Bioorganic & Medicinal Chemistry Letters 19 (2009) 2154–2157

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Ganoderic acid DM: Anti-androgenic osteoclastogenesis inhibitor

Jie Liu [a], Jun Shiono [a], Kuniyoshi Shimizu [a], Akiko Kukita [b], Toshio Kukita [c], Ryuichiro Kondo [a,*]

[a] Department of Forest and Forest Products Science, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
[b] Faculty of Medicine, Saga University, Nabeshima 5-1-1, Saga 849-8501, Japan
[c] Faculty of Dental Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 4 December 2008
Revised 16 February 2009
Accepted 27 February 2009
Available online 4 March 2009

Keywords:
Ganoderma lucidum
Anti-androgen activities
Prostate cancer
Ganoderic acid DM
Androgen receptor
Osteoclast

ABSTRACT

Prostate cancer is the most common cancer in men in Western countries, with a high incidence of bone metastasis. Ganoderic acid DM, with 5α-reductase inhibitory and androgen receptor (AR) binding activity, isolated from the ethanol extracts of *Ganoderma lucidum,* can inhibit prostate cancer cell growth and block osteoclastogenesis.

Prostate cancer is the most frequently diagnosed male cancer and second leading cause of cancer deaths in North America.[1] Androgens play an important role in the proliferation, differentiation, maintenance, and function of the prostate.[2] Evidence shows that androgens are also involved in the development and progression of prostate cancer.[3] Androgens action is mediated by the 5α-reductase and androgen receptor (AR). 5α-Reductase catalyzes testosterone to the active androgen dihydrotestosterone (DHT). Androgen receptor binds to DHT, translocates into nucleus and regulates androgen responsive genes implicated in the development of prostate cancer.[4] A unique requirement for prostate cancer is the initial reliance on androgens for growth and to avoid apoptosis.[5] Because of this requirement, standard therapies block the action of androgens or remove the testicular androgens from the patient (endocrine therapy). These therapies include lower testosterone levels (androgen ablation); treatment with anti-androgens, such as flutamide or bicalutamide, to block DHT binding to the AR (anti-androgen therapy); and maximal androgen blockade (MAB), in which anti-androgen treatment and androgen ablation therapy are combined.

Prostate 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.[6] Osteoclast activities are important for the development of bone metastasis in prostate cancer.[7] Because of the facts that increased osteoclastic activity is associated with tumor growth in bone microenvironment, anti-resorptive therapies have been used in cancer patients to block the tumor development in bone.

On the other hand, more and more attentions begin to focus on the phytotherapeutic agents that may be more effective and safer.[8] Growing epidemiological data have reported that Asian men are less susceptible to prostate cancer than Europeans and Americans, which may be due to their high dietary intake of phytoestrogens.[9,10] Our group previously has obtained a series of triterpenoids from *Ganoderma lucidum* (Leyss.:Fr.) Karst. (Ganodermataceae), which have a suppressive effect on proliferation of the androgen-dependent prostate cancer cell line LNCaP cells[11] and estrogen-like activity on proliferation of the estrogen-dependent MCF-7 cells.[12] In the present study, we further investigated ganoderic acid DM, the active constituent derived from the fungi, *G. lucidum*. We report here that ganoderic acid DM has the potential to inhibit 5α-reductase activity, bind to the AR, inhibit the growth of prostate cancer cell, and suppress the osteoclastic differentiation by using RAW 264 cell.

In our previous screening of 19 edible and medicinal mushrooms, we discovered that the ethanol extracts of the fruiting body of *G. lucidum* showed the strongest 5α-reductase inhibitory activity. In addition, the treatment of the ethanol extracts prepared from *G. lucidum* at 1.5 and 15 mg/kg/day significantly inhibited the growth of the ventral prostate induced by testosterone in rats.[13] To clarify the active principles of the ethanol extracts of *G. lucidum*, 5α-reductase inhibitory activity-guided fractionation was carried out. The ethanol

* Corresponding author.
E-mail address: ryukondo@agr.kyushu-u.ac.jp (R. Kondo).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.02.119

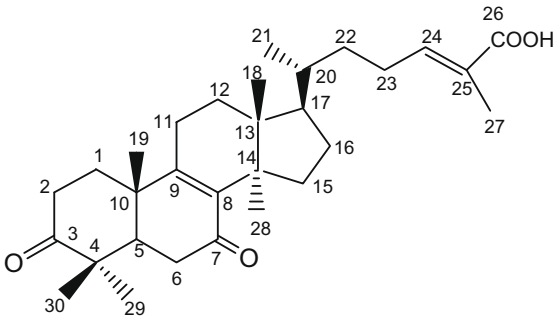

Figure 1. Structure of ganoderic acid DM.

extracts were roughly separated into three fractions (Fr. A, B, C). Only Fr. B showed the suppression effect on the ventral prostate growth induced by testosterone in rats. *G. lucidum* has been reported to produce many bioactive oxygenated triterpenoids. Over 120 species of triterpenoids have been isolated so far from *G. lucidum* and the genus *Ganoderma*.[14] Considering the results of our TLC analysis (data not shown), it is likely that most of the triterpenoids were present in Fr. B. Therefore, we focused on the triterpenoids in the ethanol extracts of *G. lucidum*. Anti-androgenic assay (5α-reductase inhibitory activity and androgen receptor-binding)-guided fractionation led to the isolation of one of active triterpenoid from Fr. B, identified as ganoderic acid DM (Fig. 1).

The inhibition of 5α-reductase by ganoderic acid DM was concentration-dependent, as shown in Figure 2. As the concentrations of ganoderic acid DM increased, the residual enzyme activity decreased. The IC_{50} of ganoderic acid DM was 10.6 μM. It should be noted that finasteride,[15] which is known as a potent steroidal inhibitor, showed an IC_{50} of 0.73 μM in our assay system. α-Linolenic acid, a natural compound with 5α-reductase inhibitory activity was also used as a positive control, showed an IC_{50} of 116 μM in our assay system. Ganoderic acid DM showed stronger 5α-reductase inhibitory activity than the natural 5α-reductase inhibiter, α-linolenic acid.[15] In the history of 5α-reductase inhibitor study, the inverted steroid-based inhibitors have been extremely important drugs for hormone dependent cancers.[16,17] These 5α-reductase inhibitors, the molecules bind in the active site of the enzyme such that the steroid A-ring mimics the A-ring functionality of testosterone or some intermediate along the reaction pathway; the potency and selectivity is determined in part by

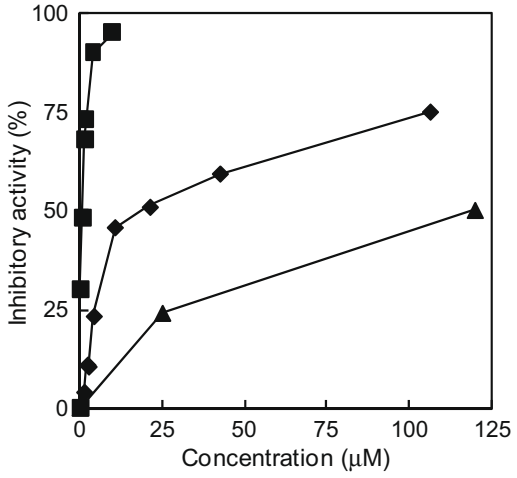

Figure 2. Effect of ganoderic acid DM (♦), finasteride (■) and α-linolenic acid (▲) on the activity of 5α-reductase.

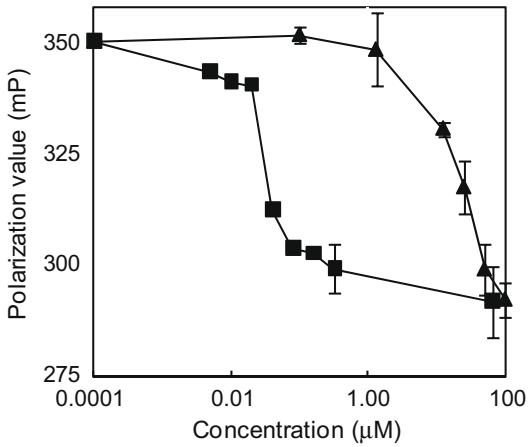

Figure 3. Effect of ganoderic acid DM and DHT on the competitive binding activity on AR. (■: DHT, ▲: ganoderic acid DM). Results are given as the mean ± S.D., $n = 3$.

appropriate substitution of the D-ring.[18] Ganoderic acid DM has the unsaturated C-26 carboxy ($\Delta^{24,25}$) at the 17 side chain and C3-carbonyl, which possibly mimic the A-ring of testosterone.

Also, the blocking of DHT from binding to the androgen receptors by ganoderic acid DM has been examined. Thus, we directly assessed the ability of ganoderic acid DM to bind to the AR. As shown by the semilog scale relative to the concentration to polarization (Fig. 3), the polarization value (mP) was decreased when the concentration of ganoderic acid DM was increased. Fifty percent of the maximal shift of the highest polarization value is represented by 50% of binding to AR-LBD. A higher concentration of ganoderic acid DM (15 μM) than that of DHT (0.018 μM) was required to bind to 50% of AR-LBD.

The LNCaP (lymph node carcinoma of the prostate) human prostate cancer cell line is a well-established androgen-dependent cell line.[19] LNCaP cells retain most of the characteristics of human prostatic carcinoma, like the dependence on androgens and the presence of ARs. For these reasons, the LNCaP cell line becomes an attractive model for in vitro studies of the biology of human prostate cancer.[20] LNCaP cells were incubated with varying concentrations of ganoderic acid DM (10–50 μM) and with or without testosterone or DHT for three days. The NR assay was performed to measure cell viability. Treating LNCaP cells with ganoderic acid DM resulted in dose-dependent inhibition of cell growth (Fig. 4A). In the absence of ganoderic acid DM, testosterone alone apparently stimulates the LNCaP cell proliferation about 150% on average more than the untreated control (Fig. 4B). DHT alone apparently stimulates the LNCaP cell number about 170% on average more than the untreated control (Fig. 4C). Ganoderic acid DM inhibited androgen induced proliferation of LNCaP cells at all concentration range (10–50 μM). Ganoderic acid DM inhibited the androgen induced cell growth in dose-dependent inhibition (Fig. 4B and C).

The prostatic carcinoma cell lines PC-3 have been characterized as true prostatic carcinoma cell lines in 1979.[21] PC-3 has a greatly reduced dependence upon serum for growth when compared to normal prostatic epithelial cells, does not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors and did not express PSA. The ability of ganoderic acid DM on the other prostate cancer cell line was also evaluated. The PC-3 cells were treated with ganoderic acid DM at concentration range of 1–50 μM. Ganoderic acid DM also inhibited proliferation of PC-3 cell at high concentration (12.5–50 μM) (Fig. 5). Interestingly, a same concentration range of ganoderic acid DM was needed to inhibition of LNCaP cells and PC-3 cell line, suggesting non-selectivity toward prostate cancer cells.

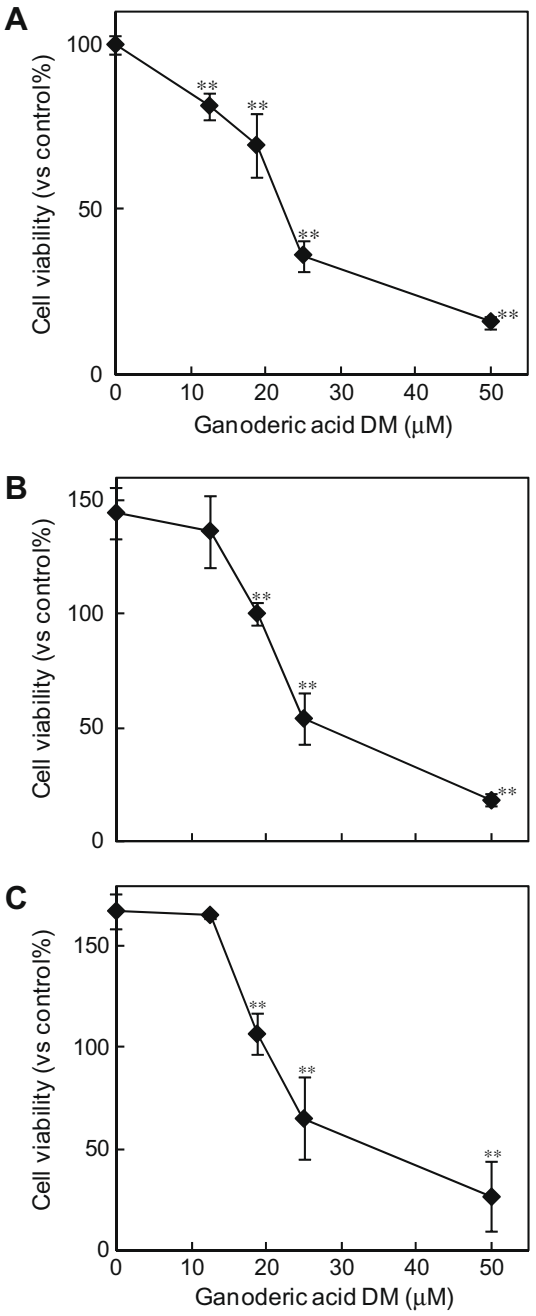

Figure 4. The inhibition effect of ganoderic acid DM on the LNCaP cell growth. Results are given as the mean ± S.D., $n = 3$. (A) No addition of androgen; (B) addition of 100 nM testosterone; (C) addition of 100 nM dihydrotestosterone. $^{**}P < 0.01$ against corresponding cell viability without ganoderic aicd DM.

We have already reported the biological activity of ganoderol B.[22] Although the structures of ganoderol B and ganoderic acid DM are quite similar but still different, and both of them were isolated from the ethanol extract of *G. lucidum*, they showed different 5α-reductase inhibitory activity and mechanism on proliferation of PC-3 cell line. Ganoderol B showed no inhibition on proliferation of PC-3 cell line, but ganoderic acid DM showed a dose-dependent manner on PC-3 proliferation. This result suggested that ganoderic acid DM suppressed cell proliferation somewhere in AR signaling pathway other than DHT formation at least in part. Hence, it seems that a little change in the 17β-chain and C-3 affect the bioactivity of triterpenoids in a large way.

To investigate whether ganoderic acid DM also affect the differentiation of osteoclasts, we utilized the preosteoclastic cell line RAW 264 cell to study the effect of ganoderic acid DM on the osteoclast-linage. Ganoderic acid DM clearly suppressed osteoclastogenesis from the RAW 264 cell (Fig. 6). At the concentration of 12.5 μM, ganoderic acid DM inhibited the 40% RANKL-induced RAW 264 cell differentiation to osteoclasts, but not affected the cell number. At the concentration of 25 μM, 72% RAW 264 cells were survived, but no RANKL-induced RAW 264 cell differentiation to osteoclasts were observed. This result suggested that ganoderic acid DM selectively inhibits the osteoclasts forming, at the concentration without cytotoxicity.

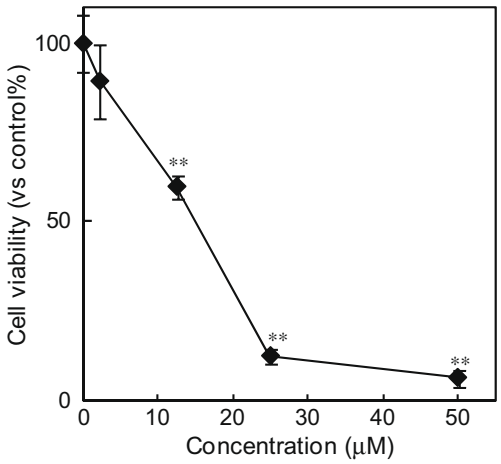

Figure 5. The inhibition effect of ganoderic acid DM on the PC3 cell growth. Results are given as the mean ± S.D., $n = 3$. $^{**}P < 0.01$ versus vehicle control.

It is critical to understand the mechanisms by which ganoderic acid DM inhibits the proliferation of prostate cancer cell. For early prostate cancer, which is androgen-dependent, reducing the levels of circulation androgens to suppress AR/androgen signaling is an effective treatment choice. For example, finasteride, a 5α-reductase inhibitor, which prevents conversion of testosterone to its more active form DHT in the prostate, is an effective drug for early prostate cancer. However, most prostate cancer patients eventually develop androgen-independent tumors that are resistant to this form of therapy. A significant body of evidence has suggested that in many androgen-independent prostate cancer cases, AR is highly expressed and hypersensitive to low, castrated-level of androgens or even can be activated by nonandrogens to induce tumor cell growth. Therefore, the strategy targeting the inactivation of AR

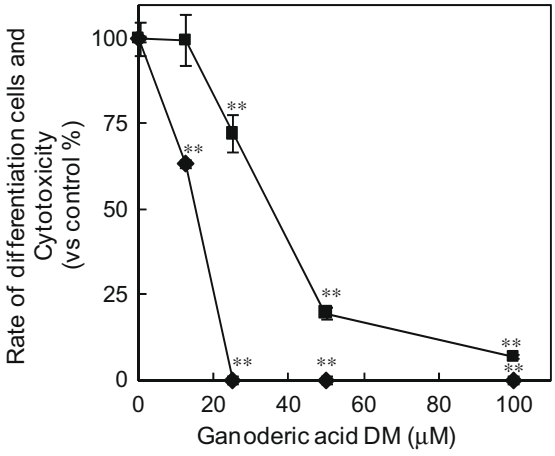

Figure 6. The osteoclastogenesis and cytotoxicity of ganoderic acid DM. (♦: osteoclastogenesis; ■: cytotoxicity). Results are given as the mean ± S.D., $n = 4$. $^{**}P < 0.01$ versus vehicle control.

function is particularly attractive to treat hormone refractory prostate tumors. On the basis of these observations, we examined the 5α-reductase inhibitory activity and AR binding activity of ganoderic acid DM. We found that ganoderic acid DM strongly inhibited the 5α-reductase activity and bound to the AR, which suggested that AR signaling pathway was suppressed by this compound. Whether this compound inhibits cell proliferation is also a key question. As expected, ganoderic acid DM significantly inhibited LNCaP cell growth. Moreover, it also inhibited PC-3 cells proliferation. The fact that ganoderic acid DM inhibited the proliferation of both LNCaP and PC-3 cell suggests that this compound have the potential to serve as an effective therapy for androgen-dependent and androgen-independent prostate cancer.

The most common skeletal manifestation of malignancy is focal osteolysis in association with metastases. In order for tumor cells to grow and invade mineralized bone, osteolysis must occur. Osteoclasts appear uniquely adapted to produce the microenvironment and the biochemical milieu that are needed to resorb bone.[23] The bulk of the evidence suggests that most tumor cells act indirectly by co-opting the physiologic mechanisms that normally favor bone resorption. Thus, they release agents such as hormones, eicosanoids, growth factors, and cytokines into the bone microenvironment, which act on osteoblastic stromal cells to enhance the production of osteoclast activating factors.[24,25] 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.[26] Concomitantly, eliminating the forming of osteoclasts means the bone metastasis could be repressed. In this study, we found that ganoderic acid DM inhibited the osteoclasts differentiation.

Altogether our results illustrate the ability of a natural product-derived substance, ganoderic acid DM, to inhibit the proliferation of prostate cancer and osteoclasts differentiation. Thus makes ganoderic acid DM can be used in therapeutics of prostate cancer by inhibiting the cancer cell proliferation and bone metastases by inhibiting the osteoclast differentiation.

A portion of the ethanol extracts (50 g) was fractionated into three fractions (Fr. A–C) by column chromatography eluting with an *n*-hexane–EtOAc step-gradient. A part of Fr. B (5 g) was fractionated by the preparative reversed phased HPLC and afforded ganoderic acid DM (150 mg, yield, 0.3% in ethanol extracts of *G. lucidum*). Ganoderic acid DM was identified by comparing MS, NMR and optical rotation matched with published data (Fig. 1).[27]

Rat microsomes were prepared by the method previously reported by Liu et al.[28]

The 5α-reductase inhibitory activity was measured by a method previously reported by Liu et al.[28]

The ability of ganoderic acid DM to interact with the AR was evaluated using the utilizing a fluorescence polarization (FP) method previously reported by Liu et al.[29]

Human LNCaP (AR positive, androgen-dependent prostate cancer cell), PC-3 (AR negative, androgen-independent prostate cancer cell) were obtained from the American Type Culture Collection. The cells were grown in RPMI 1640 containing 10% fetal bovine serum. All cell lines were grown at 37 °C in a humidified atmosphere with 5% CO_2. The cells were used between passages 5–30 at a split ratio of 1:3 in each passage. The cells were plated into a 24-well plate with a 1×10^5 cell/well density supplemented with 5% steroid-depleted (DCC-stripped) cFBS. Twenty-four hours later, the cells were treated with either vehicle control or androgens (T or DHT) in the presence or absence of each concentration of sample for another three days. Cell proliferation was determined by the 3-amino-7-dimethylamino-2-methyl-phenazine method.

The osteoclast precursor cell line, RAW 264 cell, was cultured in α-MEM containing 10% FBS (6.8×10^3 cells in 150 μl/well in 96 well culture plates) for three days in the presence of sRANKL (30 ng/ml) and TNF-α (10 ng/ml) as described by Watanabe et al.[30]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.119.

References and notes

1. Jemal, A.; Tiwari, R. C.; Murray, T.; Ghafoor, A.; Samuels, A.; Ward, E.; Feuer, E. J.; Thun, M. J. *J. Clin.* **2004**, *54*, 8.
2. Niu, Y. J.; Ma, T. X.; Zhang, J.; Xu, Y.; Han, R. F.; Sun, G. *Asin. J. Androl.* **2003**, *51*, 19.
3. DePrimo, S. E.; Diehn, M.; Nelson, J. B.; Reiter, R. E.; Matese, J.; Fero, M.; Tibshirani, R.; Brown, P. O.; Brooks, J. D. *Genome Biol.* **2002**, *3*. Research00321.
4. Gelmann, E. P. *J. Clin. Oncol.* **2002**, *20*, 3001.
5. Denmeade, S. R.; Lin, X. S.; Isaacs, J. T. *Prostate* **1996**, *28*, 251.
6. Hofbauer, L. C.; Schoppet, M. *JAMA* **2004**, *292*, 490.
7. Roodman, G. D. *N. Engl. J. Med.* **2004**, *64*, 9209.
8. Capodice, J. L.; Katz, A. E. *World J. Urol.* **2006**, *24*, 378.
9. Denis, L.; Morton, M. S.; Griffiths, K. *Eur. Urol.* **1999**, *35*, 377.
10. McCann, M. J.; Gill, G. I.; McGlynn, H.; Rowland, I. R. *Nutr. Cancer* **2005**, *52*, 1.
11. Liu, J.; Tamura, S.; Kurashiki, K.; Shimizu, K.; Noda, K.; Konishi, F.; Kumamoto, S.; Kondo, R. *Chem. biodivers*., in press.
12. Shimizu, K.; Miyamoto, I.; Liu, J.; Konishi, F.; Kumamoto, S.; Kondo, R. *J. Wood. Sci.*, in press.
13. Fujita, R.; Liu, J.; Shimizu, K.; Konishi, F.; Noda, K.; Kumamoto, S.; Ueda, C.; Tajili, H.; Kaneko, S.; Suimi, Y.; Kondo, R. *J. Ethnopharmacol.* **2005**, *102*, 107.
14. Shiao, M. S. *Chem. Rec.* **2003**, *3*, 172.
15. Liu, J.; Kurashiki, K.; Shimizu, K.; Kondo, R. *Biol. Pharm. Bull.* **2006**, *29*, 392.
16. Zeelin, F. J. *Adv. Drug Res.* **1992**, *22*, 149.
17. Metcalf, B. W.; Levy, M. A.; Holt, D. A. *Trends Pharmacol. Sci.* **1989**, *10*, 491.
18. McDonald, I. A.; Nyce, P. L.; Muench, D. M.; Gates, C. A.; Blohm, T. R.; Laughlin, M. E.; Werntraub, P. M. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 847.
19. Horoszewicz, J. S.; Leong, S. S.; Kawinski, E.; Karr, J. P.; Rosenthal, H.; Chu, T. M.; Mirand, E. A.; Murphy, G. P. *Cancer Res.* **1983**, *43*, 1809.
20. Negri-Cesi, P.; Motta, M. *Biochem. Mol. Biol.* **1994**, *51*, 89.
21. Kaighn, M. E.; Narayan, S.; Ohnuki, Y.; Lechner, J. F.; Jones, L. W. *Invest. Urol.* **1979**, *17*, 16.
22. Liu, J.; Shimizu, K.; Konishi, F.; Kumamoto, S.; Kondo. R. *Bioorg. Med. Chem.* **2007**, *15*, 4966.
23. Eilon, G.; Mundy, G. R. *Nature* **1978**, *276*, 726.
24. Kong, Y. Y.; Boyle, W. J.; Penninger, J. M. *Immunol. Today* **2000**, *21*, 495.
25. Lee, S. K.; Lorenzo, J. A. *Endocrinology* **1999**, *140*, 3552.
26. Suda, T.; Takahashi, N.; Udagawa, N.; Jimi, E.; Gillespie, M. T.; Martin, T. J. *Endocrinol. Rev.* **1999**, *20*, 345.
27. Wang, F.; Cai, H.; Yang, J. S.; Zhang, Y. M.; Hou, C. Y.; Liu, J. Q.; Zhao, M. J. *Yaoxue Xuebao* **1997**, *32*, 447.
28. The assay was conducted with microsomes from rat livers as previously reported.[15] Ganoderic acid DM was incubated with microsomes and NADPH for ten minutes, the percentage of inhibitory activity was calculated by the extent [4-^{14}C] testosterone to [4-^{14}C] DHT.
29. The assay was conducted with androgen receptor ligand-binding domain as previously reported.[22] Androgen receptor ligand-binding domain (25 nM, 20 μl) was incubated with each concentration of ganoderic acid DM or DHT for 4 h. The polarization was then measured on a beacon 2000 fluorescence polarization instrument using 485 nm excitation and 535 nm emission interference filters in polarization mode.
30. Watanabe, T.; Kukita, T.; Kukita, A.; Wada, N.; Toh, K.; Nagata, K.; Nomiyama, H.; Iijima, T. J. *Endocrinology* **2004**, *180*, 193.