Methyl Ganoderic Acid DM: A Selective Potent Osteoclastogenesis Inhibitor

Jie Liu[1], Jun Shiono[1], Yukiko Tsuji[2], Kuniyoshi Shimizu[1],* and Ryuichiro Kondo [1]

[1]*Department of Forest and Forest Products Science, Faculty of Agriculture, Kyushu University, Fukuoka, 812-8581, Japan*

[2]*Bio-Architecture Center, Kyushu University, Fukuoka, 812-8581, Japan*

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. Ganoderic acid DM, the main component of *Ganoderma lucidum*, has been known for its medicinal effects such as anti-androgen and anti-proliferative activities. In this study, we investigated the inhibitory effects of ganoderic acid DM and its analog (methyl ganoderic acid DM and 7-oxo-methyl ganoderic acid Z) on osteoclastogenesis using RAW264 cell *in vitro*. Methyl ganoderic acid DM blocked osteoclastogenesis completely at 12.5 μM with low cytotoxicity less than 30%. On the other hands, ganoderic acid DM blocked osteoclastogenesis completely at the higher concentration of 50 μM, but 7-oxo-methyl ganoderic acid Z did not up to 100 μM. These results implicated the carbonyl group at C-3 is essentially for selective osteoclastogenesis inhibitory activity, and methyl esters at C-26 should play an important role in enhancing its osteoclastogenesis inhibitory activity.

Keyword: *Ganoderma lucidum*, methyl ganoderic acid DM, osteoporosis, rheumatoid arthritis.

INTRODUCTION

The balance between bone resorption (by osteoclasts) and bone formation (by osteoblasts) maintains bone homeostasis in a process called bone remodeling [1]. Bone is obviously resistant to dissolution, at least outside the body. Inside the body's highly active milieu, however, bone is remodeled at such a high speed that approximately 10% of the total bone content is replaced per year in adult humans [2]. Incremental changes in the rate of bone resorption can lead to bone disruption. This striking contrast emphasizes what an extraordinary and specific role osteoclasts play in the active maintenance of the bony skeleton. Large tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) that are hematopoietic in origin, osteoclasts are capable of resorbing bone [3, 4]. These multinucleated cells help dynamically remodel bones in coordination with osteoblasts, which deposit bone matrix. The excessive osteoclastic bone resorption relative to osteoblastic bone formation is often associated with osteopenic diseases including osteoporosis and rheumatoid arthritis. Osteoclastogenesis progresses through multiple stages, including differentiation, fusion, and activation (maturation) regulated by various factors, including cytokines, hormones, and other cells in the bone microenvironment. So the inhibition of osteoclast differentiation also has great clinical implications.

Osteoporosis is a very common disease accompanied by a high level of bone resorption, especially for postmenopausal women. Estrogen treatment, or hormone replacement therapy, is considered by many physicians to be the best method to prevent bone loss [5]. However, many women do not tolerate the numerous side effects, or are concerned about the possible increased risk of uterine and/or breast cancer [6-8]. There thus remains a need for highly efficacious anti-resorptive agents with excellent safety and tolerability profiles. Several recent reports whose goal is to identify patterns for preventing osteoporosis through daily diet examined the effects of food components and their bioactive components on bone metabolism [9-10]. We also focused on identifying the lead compound for developmenting inhibitors of osteoclastogenesis among medicinal food stuffs.

The fungus *G. lucidum* (Reishi, Mannentake, or Lingzhi) has been used for centuries in East Asia to treat various human diseases such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, and cancers [11, 12]. Its dried powder was especially popular as a cancer chemotherapy agent in the Imperial Court of ancient China [13]. *G. lucidum* has been reported to produce many bioactive oxygenated triterpenoids. Up to now, over 120 kinds of triterpenoids have been isolated from *G. lucidum* and the genus *Ganoderma.* Some of the triterpenoids such as ganoderic and lucidic acids, isolated from *Ganoderma*, have demonstrated cytotoxicity against mouse sarcoma and mouse lung carcinoma cells *in vitro* [14]. As part of our continuing search for biologically active anti-osteoporotic compounds, we found that osteoclast differentiation was inhibited by the ethanol extracts of *G. lucidum* and ganoderic acid DM (**1**) which was isolated as one of the active compounds by bioassay-guided fractionation [15]. Ganoderic acid DM (**1**) especially suppresses the expression of c-Fos and nuclear factor of activated T cells c1 (NFATc1). This suppression leads to the inhibition of dendritic cell-specific transmembrane protein (DC-STAMP) expression and reduces osteoclast fusion. These results prompted us to investigate the ability of gano-

*Address correspondence to this author at the Department of Forest and Forest Products Science, Faculty of Agriculture, Kyushu University, Fukuoka, 812-8581, Japan; Tel: +81-92-642-3002; Fax: +81-92-642-3002; E-mail: shimizu@agr.kyushu-u.ac.jp

deric acid DM (**1**) analogs (**2**, **3**) (Fig. **1**) to inhibit osteoclast differentiation to determine what structural elements are important for the potent inhibition of osteoclast differentiation.

MATERIALS AND METHOD

Chemicals

The chemicals used were Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), trimethyl-silyldiazomethane (Tokyo chemical industry CO. LTD, Japan), activated charcoal, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), WST-1[4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate] (Wako, Osaka, Japan), glutamine (Nissui, Tokyo, Japan), penicillin, streptomycin, and trypsin (Invitrogen, Carlsbad, CA, USA). Fetal bovine serum (FBS), 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). Ganoderic acid DM (**1**) [16]. was available from our previous works. The ^{13}C NMR spectra of methyl ganoderic acid DM (**2**) and 7-oxo-methyl ganoderic acid Z (**3**) [17] were measured. The molecular formula of these two compounds were estimated from its liquid chromatographs mass spectral-ion trap-time of flight (LCMS-IT-TOF) spectrum (**2**: [M+H$^+$] at m/z 483.3469, calculated for $C_{31}H_{46}O_4$, 482.3396; **3**: [M+H$^+$] at m/z 485.3504, calculated for $C_{31}H_{48}O_4$, 484.3553).

Methyl ganoderic acid DM (**2**): ganoderic acid DM (10 mg) in methanol (1 ml) -benzene (3 ml) was added to trimethylsilyldiazomethane (1.5 ml) at room temperature. The mixture was stirred for 30 min at room temperature and concentrated to give the corresponding methyl esters of ganoderic acid DM as a white powder, then followed by preparative HPLC (column: Inertsil ODS-3(20 mm i.d. x 250 mm, GL Science, Inc. USA), methanol: water=80:20, flow rate: 10 ml/min) afforded methyl ganoderic acid DM (**2**) (Rt: 23 min). The molecular formula of methyl ganoderic acid DM (**2**) was determined to be $C_{31}H_{46}O_4$ on the basis of the ion peak at m/z 483.3469 [M+H$^+$] in LCMS-IT-TOF spectrum. The ^{1}H NMR spectrum (Table **1**) of **2** indicated the presence of eight methyl singlets at δ 0.53, 0.78, 0.94, 0.96, 1.18, 1.68, 3.57, 6.60, an olefinic proton triplet at δ 6.60. The ^{13}C NMR spectrum (Table **1**) of **2** showed the presence of eight methyls (δ 5.86, 12.31, 17.87, 18.54, 21.36, 24.87, 25.32, 51.65), nine methylenes (δ 23.79, 25.58, 34.34, 28.54, 30.08, 31.83, 35.33, 34.82, 37.13), three methines (δ 36.17, 48.93, 50.36), four quaternary carbons (δ 39.38, 44.92, 47.2, 47.76), four olefinic carbons (δ 127.19, 139.48, 142.97, 162.67), two ketone carbons (δ 198.03, 214.63), and one carboxyl carbon (δ 168.71). Compare with the spectrum with **1**, these ^{1}H NMR and ^{13}C NMR data (Table **1**) led us to suggest that compound **2** was the methyl esters of compound **1**.

7-Oxo-methyl ganoderic acid Z (**3**): Conversion of C-3 keton of **2** to C-3 hydroxyl group was carried out by reduction with NaBH$_4$. NaBH$_4$ (10 mg) was added to **2** (10 mg) in methanol (3 ml) at room temperature. The mixture was stirred for 30 min at room temperature and the reaction was

stopped by added acetic acid (1 ml). The mixture was concentrated *in vacuo*, then followed by preparative HPLC (column: Inertsil ODS-3, methanol: water=80:20, flow rate: 10 ml/min) afforded 7-oxo-methyl ganoderic acid Z (**3**) (Rt: 11 min) as a white powder. The molecular formula of 7-oxo-methyl ganoderic acid Z (**3**) was determined to be $C_{31}H_{48}O_4$ on the basis of the ion peak at m/z 485.3504 [M+H$^+$] in LCMS-IT-TOF spectrum. The ^{13}C NMR spectrum (Table **1**) of **3** revealed total 31 carbon signals including 8 methyls, 9 methylenes, 4 methines, 4 quatemary carbons, 4 olefinic carbons, 1 carboxyl, 1 ketonecarbonyl, and 1 hydroxyl. Comparison of the ^{13}C chemical shifts of **3** with those of **2** indicated that the structure of compound **3** was 3β-hydroxy-7-oxo-5α-lanosta-8, 24(*E*)-dien-26-oic acid.

Cell Cultures

RAW264 cells were maintained in DMEM with 10% FBS. All media were supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Incubations were performed at 37ºC in 5% CO_2 in humidified air. For osteoclast generation and other experiment, α-MEM medium 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 [18]. 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-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene 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 three hours. Following incubation, plates were slightly shaken and immediately read at 450 nm with a scanning multiwell spectrophotometer.

Statistical Analysis

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.

RESULTS

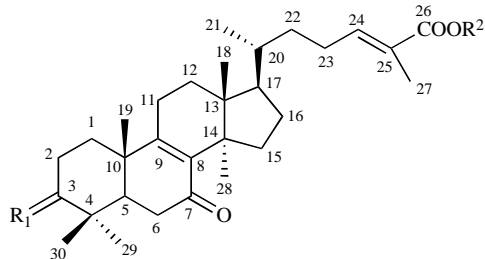In our previous work, we found the extremely high potential of the ethanol extracts of *G. lucidum* as a regulator

Table 1. NMR Spectral Data for Compounds 2 and 3 in CDCl₃

Position	δ_H 2	δ_H 3	δ_C 2	δ_C 3
1	1.79 (1H, m)	1.46 (1H, m)	35.33	34.79
	2.11(1H, m)	1.86 (1H, m)		
2	2.46 (1H, dt J=5.7,15.0)	1.73 (1H, m)	34.34	27.43
	2.70 (1H, dt J=12.9, 15.9)	1.69 (1H, m)		
3		3.30(1H, dd, J=5.7, 11.5)	214.63	77.94
4			47.20	38.92
5	1.48 (1H, dd J=3.2, 14.5)	1.63(1H, dd, J=5.4, 12.0)	50.36	49.82
6	2.35 (1H, m)	2.39 (1H, m)	37.13	36.65
	2.54 (1H, m)	2.41 (1H, m)		
7			198.03	199.05
8			139.48	138.89
9			162.67	164.78
10			39.38	39.76
11	2.36 (1H, m)	2.35 (1H, m)	23.79	23.64
		2.31 (1H, m)		
12	1.79 (1H, m)	1.82 (1H, m)	30.08	30.12
13			44.92	44.95
14			47.76	47.75
15	1.34 (1H, m)	2.07 (1H, m)	31.83	31.97
	1.97 (1H, m)	1.73 (1H, m)		
16	2.09 (1H, m)	1.96 (1H, m)	28.64	28.76
	1.69 (1H, m)	1.35 (1H, m)		
17	2.16 (1H, m)	1.44 (1H, m)	48.93	48.92
18	0.69 (3H, s)	0.70 (3H, s)	15.86	15.80
19	1.34 (3H, s)	1.21 (3H, s)	17.87	18.36
20	1.43 (1H, m)	1.47 (1H, m)	36.17	36.22
21	0.94 d (3H, d, J=6.1)	0.97 (3H, d, J=5.7)	18.54	18.60
22	2.48 (1H, m)	1.58 (1H, m)	34.82	34.88
		1.20 (1H, m)		
23	2.25 (1H, m)	2.25 (1H, m)	25.58	25.62
		2.12 (1H, m)		
24	6.88 (1H, dd, J=8.4, 7.7)	6.76 (1H, t, J=7.0)	142.97	143.08
25			127.19	127.18
26			168.71	168.71
27	1.84 (3H, s)	1.82 (3H, s)	12.31	12.34

Table 1. contd….

Position	δ_H		δ_C	
	2	**3**	**2**	**3**
28	0.94 (3H, s)	0.93 (3H, s)	24.87	24.98
29	1.12 (3H, s)	1.00 (3H, s)	25.32	27.41
30	1.10 (3H, s)	0.88 (3H, s)	21.36	15.29
31	3.58 (3H, s)	3.71 (3H, s)	51.65	51.69

of bone resorption [15]. Bioassay-guided fractionation led us to isolate ganoderic acid DM (**1**) as an inhibitor of osteoclastogenesis using RAW cells.

Triterpenoids	R^1	R^2
Ganoderic acid DM (**1**)	O	H
Methyl ganoderic acid DM (**2**)	O	CH$_3$
7-Oxo-methyl ganoderic acid Z (**3**)	OH / H	CH$_3$

Fig. (1). Structures of triterpenoids (**1-3**).

In general, dissociated form of carboxylic acids such as ganoderic acids is very unlikely to penetrate plasma membrane lipid bilayer. In neutral pH condition in medium, only small amount of the dissociated form of ganoderic acid DM (**1**) may enter into cytoplasm of RAW cells revealing the inhibition of osteoclastogenesis. If the specific lanostane-moiety in ganoderic acid DM (**1**) is essential for inhibition of osteoclastogenesis, but not carboxylic moiety at C-26, the change to the undissociated form of carboxylic moiety, more specifically, methyl ester-form (**2**) show more potent inhibitory activity than that of **1**. Furthermore, if so, the structural modification of functional group in lanostane-moiety of **2** decreases its inhibitory activity.

Thus, we focused on ganoderic acid DM (**1**) and its structural related compounds (**2, 3**). To investigate whether these analog affect the differentiation of osteoclasts, we utilized the preosteoclastic cell line, RAW 264 cell, to study the effect of analog (**2, 3**) on the osteoclast-lineage. The cells were treated with each sample for 3 days. The results clearly indicated that not all compounds depressed the osteoclast differentiation (Figs. **2** and **3**). Interestingly, among these three compounds, methyl ganoderic acid DM (**2**) showed the strongest inhibitory activity to osteoclast differentiation than ganoderic acid DM (**1**) and 7-oxo-methyl ganoderic

acid Z (**3**). Ganoderic acid DM (**1**) completely blocked the osteoclast differentiation at the concentration of 50 μM. 7-Oxo-methyl ganoderic acid Z (**3**) showed a low inhibitory activity on osteoclast differentiation even at the high concentration of 100 μM. Surprisingly, at the concentration of 12.5 μM, the osteoclast differentiation was completely blocked by methyl ganoderic acid DM (**1**). In contrast to methyl ganoderic acid DM (**2**), ganoderic acid DM (**1**) and 7-oxo-methyl ganoderic acid Z (**3**) showed no effect on osteoclast differentiation at this concentration. Almost complete inhibition of differentiation for ganoderic acid DM (**1**) was observed at 50 μM, but still remain 75% viable cells. The behavior of ganoderic acid DM (**1**) seemingly indicated that its inhibitory activity of differentiation not completely comes from its cytotoxicity. Like ganoderic acid DM (**1**), almost complete inhibition of differentiation for methyl ganoderic acid DM (**2**) was observed at 12.5 μM, but still remain more than 70% viable cells observed up to 20 μM. It should be noted that the limitation of solubility of methyl ganoderic acid DM (**2**) prevented future investigation more than 25 μM. Thus, we became aware that the inhibitory activity of methyl ganoderic acid DM (**2**) on differentiation was caused by not its cytotoxicity, but more like specific inhibition of differentiation. Furthermore, as speculated above, the easier penetration of **2** to inside of cells than **1** might enhance its inhibitory activity of osteoclastogenesis. This behavior, more specifically, selective inhibitory activity against differentiation, not viability, was important because safety is primarily consideration.
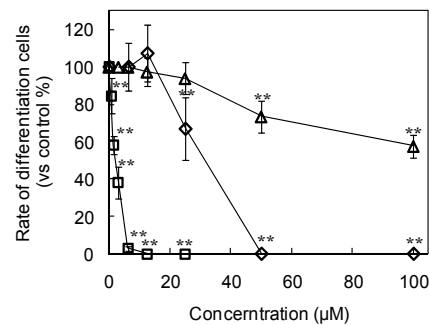

Fig. (2). The effects of ganoderic acid DM (**1**), methyl ganoderic acid DM (**2**) and 7-oxo-methyl ganoderic acid Z (**3**) on the osteoclastogenesis. (◊:ganoderic acid DM (**1**), □: methyl ganoderic acid DM (**2**), △: 7-oxo-methyl ganoderic acid Z (**3**)) Results are given as the mean±S.D., n=4. ** *P* <0.01 vs vehicle control.
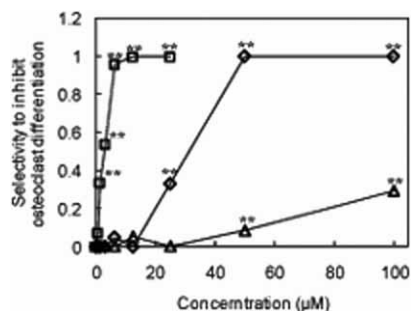
Fig. (3). The effects of ganoderic acid DM (**1**), methyl ganoderic acid DM (**2**) and 7-oxo-methyl ganoderic acid Z (**3**) on selectivity to inhibit the osteoclastogenesis. (◊:ganoderic acid DM (**1**), □: methyl ganoderic acid DM (**2**), △: 7-oxo-methyl ganoderic acid Z (**3**)) Results are given as the mean±S.D., n=4. ** P <0.01 vs vehicle control.

Osteoclast differentiation, in response to receptor activator of NF-κB ligand (RANKL) and a tumor necrosis factor α (TNF-α), was inhibited by ganoderic acid DM (**1**) [15]. The inhibitory activity of ganoderic acid DM is caused by especially suppressing the expression of c-Fos and nuclear factor of activated T cells c1 (NFATc1). This suppression leads to the inhibition of dendritic cell-specific transmembrane protein (DC-STAMP) expression and reduces osteoclast fusion. Obviously, the mechanistic investigation of osteoclast differentiation inhibitory activity by methyl ganoderic acid DM (**1**) (Fig. **2**) needs to be determined for further application against osteoporosis-related disease.

In Fig. (**3**), we defined selectivity to inhibit osteoclast differentiation by using the equality. selectivity to inhibit osteoclast differentiation = (the rate of cell viability (%) - the rate of differentiation cell (%)) / the rate of cell viability (%).

When the selectivity to inhibit osteoclast differentiation equal 1, it means that no osteoclast differentiation cell can be found among the living cells. From Fig. (**3**), methyl ganoderic acid DM (**2**) showed the highest selectivity to inhibit osteoclast differentiation than ganoderic acid DM (**1**) and 7-oxo-methyl ganoderic acid Z (**3**). Methyl ganoderic acid DM (**2**) showed the value of 1 at 12.5 μM, ganoderic acid DM (**1**) showed the same value at 50 μM, and 7-oxo-methyl ganoderic acid Z (**3**) cannot give the same value even at 100 μM.

DISCUSSION

Methyl ganoderic acid DM (**2**) and ganoderic acid DM (**1**), which showed higher selectivity to inhibit osteoclast differentiation, have a carbonyl group at C-3. In contrast to them, 7-oxo-methyl ganoderic acid Z, which showed low selectivity to inhibit osteoclast differentiation, has a hydroxyl group at C-3. These results suggested that a carbonyl group of the C-3 is essential to elicit the selectivity to inhibit osteoclast differentiation, and a methyl group at C-26 is needed to lower the cytotoxicity to osteoclast among these three compounds.

Osteoclasts appear uniquely adapted to produce the microenvironment and the biochemical milieu that are needed to resorb bone. Most notable of these is the cell membrane–associated protein termed receptor activator of RANKL, which is a member of the TNF family of cytokines. RANKL can then bind to its cognate receptor (RANK) on osteoclast precursors and, enhance the differentiation and fusion of these cells to produce functioning multinucleated osteoclasts. Concomitantly, eliminating the forming of osteoclasts means the bone resorption could be repressed. In this study, we found that methyl ganoderic acid DM (**2**) showed highly selective inhibition of osteoclastogenesis in RAW cells. Thus makes this compound can be used in osteoclastogenesis therapeutics.

It is not clear if the ingested methyl ganoderic acid DM (**2**) is absorbed into the biological system through the intestinal tract and delivered to the place where they are needed. The colonic microfloras convert most of these ingested triterpenoids into metabolites that then reach the circulation. Further biological evaluation of these triterpenoids is needed from only one aspect, but from a whole and dynamic perspective.

If the inhibitory activity of osteoclastic differentiation by the methyl ganoderic acid DM (**2**) from *G. lucidum* is also producible in human osteoclastic cells, it would be useful in a different sense since osteoclast play a crucial role in the osteopenic diseases including osteoporosis and rheumatoid arthritis.

Altogether our results illustrate the ability of some natural product-derived substances, ganoderic acid DM (**1**) and its analog (**2**) to inhibit the osteoclasts differentiation. For this kind of triterpenoids, a carbonyl group of the C-3 is essential to elicit the inhibition of osteoclast differentiation, and a methyl group at C-26 is needed to lower the cytotoxicity to osteoclast. Methyl ganoderic acid DM (**2**) should be a potential candidate for treating osteopenic diseases, such as osteoporosis and rheumatoid arthritis. Accumulation of the knowledge of the regulatory control of osteoclastogenesis on a molecular basis may provide a more rational and scientific approach to design safe and effective osteoclastogenesis control agents.

REFERENCES

[1] Manolagas, S.C. Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev*., **2000**, *21*, 115-137.

[2] Alliston, T.; Derynck, R. Interfering with bone remodelling. *Nature* **2002,** *416*, 686-687.

[3] Suda, T.; Takahashi, N.; Martin, T.J. Modulation of osteoclast differentiation. *Endocr. Rev*., **1992**, *13*, 66-80.

[4] Asagiri, M.; Takayanagi, H. The molecular understanding of osteoclast differentiation. *Bone*, **2007**, *40*, 251-264.

[5] Anderson, J.J.; Garner, S.C. The molecular understanding of osteoclast differentiation Phytoestrogens and bone. *Baillieres Clin. Endocrinol. Metab*., **1998**, *12*, 543-557.

[6] MacDonald, B.R.; Gowen, M. Emerging therapies in osteoporosis. Best practice & research. *Clin. Rheumatol*., **2001**, *15*, 483-496.

[7] Santell, R.C.; Chang, Y.C.; Nair, M.G.; Helferich, W.G. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J. Nutr*., **1997**, *127*, 263-269.

[8] Scheiber, M.D.; Rebar, R.W. Isoflavones and postmenopausal bone health: a viable alternative to estrogen therapy? *Menopause*, **1999**, *6*, 233-241.

[9] Mühlbauer, R.C.; Li, F. Nutrition: Effect of vegetables on bone metabolism. *Nature,* **1999**, *401*, 343-344.

[10] Park, C.K.; Lee, Y.; Chang, E.J.; Lee, M.H.; Yoon, J.H.; Ryu, J.H.; Kim, H.H. Bavachalcone inhibits osteoclast differentiation through suppression of NFATc1 induction by RANKL. *Biochem. Pharmacol.*, **2008**, *75*, 2175-2182.

[11] Yun, T.K. Update from Asia. Asian studies on cancer chemoprevention. *Ann. N. Y. Acad. Sci.*, **1999**, *88,* 157-192.

[12] Wasser, S.P.; Weis, A.L. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. *Crit. Rev. Immunol.*, **1999**, *19*, 65-96.

[13] Mizushina, Y.; Hanashima, L.; Yamaguchi, T.; Takemura, M.; Sugawara, F.; Saneyoshi, M.; Matsukage, A.; Yoshida, S.; Sakaguchi, K. A mushroom fruiting body-inducing substance inhibits activities of replicative DNA polymerases. *Biochem. Biophys. Res. Commun.*, **1998**, *249*, 17-22.

[14] Min, B.S.; Gao, J.J.; Nakamura, N.; Hattori, M. Triterpenes from the spores of *Ganoderma lucidum* and their cytotoxicity against

Meth-A and LLC tumor cells. *Chem. Pharm. Bull.*, **2000**, *48*, 1026-1033.

[15] Miyamoto, I.; Liu, J.; Shimizu, K.; Sato, M.; Kukita, A.; Kukita, T.; Kondo, R. Regulation of osteoclastogenesis by ganoderic acid DM isolated from *Ganoderma lucidum*. *Eur. J. Pharmacol.*, **2009**, *602*, 1-7.

[16] Liu, J.; Kurashiki, K.; Shimizu, K.; Kondo, R. Structure-activity relationship for inhibition of 5α-reductase by triterpenoids isolated from *Ganoderma lucidum*. *Bioorg. Med. Chem.*, **2006**, *14*, 8654-8660.

[17] Li, C.; Li Y. M.; Sun H.H. New ganoderic acids, bioactive triterpenoid metabolites from the mushroom *Ganoderma lucidum. Nat. Prod. Res.*, **2006**, *20*(11), 985-991.

[18] Watanabe, T.; Kukita, T.; Kukita, A.; Wada, N.; Toh, K.; Nagata, K.; Nomiyama, H.; Iijima, T. Direct stimulation of osteoclastogenesis by MIP-1alpha: evidence obtained from studies using RAW264 cell clone highly responsive to RANKL. *J. Endocrinol.*, **2004**, *180*, 193-101.