnology.

4-week-old male C57BL/6 mice were purchased from SLAC laboratory and were maintained at 22°C-24°C and 55%–60% humidity in a room with a 12/12-h light/dark cycle. All animal experiments were conducted according to the guidelines of the humane use and care of laboratory animals and were approved by the Shanghai

Jiao Tong University School of Medicine Animal Study Committee.

5.2.2 Cell culture

BMMs were isolated from 4-week-old male C57/BL6 mouse. MC3T3-E1 preosteoblast cell line was purchased from ATCC, and mouse mesenchymal stem cells (MSCs) derived from C57BL/6 were from Cyagen Biosciences. All the cells were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (full α-MEM) at 37°C in a 5%-humidified CO_2 incubator.

5.2.3 Primary bone marrow-derived macrophages cells isolation

BMMs isolated from a 4-week-old C57/BL6 mouse were prepared as previously described[32]. Briefly, all the bone marrow cells in the femur and tibiae of mouse were flushed out by α-MEM and then incubated in full α-MEM overnight. The cells in the supernatant were collected and then cultured in full α-MEM containing 30 ng/mL M-CSF for proliferation. After reaching 90% confluence, cells adhering to the bottom of the dish classified as BMMs were harvested by cell scraper. BMMs were induced to osteoclasts by full α-MEM plus M-CSF (30ng/mL) and RANKL (50ng/mL).

5.2.3 MTT assay

Effects of rhein derivatives on cell proliferation or cytotoxicity were determined by MTT assay. BMMs were plated in 96-well plates in full medium plus 30ng/mL M-CSF for proliferation at a density of 3×10^3 cells/well. The next day, cells were treated with indicated concentrations of compounds in triplicate for two days. 10 μL MTT (5mg/mL) was then added to each well, and the plates were incubated at 37°C for an additional 2 h. Then the supernatant were removed and 100μL DMSO was

added to each well to resolve the crystals, and the optical density (OD) was then measured with an Infinite F200 PRO absorbance microplate reader (Tecan) at 570 nm. Cell viability was calculated relative to the control.

5.2.4 Tartrate-Resistant Acid Phosphatase (TRAP) Colorimetric Assay

BMMs were seeded in 96-well plates at a density of 3×10^3 cells/well. Cells were exposed to in full medium plus 30ng/mL M-CSF and 50ng/mL RANKL in combinations with indicated concentrations of compounds in triplicate for two days. Cells were lysised and TRAP activity were measured by TRAP activity assay kit according to the manufacturer's instructions. Briefly, cell culture mediums were removed and cells were washed by PBS for 3 times. Then cells were lysised by passive lysis buffer (Promega) for 15min at 37°C and the supernatant were collected and incubated with para-nitrophenylphosphate (p-NPP) in the presence of disodium tartrate for 45min. The reaction was subsequently stopped with the addition of NaOH. The TRAP activity was then quantified by measuring optical density at 405 nm with Tecan absorbance microplate reader. The total protein levels were determined by BCA protein assay kit and the TRAP activity was normalized to the protein levels.

5.2.5 In vitro osteoclastogenesis assay

BMMs were cultured in 96-well plates in full medium containing M-CSF and allowed to adhere overnight. The medium was replaced and the cells were treated with 30 ng/mL M-CSF and 50ng/mL RANKL for additional five days. The medium was replaced for each two days. TRAP staining was then performed using a leukocyte acid phosphatase kit (Sigma) according to the manufacturer's instructions.

TRAP-positive multinucleated cells (≥3 nuclei) were scored as osteoclasts.

5.2.6 Bone resorption assay

For the bone resorption assay, BMMs were seeded on bovine femur bone slices in 96-well plates and the next day cells were induced with 30ng/mL M-CSF and 50ng/mL RANKL in combination with indicated concentrations of compound for five days. The medium was replaced for each two days. Cells were then fixed with 2.5% glutaraldehyde. Bone slices were stained by 0.5% toluidine blue and then imaged using an Olympus microscope with 200×magnification. Three view fields were randomly selected for each bone slice for further pit area analysis which were quantified using Image J software.

5.2.7 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA extraction and mRNA expression analysis by qRT-PCR were performed as described previously. mRNA levels of NFATc1, c-fos, TRAP, MMP-9, cathepsin K and β-actin were quantified by qRT-PCR using specific primers. The primer sequences were summarized below (**Table 3**). All values were reported as mean ± S.D. of triple measurements of each cDNA sample. mRNA levels were normalized to β actin mRNA.

(Table 3)

5.2.8 Statistical analysis

All data were expressed as mean ± SD and presented as the mean of triplicate points. Similar independent experiments were repeated three times with three replicates. A two-tailed non-paired Student's *t* test was used to compare differences,

and statistical significance was displayed as * $P < 0.05$ ** $P < 0.01$ or *** $P < 0.001$; # $P < 0.05$ ## $P < 0.01$ or ### $P < 0.001$.

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Supplementary Material

Rhein amide derivative d6 showed none cytotoxicity on MC3T3-E1 osteoblast and MSCs.

Compound d6 at indicated concentrations were incubated with MC3T3-E1 **(A)** and MSCs **(B)** for 2 days. Cell viability was then determined by MTT assay and ns represents no significance.

Supplementary data related to this article can be found at

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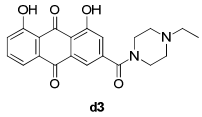
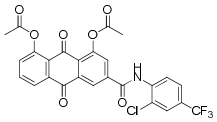
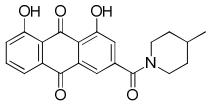
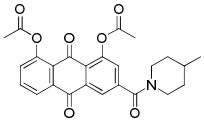
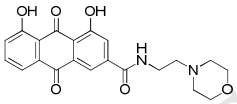
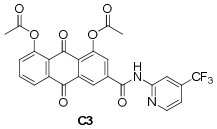
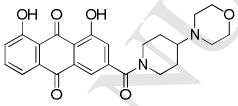
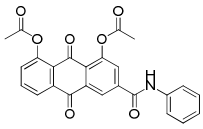
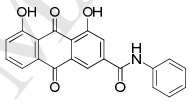
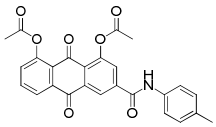
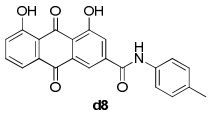
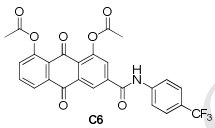
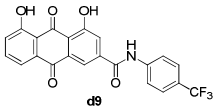
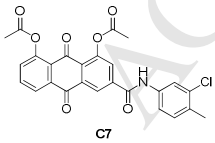
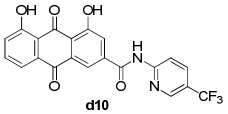
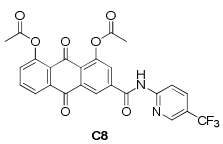
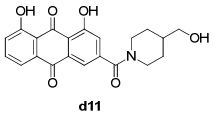
Table 1 The effects of rhein derivatives on BMMs viability

Compounds	Viability[a]（%）	Compounds	Viability[a]（%）
control	100	 d3	24±3
 C1	17±2	 d4	103±2
 C2	8	 d5	15±1
 C3	37±1	 d6	92±2
 C4	28±1	 d7	53±1
 C5	28±1	 d8	62±3
 C6	51±3	 d9	57±2
 C7	75±1	 d10	49±3
 C8	35±1	 d11	79±1

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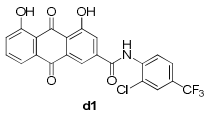
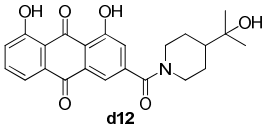
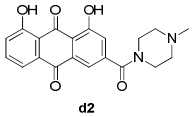
 d1	17±2	 d12	92±2
 d2	122±15	rhein	97±7
		emodin	109±6

[a] Cell viability of tested compounds were determined relative to the control at 5 μM by MTT assay.

Table 2 The inhibitions of rhein derivatives on osteoclasts differentiation determined by TRAP activity.

Compounds	**Trap activities Inhibition**[a]
c7	0
d2	0
d4	0
d6	98%
d11	85%
d12	65%
rhein	47%
emodin	45%

[a] TRAP activity was determined with a commercially available kit .

Table 3: Sequences of all primers in quantitative RT-PCR analysis.

Gene	**Primer sequence**
NFATc1	Forward 5’ - ACCACCTTTCCGCAACCA - 3’
	Reverse 5’ - TTCCGTTTCCCGTTGCA - 3’
c-fos	Forward 5’ - AGGCCCAGTGGCTCAGAGA - 3’
	Reverse 5’ - GCTCCCAGTCTGCTGCATAGA - 3’
TRAP	Forward 5’ - TCCCCAATGCCCCATTC - 3’
	Reverse 5’ - CGGTTCTGGCGATCTCTTTG - 3’
MMP-9	Forward 5’- CAAACCCTGCGTATTTCC - 3’
	Reverse 5’ - AGAGTACTGCTTGCCCAGGA - 3’
CtsK	Forward 5’ - GAAGAAGACTCACCAGAAGCAG - 3’
	Reverse 5’ - TCCAGGTTATGGGCAGAGATT - 3’
β actin	Forward 5’ - CTGTCCCTGTATGCCTCTG - 3’

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Reverse 5’ - ATGTCACGCACGATTTCC - 3’

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Figure Caption

Fig. 1. Chemical structures of natural osteoclast inhibitors.

Fig. 2. Novel rhein amide derivatives

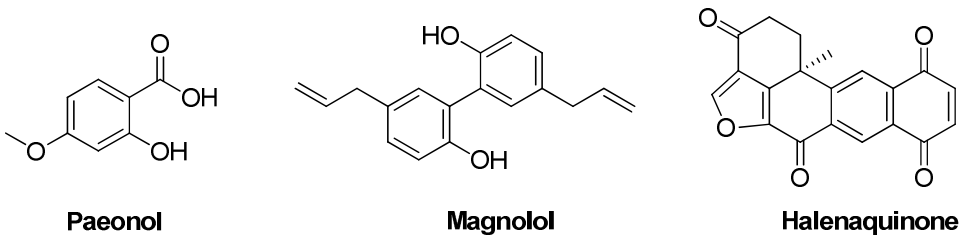
Fig. 3. Rhein amide **d6** inhibited the TRAP activity and the formation of osteoclast induced by M-CSF and RANKL in BMMs without any cytotoxicity. (**A**) Effect of compound **d6** on TRAP activity induced by M-CSF and RANKL. Compound **d6** in increasing dose, emodin and rhein at indicated concentrations were incubated with BMMs for 2 days in combination with M-CSF and RANKL. Cell lysis was determined with TRAP activity using a TRAP colorimetric assay. **P<0.01 and ***P<0.001vs control; (**B**) Effect of compound **d6** on BMMs proliferation. Compound **d6** in increasing dose, emodin and rhein at indicated concentrations were incubated with BMMs with M-CSF for 2 days. Cell viability was then determined by MTT assay; (**C**) Effect of compound **d6** on osteoclast activation and differentiation. BMMs were induced to differentiate into osteoclasts by M-CSF and RANKL. Compound **d6** in increasing dose, emodin and rhein at indicated concentrations were incubated with BMMs for 5 days and then TRAP staining was applied to identify TRAP-positive multinucleated cells which indicated mature osteoclasts; (**D**) The numbers of TRAP-positive multinucleated cells of different treatments as shown in **C** were calculated and presented graphically. The number of osteoclasts in control was set as 100%, the data was shown as the percents of the control. All experiments were carried out at least three times. ***P<0.001vs control.

Fig. 4. Rhein amide derivative **d6** inhibited bone resportion of osteoclasts induced by RANKL in BMMs. (**A**) Effect of **d6** on bone resorption of osteoclasts. BMMs were plated on bone slices in 96-well plate and then differentiate into osteoclasts by M-CSF and RANKL. Compound **d6** at indicated concentrations with BMMs for 5 days and then bone slices were stained by 0.5% toluidine blue to identify bone resorption areas by mature osteoclasts. Image was shown as 200×magnification; (**B**) The pit areas were quantified using Image J software and presented graphically. The pit area in control was set as 100%, the data was shown as the percents of the control. **P<0.001vs control.

Fig.. 5. Rhein derivative **d6** decreased the mRNA expressions of osteoclast marker genes. Osteoclasts marker gene levels of (**A**) NFATc1, (**B**) c-fos, (**C**) TRAP, (**D**) MMP-9, (E) cathepsin K were determined by quantitative RT-PCR analysis of compound **d6** at indicated concentrations in combination with M-CSF and RANKL in BMMs. The results were normalized to β-actin expression and expressed as fold change relative to gene expression in control cells. ***P<0.001 vs control.

Scheme Captions

Scheme 1. Synthesis of compounds c and d.. Reagents and conditions: (1) acetic anhydride, Zn(CF3SO3)2, 130 ℃, 2 h; (2) i: oxalyl chloride, DMF, DCM, 1 h, rt; ii: various anilines/ aliphatic amines, DCM, N(Et)3, 3 h, rt, ; (3) LiOH, H2O, MeOH, 40 ℃, 1 h.



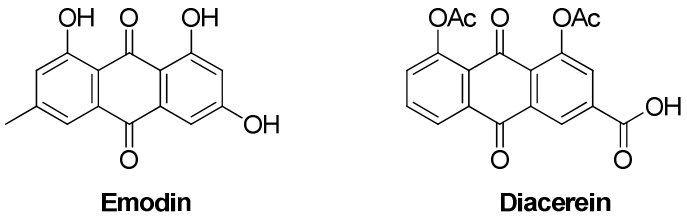


Fig. 1

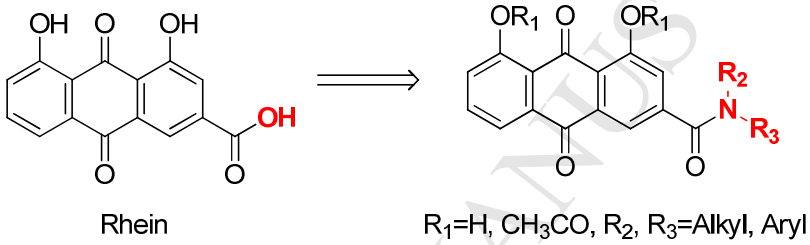


Fig. 2

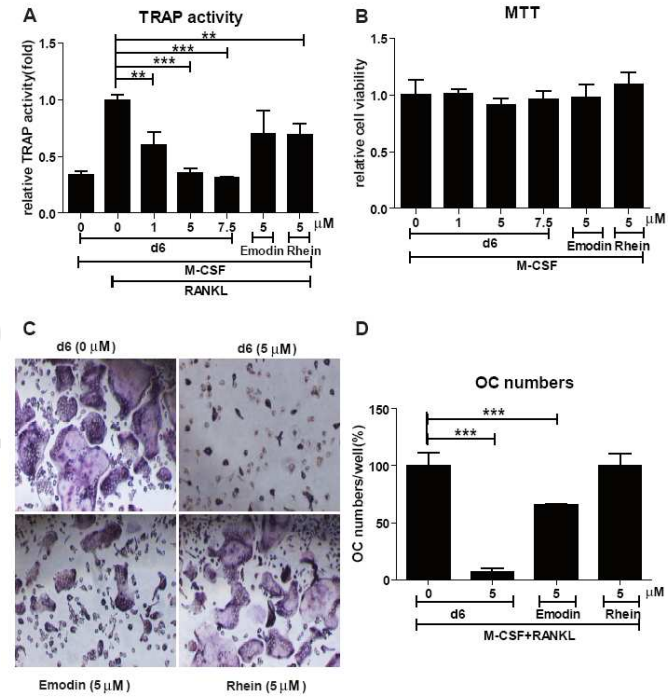


Fig. 3

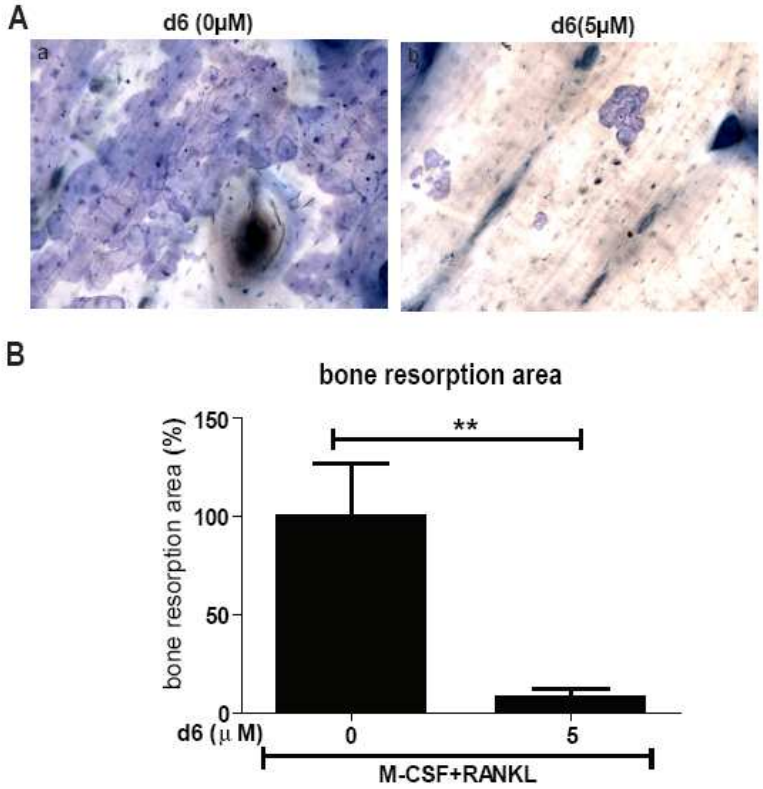

A
d6 (0μM)
d6(5μM)
a
b
B
bone resorption area
bone resorption area (%)
150
100
50
0
**
d6 (μ M)
0
5
M-CSF+RANKL

Fig. 4

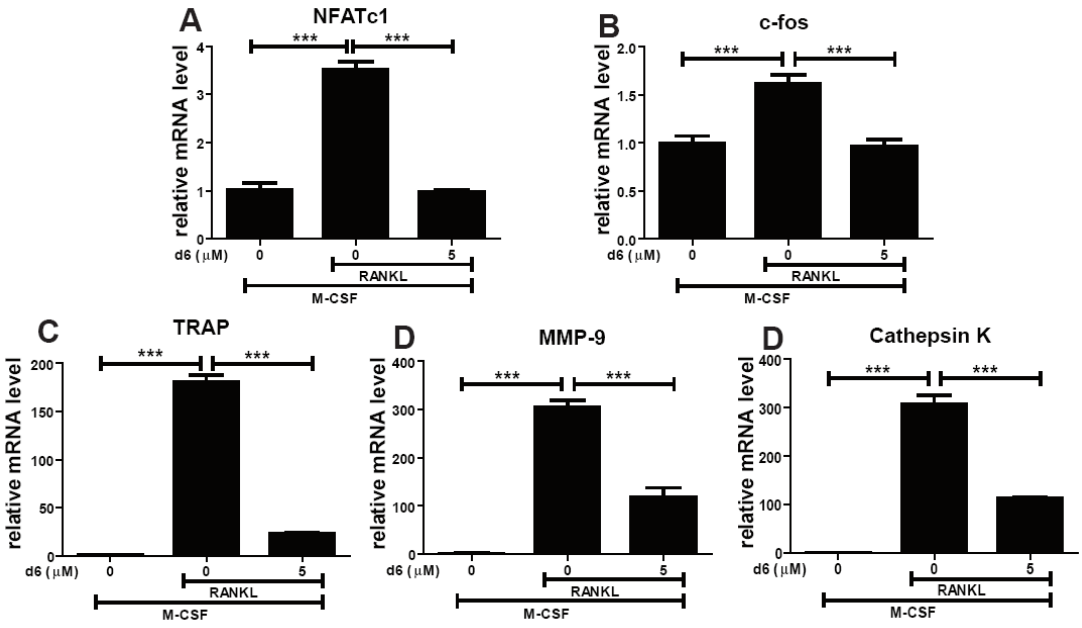
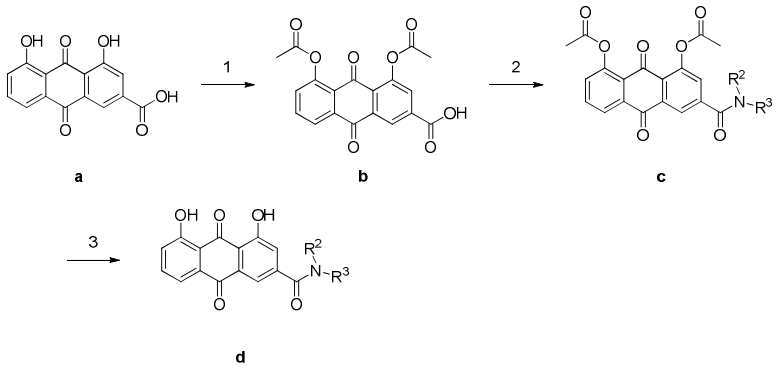

A
NFATc1
relative mRNA level
***
***
d6 (μM)
0
0
5
RANKL
M-CSF
B
c-fos
relative mRNA level
***
***
d6 (μM)
0
0
5
RANKL
M-CSF
C
TRAP
relative mRNA level
***
***
d6 (μM)
0
0
5
RANKL
M-CSF
D
MMP-9
relative mRNA level
***
***
d6 (μM)
0
0
5
RANKL
M-CSF
D
Cathepsin K
relative mRNA level
***
***
d6 (μM)
0
0
5
RANKL
M-CSF

Fig. 5



Scheme 1

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Highlights:

1. 20 rhein derivatives were designed, synthesized and characterized;

2. Compounds were evaluated against osteoclast differentiation and bone resorption;

3. The most active compound **d6** was reported with high activity and low toxicity.

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