Methyl Ganoderic Acid DM: A Selective Potent Osteoclastogenesis Inhibitor

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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-

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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), trimethylsilyldiazomethane (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 ^1H 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 ^1H 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 $CDCl_3$

	δ_H		δ_C	
Position	**2**	**3**	**2**	**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

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