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The anti-androgen effect of ganoderol B isolated from the fruiting body of *Ganoderma lucidum*

Jie Liu,[a] Kuniyoshi Shimizu,[a] Fumiko Konishi,[b] Shoichiro Kumamoto[b] and Ryuichiro Kondo[a,*]

[a]*Department of Forest and Forest Products Science, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan*
[b]*Research Laboratories, Chlorella Industry Co. Ltd, Res Labs, Fukuoka 833-0056, Japan*

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Abstract—The anti-androgenic activity of the ethanol extract of the fruiting body of *Ganoderma lucidum* has been previously reported. Ganoderol B with 5α-reductase inhibitory activity and the ability to bind to androgen receptor (AR) can inhibit androgen-induced LNCaP cell growth and suppress regrowth of the ventral prostate induced by testosterone in rats. The down-regulation of AR signaling by ganoderol B provides an important mechanism for its anti-androgenic activity. In view of the fact that PSA (prostatic specific antigen, a well-accepted prognostic indicator of prostate cancer) is down-regulated, an important implication of this study is that ganoderol B intervention strategy aimed at toning down the amplitude of androgen signaling could be helpful in controlling morbidity of prostate cancer. In conclusion, our result suggests that ganoderol B might be useful in prostate cancer and benign prostatic hyperplasia (BPH) therapy through suppressing the function of androgen and its receptor.

1. Introduction

Prostate cancer is one of the most frequently diagnosed malignancies and is the second leading cause of cancer death in American men. Androgen and the androgen receptor (AR) play important roles in this malignancy, and androgen ablation has been the main therapeutic option for the treatment of locally advanced or metastatic prostate cancer. Benign prostatic hyperplasia (BPH) is a ubiquitous condition in the aging males, such that the incidence of BPH detected at autopsy increases from approximately 30% at age 50 to >80% at age 80.[1] Unfortunately, the urinary symptoms attributed to BPH lead to significant erosion in the quality of life for affected men, and many undergo surgery as a result.[2]

The AR is a transcriptional activator which in a multistep process transduces extracellular signals to target tissues. The AR regulates transcription in response to androgens and plays a key role in the regulation of prostate growth and the maintenance of prostatic function. The AR is structurally organized into three domains: (1) the ligand-binding domain at the C-terminus of the protein, a moderately conserved region; (2) the cystern-rich and well-conserved DNA-binding domain, which consists of two zinc finger elements; and (3) the variable N-terminus, which is involved in trans-activation. AR transduces androgen signals in prostate cells to regulate the physiological and pathological development of the gland.[3] It is classically understood that after ligand binding {mainly dihydrotestosterone (DHT)}, the ligand–AR receptor complex with associated proteins translocates into the nucleus, binds to the consensus sequence of androgen response elements,[4] and regulates the expression of androgen-responsive genes (ARGs).[5] Prostate specific antigen (PSA) is an ARG known to be under the control of the AR and is a well-accepted marker for the diagnosis and prognosis of prostate cancer. Amplification of AR can lead to the development of prostatic diseases or androgen-refractory prostate cancer. Therefore, the first step in the process of transcriptional regulation is the binding of DHT to the AR. The androgen antagonist can suppress DHT-induced prostate regrowth. Clinically, androgen ablation therapy is used to reduce AR ligand production or to block AR-mediated signaling. Anti-androgens compete with DHT for AR binding and block AR-mediated signaling. A non-steroidal

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* Corresponding author. Tel.: +81 92 642 2811; fax: +81 92 642 2811; e-mail: kondo@agr.kyushu-u.ac.jp

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anti-androgen, flutamide, has also been used in the treatment of BPH.

On the other hand, the principal circulating androgen is testosterone. In several androgen targets, like the prostate, testosterone is converted to DHT, which is the most potent natural androgen. The microsomal enzyme steroid 5α-reductase [EC 1.3.99.5] catalyzes the NADPH-dependent reduction of the $\Delta^{4,5}$ double bonds of a variety of 3-oxo-Δ^4 steroids.[6] Although the etiology of BPH is unclear, the permissive role of DHT in the hyperplastic growth of the prostate is well established. Patients with BPH have been reported to have higher than normal concentrations of DHT in the prostate.[7,8] Androgen ablation therapy of prostate cancer reduces the levels of circulating androgens and then inhibits tumor proliferation and induces apoptosis of tumor cells.[9] DHT has also been implicated in acne,[10] female hirsutism,[11] and male-pattern baldness.[12] Thus, inhibiting the 5α-reductase activity can reduce the formation of DHT and be a useful way of minimizing certain androgen-responsive conditions. Finasteride, a 5α-reductase type 2 inhibitor, has been used to treat patients with BPH. Finasteride slows the progression of BPH by suppressing DHT synthesis.[13] As a result, the inhibition of 5α-reductase and binding of AR have become a pharmacological strategy for the treatment of BPH, prostate cancer, as well as other DHT-related disorders such as acne and male-pattern baldness.

For thousands of years, mushrooms have been known to be a source of medicine. In our previous screening of mushrooms, we discovered that the ethanol extract of *Ganoderma lucidum* (Leyss.:Fr.) Karst. (Ganodermataceae) showed the strongest 5α-reductase inhibitory activity. Also, treatment with *G. lucidum* itself or with the ethanol extract prepared from it significantly inhibited the growth of the ventral prostate induced by testosterone in rats.[14,15] *G. lucidum*, known as Ling Zhi in China and Reishi in Japan, is a wood-rotting fungus generally found growing on tree stumps. Over one hundred oxygenated triterpenes have been isolated from this mushroom,[16] and these compounds have been proven to display wide-ranging biological activity, for example, cytotoxic,[17] histamine release inhibiting,[18] angiotensin converting enzyme inhibiting,[19] antitumor promoting,[20] and cholesterol synthesis inhibiting[21] effects.

In the course of our search for the anti-androgenic components from *G. lucidum*, we have isolated ganoderol B from the ethanol extract of *G. lucidum*. We report here that ganoderol B has the potential to inhibit 5α-reductase and bind to the AR, inhibit the androgen-induced cell growth of LNCaP, and suppress the ventral prostate growth induced by testosterone in castrated rats.

2. Results and discussion

In our previous screening of 19 edible and medicinal mushrooms, we discovered that the ethanol extract of the fruiting body of *G. lucidum* showed the strongest 5α-reductase inhibitory activity.[14,15] In addition, the treatment of the ethanol extract 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.[15] To clarify the active principles of the ethanol extract of *G. lucidum*, 5α-reductase inhibitory activity-guided fractionation was carried out. The ethanol extracts were roughly separated into three fractions (Fr. A, B, C), which were analyzed by TLC. 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*.[16] 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 extract of *G. lucidum*. Anti-androgenic assay (5α-reductase inhibitory activity and androgen-receptor binding)-guided fractionation led to the isolation of active triterpenoid from Fr. B, identified as ganoderol B (Fig. 1) comparing with published data.[22] Ganoderol B was isolated as an inhibitor of melanin formation in the skin for lightening the skin exposed to UV radiation of sun light, prevents formation of stains, and covers freckles,[23] and as cholesterol synthesis inhibitor.[24] In this study, we investigated the effects of ganoderol B on steroid 5α-reductase activity, androgen-receptor binding, androgen-induced LNCaP cell growth, and testosterone-induced growth of the prostate in castrated rats.

The inhibition by ganoderol B of 5α-reductase prepared from the rat liver was concentration-dependent, as shown in Figure 2. As the concentrations of ganoderol B increased, the residual enzyme activity decreased. The inhibitory concentration leading to 37% activity loss was estimated to be 113 μM. The IC_{50} of ganoderol B was not detected, because this compound cannot dissolve in the assay buffer over 120 μM. It should be noted that finasteride,[25] which is known as a potent steroidal inhibitor, showed an IC_{50} of 0.73 μM in our assay system. In our previous paper, Fr. B showed the very strong 5α-reductase inhibitory activity. From Fr. B, we isolated three 5α-reductase inhibitors, ganoderic acid TR, ganoderic acid DM, and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one. Compared with these three active compounds, which showed the IC_{50} of 8.5 μM (ganoderic acid TR), 10.6 μM (ganoderic acid DM), and 41.9 μM (5α-lanosta-7,9(11),24-triene-15α,26-dihy-

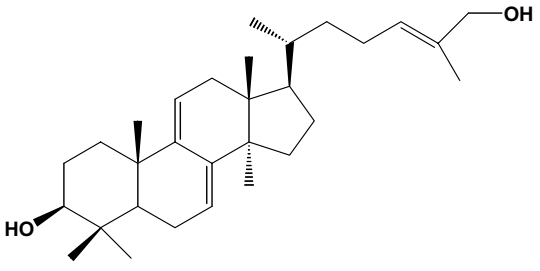

Figure 1. Structure of ganoderol B.

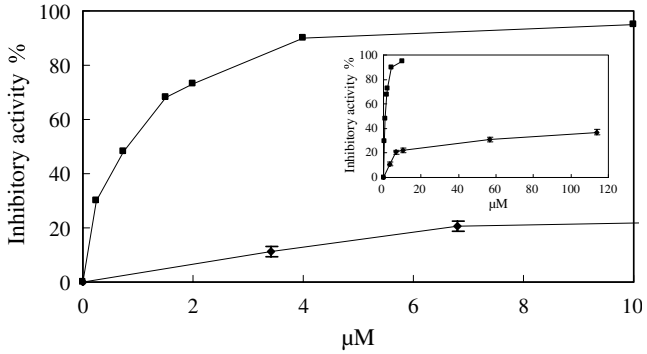

Figure 2. Effect of ganoderol B on the activity of 5α-reductase. (■: finasteride, ♦: ganoderol B). Assay was conducted with microsomes from rat livers as described under Section 3; finasteride or ganoderol B was incubated with microsomes and NADPH for 10 min, the percentage of inhibitory activity was calculated by the extent of conversion from [4-^{14}C]testosterone to [4-^{14}C]DHT.

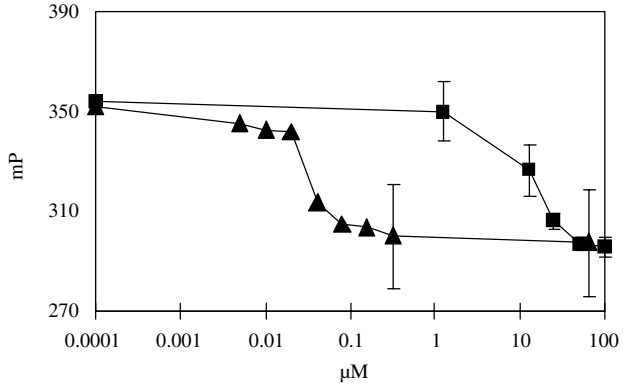

Figure 3. Effect of ganoderol B and DHT on the competitive binding activity on AR. (■: ganoderol B, ▲: DHT). Assay was conducted with AR–LBD as described under Section 3. AR–LBD (25 nM, 20 μl) was incubated with each concentration of ganoderol B or DHT for 4 h. The polarization was then measured on a beacon 2000 fluorescence polarization instrument using 485 nm excitation and 535 nm emission interference filters in polarization mode. The polarization values (mP) were plotted against increasing concentrations of the test extracts. The semilog scale relative to the concentration to polarization was used in the figure.

droxy-3-one), respectively,[14,26] the 5α-reductase inhibitory activity of ganoderol B is weak. In the history of 5α-reductase inhibitor study, the inverted steroid-based inhibitors have been extremely important drugs for hormone-dependent cancers.[27,28] In 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 appropriate substitution of the D-ring.[29] Ganoderol B has the unsaturated hydroxyl ($\Delta^{24,25}$) at the 17 side chain and 3β-hydroxyl, which possibly mimic the A-ring of testosterone.

Also, the blocking of DHT from binding to the androgen receptors of ganoderol B has been examined. Thus, we directly assessed the ability of ganoderol B to bind to the AR. As shown by the semilog scale relative to the concentration to polarization (Fig. 3), the polarization value decreased when the concentration of ganoderol B 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 ganoderol B (15 μM) than that of DHT (0.018 μM) was required to bind to 50% of AR–LBD.

Considering the results of 5α-reductase inhibitory activity and androgen-receptor binding experiments, ganoderol B inhibits 5α-reductase activity and binds to the androgen receptor. The effect of ganoderol B on the proliferation of prostate cancer cell is shown in Figure 4. The LNCaP (lymph node carcinoma of the prostate) human prostate cancer cell line is a well-established androgen-dependent cell line.[30] LNCaP cells retain most of the characteristics of human prostatic carcinoma, like the dependence on androgens, the presence of ARs, and the production of acid phosphatase and PSA. More importantly, LNCaP cells express only 5α-reductase 1 but not 5α-reductase 2.[31] For these reasons, the LNCaP cell line becomes an attractive model for in vitro studies of the biology of human prostate cancer.[32] LNCaP cells were incubated with varying concentrations of ganoderol B (9.25–25 μM) and with or without testosterone or

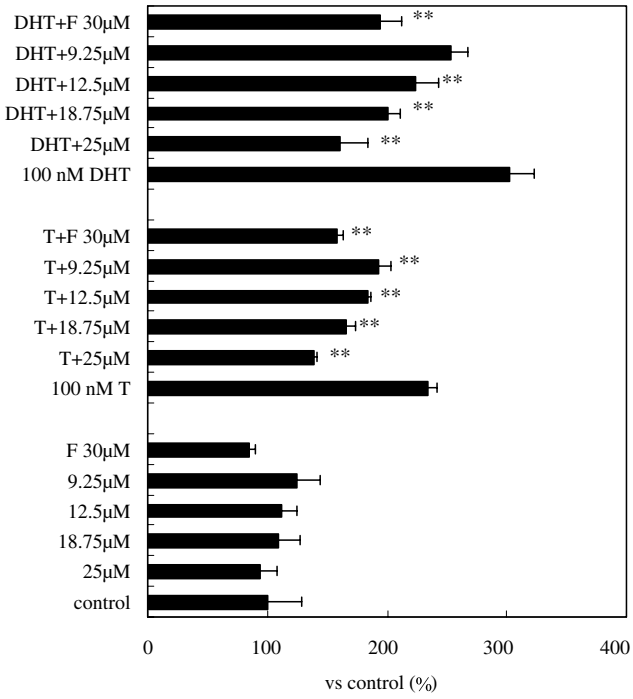

Figure 4. The inhibition effect of ganoderol B on the LNCaP cell growth. $n = 3$. T, testosterone; DHT, dihydrotestosterone; F, flutamide. $^{*}p < 0.05$ against corresponding control, T or DHT, $^{**}p < 0.01$ against corresponding control, T or DHT. Assay was conducted with LNCaP cells as described under Section 3. Cells were grown in 5% cFBS medium for 24 h before incubating simultaneously with substrate and inhibitors for 72 h. Cell number was determined by NR assay.

DHT for 3 days. The NR assay was performed to measure cell viability. In the absence of ganoderol B, testosterone alone apparently stimulates the LNCaP cell number about 140% on average more than the untreated control, and DHT alone apparently stimulates the LNCaP cell number about 200% on average more than

the untreated control. Ganoderol B showed no inhibition of LNCaP cell growth when cultured without testosterone or DHT. Interestingly, treating LNCaP cells with ganoderol B in the presence of testosterone or DHT resulted in dose-dependent inhibition of cell growth. These results suggested that the inhibition of cell growth in the presence of testosterone or DHT was not the result of the cell cytotoxic effect, but came from an anti-androgen effect such as 5α-reductase inhibition and the binding to AR.

The results described above suggested that ganoderol B may affect androgen-stimulatory effects. Therefore, we began to assess whether the inhibitory effect was because of an alteration in AR expression, and real time PCR was used to measure the steady levels of AR and PSA messenger RNA (mRNA). Figures 5a and b show that AR and PSA mRNA expression were down-regulated by ganoderol B treatment in a dose-dependent fashion on the androgen-reduced cell proliferation. No up-regulation of PSA mRNA expression was observed when the ganoderol B was added to cell without androgen. Ganoderol B acts as an antagonist on the AR. This result shows that ganoderol B is able to down-regulate the expression of PSA in human prostate cancer cells via a mechanism involving disruption of the androgen signal transduction pathway. As PSA is a well-accepted diagnostic and prognostic biomarker of prostate cancer progression, the down-regulation of PSA by ganoderol B has significant clinical implication. In patients treated with ganoderol B, the monitoring of PSA in the circulation could potentially be evaluated as a barometer to gauge the efficacy of intervention.

From the results of 5α-reductase inhibitory activity, androgen-receptor binding experiments, and LNCaP cell proliferation effect, ganoderol B showed the ability to inhibit the 5α-reductase activity, bind to the androgen receptor, and inhibit the androgen-induced growth of prostate cancer cells in vitro through the suppression of expression of AR and PSA. It still cannot be concluded that ganoderol B suppresses the growth of the ventral prostate because some problems remain, such as the absorption into the blood, its metabolism in the blood, its penetration through the cell membrane, etc. So we designed a growth suppression experiment on the rat prostate. In the rats that received testosterone, the administration of ganoderol B reduced the increased

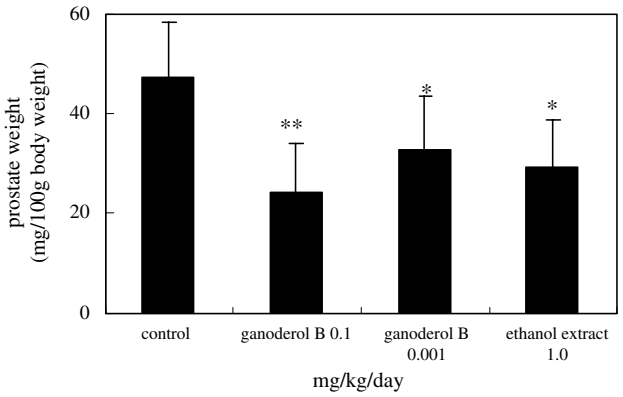

Figure 6. Effects of ganoderol B and *G. lucidum* on testosterone-induced regrowth of the castrated rat prostate. Each column represents means ± SEM, $n = 4$. C, castrated rat; T, testosterone; ethanol extract, ethanol extract of *G. lucidum*. $^{*}p < 0.05$ against C + T, $^{**}p < 0.01$ against corresponding C+T. The testes of SD rats were removed at four weeks of age under light anesthesia with pentobarbital. After 4 days, testosterone (100 μg/body) was injected sc into the rats once daily for 7 days. Ganoderol B or *G. lucidum* suspended in 0.5% methylcellulose was orally administered once daily for 7 days. After 7 days, rat prostates were removed and their weights determined.

weight of the ventral prostate (Fig. 6). More specifically, 4 days after castration, the weights of the rat prostates were markedly reduced, and the prostate size was recovered by s.c. injections of testosterone. Administration of ganoderol B at the concentrations of 0.1 and 0.001 mg/kg/day can have 49% and 31% prostate growth inhibition, respectively. The treatment of the ethanol extract of *G. lucidum* at 1.0 mg/kg/day significantly inhibited the growth of the ventral prostate (28%) induced by testosterone in rats. Also, the isolation yield of isolation of ganoderol B in the ethanol extract of *G. lucidum* is about 0.1%. Considering the isolation yield of ganoderol B, we can conclude that ganoderol B is one of the active compounds in the ethanol extract of *G. lucidum* that contribute to the anti-androgen effects. However, it should be noted that *G. lucidum* has a variety of triterpenoids with structures similar to ganoderol B, so it is evident that not only ganoderol B but also other structurally similar compounds must contribute to the anti-androgenic activity. Because ganoderol B exhibits inhibitory activity on 5α-reductase and binding activity to AR, and inhibits the androgen-induced growth of LNCaP, the prostate growth inhibition might be related to the 5α-reductase inhibition, the

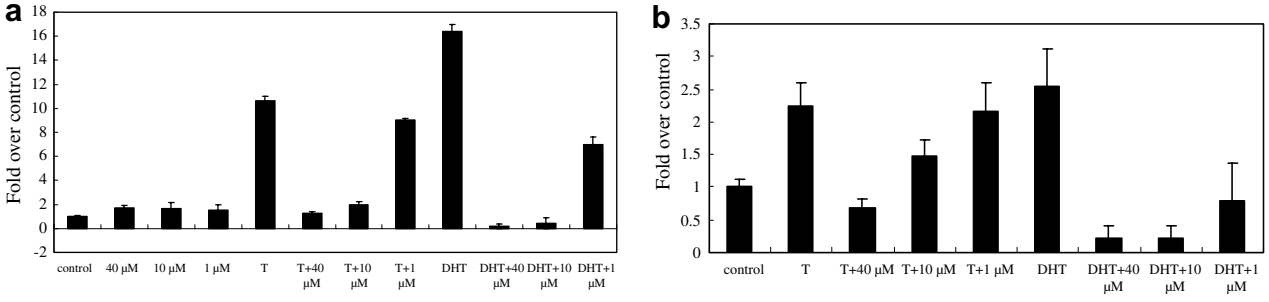

Figure 5. Effect of ganoderol B on PSA and AR expression. (a) Changes in PSA mRNA, as determined by quantitative RT-PCR. (b) Changes in AR mRNA, as determined by quantitative RT-PCR. The PSA/AR mRNA level was determined by RT-PCR analysis after exposure of the cells for 72 h to various concentrations of ganoderol B in medium, with or without 100 nM androgen.

competitive binding to AR with DHT, and the suppression of expression of mRNA AR and mRNA PSA.

Androgens, acting through the AR, are required for prostate development and normal prostate function. Androgen action can be considered to function through an axis involving testosterone and DHT. Testosterone and DHT exert their biological effects through binding to AR, and transcriptional activation of AR is modulated by the interaction of AR with coregulators and by the phosphorylation of AR and AR coregulators in response to growth factors. Approximately, 80–90% of prostate cancers are dependent on androgen at the initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and the inhibition of AR. The dependence of prostate cancer on androgens has raised the question of whether DHT is required for the onset, maintenance, or progression of adenocarcinoma of the prostate. The notion that 5α-reductase inhibitors could be useful in the treatment of androgen-related disease emerged in the early 1970s as the genetic phenotype of 5α-reductase type 2 deficiency was described and the role of DHT as the primary mediator of androgen action in many tissues was demonstrated. In the prostate, interestingly, type-1 5α-reductase activity is 3–4 times greater in malignant tissues than in benign prostate tissues, but type-2 5α-reductase activity in these 2 diseases is similar. Furthermore, the blockade of 5α-reductase, combined with an androgen-receptor antagonist, produces a cumulative inhibition of the cultured growth of prostate cancer cells. Thus began the search for inhibitors of 5α-reductase which has continued to this day. It has also become apparent that several classes of 5α-reductase inhibitors demonstrate time-dependent inhibition of 5α-reductase, which further complicates the comparison of in vitro potency.

For many years, androgen ablation or endocrine therapy has remained the mainstay of treatment for prostate cancer. Unfortunately, cancer cells eventually escape the steroid requirement and progress into the androgen-independent phenotype during androgen ablation therapy. In many recurrent or advanced prostate cancers, AR is still expressed, either mutated or amplified. The mutated but functionally intact AR can be activated by lower concentrations of androgens or other non-androgenic ligands such as growth factors. Therefore, AR is not only important in androgen-dependent cancer cells but also in androgen-independent cancers. In this study, we also focused on whether ganoderol B inhibits AR expression. In Figure 5, ganoderol B significantly decreases the AR expression. The ability of ganoderol B on reducing AR might make it a good chemopreventive or chemotherapeutic agent for prostate cancer.

In this study, we isolated ganoderol B with 5α-reductase inhibitory activity, AR binding activity, and androgen-induced growth suppression of LNCaP cells and the rat prostate. In summary, we hypothesize that the ganoderol B exhibits its anti-androgenic effects on androgen action in prostate cancer cells and androgen dependent prostate regrowth by two aspects including inhibition of (i) 5α-reductase activity and (ii) AR expression. Therefore, ganoderol B may have the potential to become chemopreventive or chemotherapeutic agents for prostate cancer and BPH.

3. Experimental

3.1. Chemicals

Ganoderma lucidum (BMC9049) was obtained from Bisoken Inc. (Oita, Japan). The mushroom was identified by Mr. Shuhei Kaneko, Fukuoka Prefecture Forest Research and Extension Center. The voucher specimen (BMC9049) is preserved at the herbarium of the Department of Forest and Forest Products Science, Kyushu University, in Japan. The fruiting body was dried and ground into powder before use. Unless otherwise specified, chemicals were obtained from Sigma–Aldrich Japan Co., Ltd (Tokyo, Japan). Organic solvents were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). [4-^{14}C]Testosterone was obtained from Perkin-Elmer Japan Co., Ltd (Kanagawa, Japan). Fluormone AL Green and the purified androgen receptor ligand-binding domain (Lot No. 35794, Part No. P3018) were purchased from Invitrogen Company.

3.2. Fractionation of the ethanol extracts of *G. lucidum* by silica gel column chromatography

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 HPLC and afforded **ganoderol B** (72 mg, t_R 22.38 min, yield, 0.1% in ethanol extract of *G. lucidum*). The compound was identified as ganoderol B by a comparison of the FAB-MS and NMR, and by optical rotation matched with published data.

3.3. Preparation of rat microsomes

Rat liver from female SD rats (7 weeks age) was prepared by a method previously reported by Liu et al.[33] Two mature SD female rats were killed. The liver was removed and minced tissue was homogenized in a 4-tissue volume medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5). The homogenate was then centrifuged at 10,000*g* for 10 min. The resulting supernatant from the centrifugations was further centrifuged at 105,000*g* for 1 h twice. The washed microsomes were suspended in 1-pellet volume medium A, and the dispersion of microsomes was achieved using a syringe with 18 G, 23 G, and 26 G needles in succession. The microsomal suspension was stored at −70 °C just before use.

3.4. Measurement of 5α-reductase inhibitory activity

The 5α-reductase inhibitory activity was measured by a method previously reported by Liu et al.[33] A complete reaction mixture included 1 mM dithiothreitol, 20 mM phosphate buffer (pH 6.5), 1.9 nCi [4-^{14}C]testosterone, 150 μM testosterone, 167 μM NADPH, and the enzyme

preparation (1.54 mg of protein) in a final volume of 0.3 ml. The concentration of testosterone contributed by [4-^{14}C]testosterone was negligible. Ganoderol B was added at each concentration. The incubation was carried out for 10 min at 37 °C. The incubation was started by the addition of 10 μl microsomes to pre-heated reaction solution in a tube. After 10 min, the incubation was terminated by adding 10 μl of 3 M NaOH. To extract metabolites, 1 ml of diethyl ether was added, and the tubes were capped and shaken. The organic phase was applied to a silica plate (Kieselgel 60 F_{254}). The plate was developed in ethyl acetate–*n*-hexane (7:3) at room temperature. The radioactivity profile was determined with an imaging analyzer (FLA-5000 RF, Fuji Film, Tokyo, Japan). The 5α-reductase activity was calculated from the percentage of the extent to which [4-^{14}C]testosterone was converted to [4-^{14}C]DHT.

3.5. Androgen receptor competitor assay

The ability of ganoderol B to interact with the AR was evaluated using the fluorescence polarization (FP) method. This methodology measured the capacity of these competitor chemicals to displace a high-affinity fluorescent ligand (AL Green) from the purified, recombinant ligand-binding domain (LBD) of the human AR at room temperature. Conceptually, the binding of a fluorescent molecule to another molecule can be quantified by the change in its speed of rotation. Hence the androgen receptor-fluorescent ligand–bound complex (AR–AL Green) will rotate slowly and have a high FP value. Increasing concentrations of the competing ligand will displace the AL Green from the AR. Free AL Green will then rotate more rapidly and have a low FP value. Because the measured polarization is an average of the free and bound AL Green molecules, it can be used to assess competitive displacement from the AR–LBD. Briefly, ganoderol B was prepared as stock solutions in DMSO. Chemicals were serially diluted, over at least six log order concentrations, in triplicate in 20 μl volumes in assay buffer on a 384-well plate. No final DMSO concentrations exceeded the manufacturer's recommendations and, therefore, were not anticipated to alter fluorescence. A mixture of AR (25 nM final) and AL Green (1 nM final) was added in 20 μl volumes to the serially diluted test chemicals. The plate was then incubated in the dark for approximately 4 h at room temperature. 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. The polarization values (mP) were plotted against increasing concentrations of ganoderol B.

3.6. The inhibitory effect on the prostate cancer cell

The AR-positive human prostate cancer LNCaP cells were obtained from the American Type Culture Collection. The cells were used between passages 5 and 30 at a split ratio of 1:3 in each passage. The cells were plated into a 24-well plate with a 2×10^5/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 3 days. Cell proliferation was determined by the 3-amino-7-dimethylamino-2-methyl-phenazine (NR) method. The NR solution was made at 5 mg/ml and diluted by culture medium to 5 μg/ml. The NR extract solution was made by using water and 50% ethanol (1% acetic acid). The culture medium was changed to NR solution and incubated for 3 h at 37 °C, then the NR solution was aspirated and the cells were washed by PBS twice. 500 ml of the NR extract solution was added to each well to extract for 20 min at room temperature. The absorbance of each well was measured at 540 nm.

3.7. Growth suppression of the rat prostate by ganoderol B

The assay for growth suppression of the rat prostate was performed as described by Fukuta et al.[34] The testes of SD rats were removed at four weeks of age under light anesthesia with pentobarbital. After 4 days, testosterone (100 μg/body) was injected sc into the rats once daily for 7 days. There are 4 rats for each group. Ganoderol B suspended in 0.5% methylcellulose was orally administered at concentrations of 0.1 or 0.001 mg/kg of body weight once daily for 7 days. The ethanol extract of *G. lucidum* (1.0 mg/kg body weight) was used as the positive control and was suspended in 0.5% methylcellulose and orally administered once daily for 7 days. After 7 days, rats were deprived of food and water for 18 h and sacrificed by pentobarbital. Then, their prostates were removed and their weights determined.

3.8. Statistics

Results were expressed as means SEM or SD. The statistical significance of the animal test was determined by ANOVA and by a Bonferroni-type multiple *t*-test and the statistical significance of cell proliferation was determined by the *t*-test.

3.9. Real-time polymerase chain reaction analysis

Total RNA was extracted by use of Isogen (Nippon-Gene, Toyama, Japan) from LNCaP cells after being cultured with or without ganoderol B. Complementary DNA was synthesized in a final volume of 20 μl that included 1 μg of total RNA (4–5 μl of 0.2–0.3 μg/μl total RNA), 1 μM oligo-dT 18-mer primer, 10 U Rnase inhibitor, and 10 U of AMV Reverse Transcriptase (Takara, Japan) according to the manufacturer's instructions. Real-time PCR was performed in a final volume of 10 μl with a Line Gene (Bio flux corporation, Japan). The SYBR Premix Ex Taq kit (Takara, Japan) was used according to the manufacturer's instructions with a final concentration of 0.2 μM each primer. PCR amplification was performed as follows: (i) an initial denaturation at 94 °C for 1 min, (ii) 35 cycles, with 1 cycle consisting of denaturation at 94 °C for 30 s, annealing at 58 °C at 1 min, and elongation at 72 °C for 2 min. The following primers were used: for the AR, the sense primer was 5′-TCTCAAGAGTTTGGATGGCT-3′, and the antisense

primer was 5′-TTGCACAGAGATGATCTCTGC-3′; for the PSA, the sense primer was 5′-GAGGTCCACA CACTGAAGTT-3′, and the antisense primer was 5′-CCTCCGAAGAATCGATTCCT-3′; for the β-actin, the sense primer was 5′-CACTGTGTTGGCGTA CAGGT-3′, and the antisense primer was 5′-TCAT CACCATTGGCAATGAG-3′. The ratio of gene-specific expression was defined as relative expression to the actin expression. The data were three individual runs ± SD.

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