Bioorganic & Medicinal Chemistry Letters 19 (2009) 3320–3323

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

20(*R*)-Ginsenoside Rh2, not 20(*S*), is a selective osteoclastgenesis inhibitor without any cytotoxicity

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ARTICLE INFO

Article history:
Received 27 January 2009
Revised 3 April 2009
Accepted 15 April 2009
Available online 18 April 2009

Keywords:
Ginsenosides 20(*R*)-Rh2
Bone protection
Osteoclast

ABSTRACT

Increased osteoclastic bone resorption plays a central role in the pathogenesis of many bone diseases, and osteoclast inhibitors are the most widely used treatments for these diseases. Ginsenosides, the main component of ginseng, have been known for their medicinal effects such as anti-inflammatory and anti-proliferative activities. In this study, we investigated the inhibitory effects of ginsenosides (ginsenoside 20(*R*)-Rh2 and ginsenoside 20(*S*)-Rh2) on osteoclastgenesis using RAW264 cells in vitro. Only ginsenoside 20(*R*)-Rh2 showed selective osteoclastgenesis inhibitory activity without any cytotoxicity up to 100 μM. These results implied that the stereochemistry of the hydroxyl group at C-20 may play an important role in selective osteoclastgenesis inhibitory activity.

Osteoporosis is a systemic skeletal disease characterized by loss of bone mass and micro-architectural deterioration of bone tissue. Osteoporosis is also a condition of aging and an increasing health burden in our society. Currently, osteoporosis affects more than 200 million people worldwide. Osteoporotic bone is less dense, more fragile, and more prone to fracture than normal bone. Healthy bone is maintained through continual replacement. There are two main cell types responsible for this: the osteoclasts, which break down the old bone; and osteoblasts, which form new bone. In the osteoporotic condition, bone resorption exceeds bone formation, resulting in a reduction in bone volume or density. The majority of current therapies for osteoporosis are anti-resorptive.

In addition, cancer preferentially metastasizes to bone, resulting in significant disease morbidity prior to a patient's death. It is known that cancer cells spread to bone and use the local cytokine machinery to stimulate osteoclasts, resulting in bone resorption and cancer cell growth.[1] Osteoclast activities are important to the development of bone metastasis in cancer.[2] Because of the fact that increased osteoclastic activity is associated with tumor growth in the bone microenvironment, anti-resorptive therapies have been used in cancer patients to block the tumor development in bone.

Ginseng, the root of *Panax ginseng* C.A. Meyer, Araliaceae, has been used as a traditional Chinese medicine for more than two thousand years in Asia. Ginseng and its active ingredients show widely beneficial health affects in animals and humans, including immune function effects,[3] anticancer effects,[4] a sexual function-enhancing effect,[5] and others. Increasing evidence is accumulating regarding its immune effect, its influence on circulatory function, and other effects. However, until now, only a few studies have been conducted to assess its anti-osteoporosis effect.[6] Very little is known about the principal constituents responsible for the anti-osteoporosis effect.

Many of the medicinal effects of ginseng are attributed to the triterpene glycosides known as ginsenosides. Ginsenosides are the main components responsible for the actions of ginseng; more than 30 types of ginsenosides have been identified.[7] As part of our continuing search for biologically active anti-osteoporotic and anti-metastic agents, we focused on ginsenosides. Recent reports have shown that the different stereochemistries of the ginsenosides, 20(*R*)-ginsenoside and 20(*S*)-ginsenoside, showed different pharmacological effects.[8,9] Thus, some recent reports about the stereoselective pharmacological activities of ginsenosides are worthy of attention.

Native ginseng contains trace amounts of ginsenoside Rh2 (about 0.01%). However, previous studies demonstrated that some natural ginsenosides, such as Rg3, Rb1, Rb2 and Rc, could be metabolized to Rh2 by human intestinal bacteria,[10] which suggests that Rh2 may contribute to ginseng's chemopreventive effects.

These previous findings have led us to examine the anti-osteoporosis effect of ginsenoside Rh2 and its stereoselective pharmacological effects due to the hydroxyl group at carbon-20 using RAW264 cells.[11]

To investigate whether ginsenosides affect the differentiation of osteoclasts, we utilized the preosteoclastic cell line RAW 264 to

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doi:10.1016/j.bmcl.2009.04.054

study the effect of ginsenosides on the osteoclast-lineage. The cells were treated with ginsenoside 20(*R*)-Rh2 and ginsenoside 20(*S*)-Rh2 (Fig. 1) for 3 days. The results clearly indicated that both compounds significantly depressed osteoclast formation (Figs. 2 and 3). Ginsenoside 20(*R*)-Rh2 showed a stronger inhibitory effect on osteoclast formation than ginsenoside 20(*S*)-Rh2 (Table 1). Ginsenoside 20(*R*)-Rh2 showed a lower IC_{50} (12 μM) (Table 1) on osteoclast formation than did 20(*S*)-Rh2 (32 μM) (Table 1).

It was reported that ginsenosides, specifically ginsenosides Rg3 and Rh2, inhibit tumor-cell proliferation and tumor growth, induce differentiation and apoptosis, and inhibit tumor-cell invasion and metastasis.[12–15] It has been documented that the progress of apoptosis is regulated by the expression of several transcriptional genes. One of these genes is a member of the Bcl-2 family.[16] The Bcl-2/Bax ratio, rather than the levels of individual proteins, is considered critical in determining the survival/ death of cells.[17] Hence, the ginsenosides which were observed to be associated with a decline of the Bcl-2/Bax ratio may contribute to HeLa cell apoptosis.[18] It is reasonable to consider that the inhibitory activities of ginsenosides on osteoclast formation might sometimes be a result of cytotoxicity effects. The cytotoxicity effect of ginsenoside 20(*R*)-Rh2 and ginsenoside 20(*S*)-Rh2 on osteoclastic cells was examined using WST-1 assay. The results clearly indicated that ginsenoside 20(*R*)-Rh2 did not affect the pre-osteoclast proliferation up to 100 μM (Fig. 3). Cell proliferation was observed at low concentration (up to 50 μM). On the contrary, ginsenoside 20(*R*)-Rh2 inhibited the pre-osteoclast proliferation and showed the IC_{50} at 62 μM (Table 1).

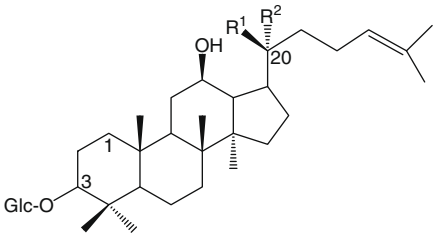

Figure 1. Structure of ginsenoside 20(*R*)-Rh2 and ginsenoside 20(*S*)-Rh2.

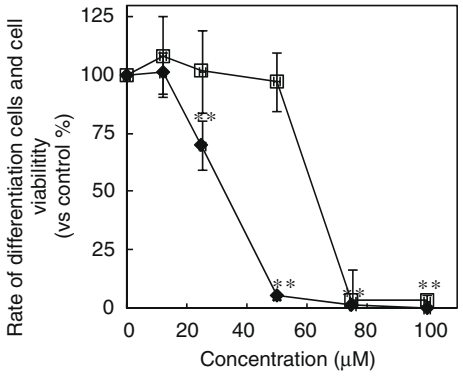

Figure 2. The effect of ginsenoside 20(*S*)-Rh2 on osteoclastgenesis and cell viability. (□: cytotoxicity; ♦: osteoclastgenesis). Results are given as the mean ± S.D., $n = 4$. ** $P < 0.01$ versus vehicle control.

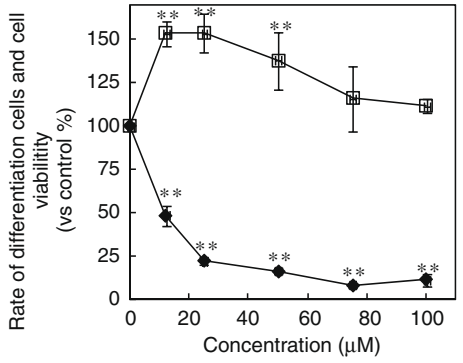

Figure 3. The effect of ginsenoside 20(*R*)-Rh2 on osteoclastgenesis and cell viability. (□: cytotoxicity; ♦: osteoclastgenesis). Results are given as the mean ± S.D., $n = 4$. ** $P < 0.01$ versus vehicle control.

Table 1
The antioxidant effects, osteoclast formation inhibition and proliferation inhibition of ginsenosides

Ginsenoside	Inhibition of osteoclast formation IC_{50} (μM)	Inhibition of osteoclast proliferation IC_{50} (μM)	DPPH radical scavenging activity % (1.6 mM)
20(*S*)-Rh2	32	62	<10
20(*R*)-Rh2	12	No inhibition up to 100 μM	<10

In the present study, we found that the stereospecificity of the hydroxyl group at the carbon-20 of ginsenosides plays a differential role in pre-osteoclast proliferation. More specifically, a slight difference in chemical structure between the ginsenoside Rh2 epimers produces a large difference in pre-osteoclast proliferation. More specifically, 20(*R*)-hydroxylation of ginsenoside Rh2 did not affect the pre-osteoclast proliferation. In contrast to 20(*R*)-Rh2, 20(*S*)-Rh2 showed strong cytotoxicity to pre-osteoclast proliferation (Fig. 2). These results indicate that the stereochemistry of hydroxylation at the C-20 of Rh2 is important for cell cytotoxicity.

Increased osteoclast activities are important to the development of bone metastasis in cancer. The data presented in this study demonstrated that ginsenoside 20(*R*)-Rh2, not 20(*S*)-Rh2, appears to inhibit the osteoclastic bone resorption via depression of the new osteoclast formation without any cytotoxicity. These results clearly demonstrated that ginsenoside 20(*R*)-Rh2 appears to be the active component in the osteoclastgenesis inhibitory effect, and has great potential in the treatment of osteoporosis and in bone metastases therapeutics with fewer side effects than other treatments. Clearly, further research is necessary to elucidate the molecular mechanisms involved in these actions, and might provide a new approach to the treatment of osteoporosis.

Recently, some studies have shown that ginsenosides exerted protective and pro-proliferation effects on diverse cells, such as neurons, endothelial cells, splenocytes and ovarian germ cells.[19,20] In these studies, ginsenosides promoted cell proliferation and life mainly via antioxidant and anti-apoptotic activity. In our experiment, we found that neither 20(*R*)-Rh2 nor 20(*S*)-Rh2 showed antioxidant activity up to 1.6 mM (Table 1). In this experiment, α-tocopherol showed 96% radical scavenging activity at the concentration of 0.2 mM. No significant differences in terms of solubility and lipophilicity were observed in these two compounds according to a series of assays. Also, these two compounds showed the same log*P* at 5.619 ± 0.429 (data cited from predicted property values of Scifinder Scholar). Osteoclast differentiation is induced by macrophage-colony stimulating factor (M-CSF) and RANKL. RANKL binds its cognate receptor RANK and induces the expression of c-Fos. The c-Fos induces NFATc1 expression, and c-Fos and NFATc1 cooperatively regulate osteoclastogenesis in response to RANKL stimulation. Because DC-STAMP is a target of RANKL stimulation, DC-STAMP expression is likely regulated by transcription factors downstream of RANKL-RANK signals, such as c-Fos and NFATc1. In our recent study, ganoderic acid DM, one of the triterpenoids

isolated from *Ganoderma lucidum*, significantly suppressed not only c-Fos induction but also NFATc1 upregulation by RANKL.[21] Ginsenoside 20(*R*)-Rh2 and/or ginsenoside 20(*S*)-Rh2 may inhibit osteoclastgenesis by this pathway. However, this remains unclear, and additional investigation is needed.

It has been reported that the cytotoxicity of ginsenosides against tumor cell lines differed between ginsenoside 20(*R*)-Rh2 and ginsenoside 20(*S*)-Rh2. Ginsenoside 20(*S*)-Rh2 showed potent cytotoxicity against tumor cell lines, with IC_{50} values of 22–33 μM, while ginsenoside 20(*R*)-Rh2 showed no potent cytotoxicity. These results seem to be consistence with our results.[22] Some studies have reported that Rh2 induces caspase-3 activity in hepatoma (SK-HEP-1) cells;[23,24] others reported that Rh2 appears to express a cytotoxic effect on intestinal cells that involves altering membrane permeability before initiating both apoptotic and necrotic cell characteristics. The cholesterol content of the culture medium which can influence the content of the cell membrane, is a potentially critical factor influencing the cytotoxic ginsenoside effect, since increasing the concentration of cholesterol of Erlich tumor cells has been shown to decrease the cytotoxicity of both Rg3 and Rh2.[25] Furthermore, a reduction in membrane cholesterol will lead to apoptosis.[26] The stereostructure-specific interaction of compounds with membranes containing cholesterol has been studied.[27] The compound-induced changes in membrane properties can be determined by turbidity and fluorescence polarization measurements of membrane preparations. Perhaps stereostructure-specific interactions with membranes may be related to the different levels of cytotoxicity between ginsenoside 20(*R*)-Rh2 and ginsenoside 20(*S*)-Rh2. Further research is needed to clarify these issues.

Further research is also necessary to study why only 20(*R*)-Rh2 showed no cytotoxicity to pre-osteoclast proliferation, but rather, if anything, growth stimulation effects at concentrations lower than 50 μM.

In conclusion, first: 20(*R*)-Rh2 showed a stronger inhibitory effect on osteoclast formation than 20(*S*)-Rh2; second: 20(*S*)-Rh2 showed a strong cytotoxicity effect on pre-osteoclast proliferation and 20(*R*)-Rh2 showed no cytotoxicity to pre-osteoclast proliferation. All of our results indicated that the effect of ginsenoside Rh2 on osteoclastgenesis and cell proliferation is stereoselective, and that the hydroxyl group at carbon-20 of Rh2 is important for not only osteoclast formation but also cytotoxicity.

The accumulation of knowledge of the biological activity of ginsenoside based on stereochemical molecular structure is likely to lead to the design of a safer and more effective osteoclastgenesis inhibitor.

The chemicals used were Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), Fetal bovine serum (FBS) (Gibco: activated charcoal, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), WST-1[4-[3-(4-iodophenly)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-bezene (Wako, Osaka, Japan), glutamine (Nissui, Tokyo, Japan), penicillin, streptomycin, and trypsin (Invitrogen, Carlsbad, CA, USA). α-MEM was purchased from GIBCO BRL (Grand Island, NY, USA), soluble RANKL (sRANKL) was purchased from PeproTech EC Ltd (London, UK), and TNF-α was obtained from Roche Molecular Biochemical (Mannheim, Germany). Tartrate-resistent acid phosphatase (TRAP) staining kit was purchased from Sigma–Aldrich (St. Louis, MO, USA). The ginsenosides were isolated as previously reported by Kim et al.[28]

Cell cultures. RAW264 cells were maintained in DMEM (Sigma) with 10% FBS. All media were supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Sigma). Incubations were performed at 37 °C in 5% CO_2 in humidified air. For osteoclast generation and other experiment, α-MEM medium (Sigma) was used.

TRAP-positive cell staining. RAW264 cells were suspended in phenol α-MEM containing 10% FBS and plated at a concentration of 6.8×10^3 cells/well into a 96-well culture dish in the presence of 30 ng/ml RANKL and TNF-α (10 ng/ml), then incubated for 24 h.[29] Then, different concentrations of each compound were added to the cultures. After 3 days of culture, the cells were fixed and stained for TRAP using the TRAP staining kit according to the manufacturer's instructions. TRAP staining cells with more than three nuclei were counted as osteoclast.

WST-1 assay. RAW264 cells were suspended in phenol α-MEM containing 10% FBS and plated at a concentration of 6.8×10^3 cells/well into a 96-well culture dish in the presence of 30 ng/ml RANKL and TNF-α (10 ng/ml), then incubated for 24 h. Then, different concentrations of each compound were added to the cultures. After 3 days of culture, the number of viable cells was compared by WST-1[4-[3-(4-iodophenly)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-bezene disulfonate] assay. Following incubation of cells for the above mentioned time, 10% WST-1 solution was added to each well and incubated at 37 °C for 3 h. Following incubation, plates were slightly shaken and immediately read at 450 nm with a scanning multiwell spectrophotometer.

DPPH (1,1-diphenyl-2-picry1hydrazyl) radical scavenging activities. DPPH and methanol were purchased from Wako Pure Chemical Industries, Ltd. α-Tocopherol, used as positive standard material, was purchased from Aldrich Chemical Co., One milliliter of the sample in methanol were added to a 250 μl of DPPH methanol solution (0.5 mM). After mixing gently and incubating at 37C for 20 min, the optical density was measured at 514 nm using UV spectrometer.

Data are reported as the mean ± S.D. Student's *t*-test for cell experiments was done to determine any significant difference between the groups. Differences between means at the 1% confidence level ($P < 0.01$) were considered to be statistically significant.

Acknowledgment

We thank Ms. E. Yamauchi for technical assistance in cell culture.

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