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The effects of ganoderma alcohols isolated from *Ganoderma lucidum* on the androgen receptor binding and the growth of LNCaP cells

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Abstract

The effects of ganoderma alcohols isolated from ethanol extracts of *Ganoderma lucidum* (Fr.) Krast (Ganodermataceae) on the androgen receptor-binding and the growth of LNCaP cells have been investigated. Less than two hydroxyl groups in 17 β -side chain are needed for binding to androgen receptor. In the case of the ganoderma alcohols with the same number of hydroxyl groups in 17 β -side chain, the one which has C-3 carbonyl group showed better binding activity to androgen receptor than that which has C-3 hydroxyl group. The unsaturation in 17 β -side chain is needed for the inhibition of the cell proliferation of androgen-induced LNCaP cells growth.

Keywords: *Ganoderma lucidum*; androgen receptor; prostate cancer; triterpenoids.

1. Introduction

Prostate cancer is the most frequently diagnosed male cancer and second leading cause of cancer deaths in North America [1]. The transcriptional program activated by the androgen receptor (AR) plays a critical role in overall function of the prostate as well as growth and survival of normal and malignant prostate tissue. Therefore, the treatment regimen for locally advanced, relapsed, or metastatic prostate cancer is based on inhibiting androgen receptor transcriptional activity. Although this approach is initially effective it is not curative, and with time prostate cancer will recur in a form resistant to further hormonal manipulations. Mounting evidence suggests that the androgen receptor is able to retain activity at this stage of the disease through aberrant mechanisms of activation. Therefore, many of the androgen receptor -regulated target genes that promote the growth and survival of prostate cancer cells are likely still expressed.

The androgen receptor is found in many tissues of both sexes but is most abundant in male sex tissues. The best characterized functions of the androgen receptor are to promote the growth and differentiation of the male urogenital structures. The androgen receptor regulates transcription in response to androgens and plays a key role in the regulation of prostate growth, the maintenance of prostatic function and the regulation of physiological and pathological development [2]. It is classically understood that after ligand binding [mainly dihydrotestosterone (DHT)], the ligand- androgen receptor complex with associated proteins translocates into the nucleus, binds to the consensus sequence of androgen response elements [3] and regulates the expression of androgen-responsive genes [4].

A unique requirement for prostate cancer is the initial reliance on androgens for growth and to avoid apoptosis [5]. Because of this requirement, androgen deprivation therapy (ADT) has become well-established in the treatment of prostate cancer. However, although many tumors initially regress after androgen deprivation therapy, most of the tumors eventually begin to re-grow at various rates in an androgen-refractory manner. This change to androgen-refractory growth may be due to an evolution of the cancer, whereby the minorities of cells that are androgen refractory before anti-androgen or androgen ablation therapy have a selective advantage relative to the androgen-sensitive cells. Of interest, studies on patient specimens [6-11] show that the androgen receptor is expressed in nearly all cancers of the prostate, both before and after androgen ablation therapy, indication that the androgen receptor -signaling pathway is still functional in these cancers, and give the result as amplification of androgen receptor can lead to the development of prostatic diseases or androgen-refractory prostate cancer.

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 5 α -reductase inhibitory activity. Also, treatment with *G. lucidum* itself or with the ethanol extracts prepared from it significantly inhibited the growth of the ventral prostate induced by testosterone in rats [12, 13]. *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 triterpenoids have been isolated from this mushroom [14], and these compounds have been proven to display wide-ranging biological activity, e.g. cytotoxic [15], histamine release inhibiting [16], angiotensin converting enzyme inhibiting [17], 5 α -reductase inhibiting [18], antitumor promoting [19] and cholesterol synthesis

inhibiting [20] effects.

These triterpenoids can be divided into two groups depending on the C-26. One group has C-26 carboxyl group, and is called as ganoderic acids. The other group has C-26 hydroxyl group, and is called as ganoderma alcohol. In the course of our search for the anti-androgenic triterpenoids isolated from the ethanol extracts of *G. lucidum*, we found that some ganoderma alcohol have the potential to bind to the androgen receptor, inhibit the androgen-induced cell growth of LNCaP. We also found some ganoderic acids that can bind to the androgen receptor and increase/inhibit the cell proliferation. In the case of the ganoderic acids, we could not find the systematic relationship between their androgen receptor binding activity and their cell proliferation because they should dissociate in neutral medium to have an anion. This may make them difficult to get into the cell and make them have different mechanism in inhibiting or increasing cell proliferation, other than the binding to androgen receptor [21]. Therefore we focused on the ganoderma alcohols to obtain the knowledge of structure-based bioactivities in this study. Some of ganoderma alcohols, isolated from *G. lucidum*, have demonstrated cytotoxicity against mouse sarcoma and mouse lung carcinoma cells in vitro. Ganoderma alcohol are highly multifunctional and the cytotoxicity of these compounds belong to their ability to block nuclear factor- κ B activation, induce apoptosis, inhibit signal transducer, and activate transcription and angiogenesis. This time we found that some ganoderma alcohol can inhibited the cell proliferation of LNCaP cell through it androgen receptor binding activity. The structure activity relationship of ganoderma alcohols against LNCaP cell was also reported here.

2. Experimental Part

2.1. Materials and Methods

G. lucidum was obtained from Bisoken (Fukuoka, Japan). The mushroom was identified by Mr. Shuhei Kaneko, Fukuoka Prefecture Forest Research and Extension Center. The fruiting body was dried and ground to powder before use. Unless otherwise specified, chemicals were obtained from Sigma Aldrich Japan (Tokyo, Japan). Organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). [4-¹⁴C] Testosterone was obtained from PerkinElmer Japan (Kanagawa, Japan).

2.2. Androgen receptor binding assay

The ability of each compound to interact with the androgen receptor was evaluated using the utilizing a 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 androgen receptor 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 androgen receptor. 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, each compound was prepared as stock solutions in DMSO. Chemicals were serially diluted, over at least six log order concentrations, in triplicate in 20 μ l 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 androgen receptor (25 nM final) and AL Green (1 nM final) was added in 20 μ l 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 each compound.

2.3. Cell culture and growth studies

The androgen receptor-positive human prostate cancer LNCaP cells were obtained from the American Type Culture Collection. 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 cells/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 (NR) method. The NR solution was made at 5 mg/ml, and diluted by culture medium to 5 μ g/ml. The NR extract solution was made by use water and 50% ethanol (1% acetic acid). The culture medium were changed to NR solution and incubated for 3 hours at 37 $^{\circ}$ C, then the NR solution were aspirated and the cells were washed by PBS twice. NR extract solution (500 ml) was added to each well to extract for 20 min at room temperature. The absorbance of each well was measured at 540 nm.

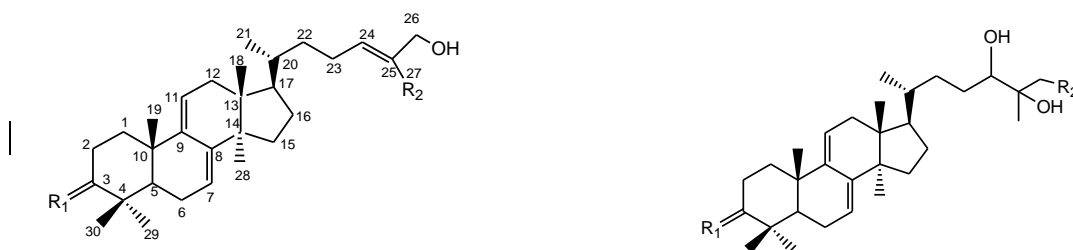
2.4. Statistics

Results were expressed as means \pm SD. The statistical significance of cell proliferation was determined by the *t*-test.

3. Results and Discussion

3.1. Chemistry

Ganoderol B (**1**), ganoderatriol (**2**), ganoderiol F (**3**), lucidumol B (**4**), ganodermanontriol (**5**), and ganoderiol A (**6**) were isolated from *G. lucidum*. These compounds were available from our previous works,¹⁸ and their structures are shown in Fig.1.



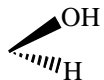
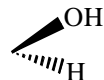
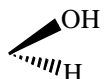
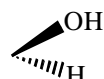
Compounds	R ₁	R ₂	Compounds	R ₁	R ₂
Ganoderol B (1)		CH ₃	Lucidumol B (4)		H
Ganodermatriol (2)		CH ₂ OH	Ganodermanontriol (5)	O	OH
Ganoderiol F (3)	O	CH ₂ OH	Ganoderiol A (6)		OH

Figure 1. Compounds isolated from ethanol extracts of *G. lucidum*

3.2. Androgen receptor binding assay

Blocking of DHT from binding to the androgen receptors of each triterpenoid has been examined. Thus, we directly assessed the ability of each triterpenoid to bind to the androgen receptor. The polarization value was decreased when the concentration of each triterpenoid were increased. Fifty percent of the maximal shift of the highest polarization value is represented by 50% of binding to AR-ligand binding domain (LBD). After made the graph of the semilog scale relative to the concentration to polarization, the IC₅₀ were calculated from the graph (Table 1).

Table 1 IC₅₀ of androgen receptor binding activity of each compound

Compounds	IC ₅₀ of Androgen receptor binding activity (μM)	The number of OH in side chain at C-17	C-3
Ganoderol B (1)	16	1	OH
Ganoderiol F (3)	25	2	C=O
Lucidumol B (4)	90	2	OH
Ganodermatriol (2)	100	2	OH
Ganodermanontriol (5)	400	3	C=O
Ganoderiol A (6)	470	3	OH
DHT (positive control)	0.02		

3.3. Prostate cancer cell proliferation inhibition

The effect of each compound on the proliferation of prostate cancer cell is shown in Fig. 2 and 3. The

LNCaP (lymph node carcinoma of the prostate) human prostate cancer cell line is a well-established androgen-dependent cell line [22]. 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. For these reasons, the LNCaP cell line becomes an attractive model for *in vitro* studies of the biology of human prostate cancer [23]. LNCaP cells were incubated with varying concentrations of each compound (5 μ M to 40 μ M) and with or without testosterone or DHT for 3 days. The NR assay was performed to measure cell viability. In the absence of each compound, testosterone alone apparently stimulates the LNCaP cell number about 230 % on average more than the untreated control, and DHT alone apparently stimulates the LNCaP cell number about 300 % on average more than the untreated control.

Compound **1**, **2** and **3** showed no inhibition of LNCaP cell growth when cultured without testosterone or DHT (Table 2, Fig. 2). Interestingly, treating LNCaP cells with compound **1**, **2** and **3** in the presence of testosterone or DHT resulted in dose-dependent inhibition of cell growth (Fig. 2). These results suggested that the inhibition of cell growth in the presence of testosterone or DHT was not caused by the cell cytotoxicity, but came from an anti-androgen effect such as binding to AR.

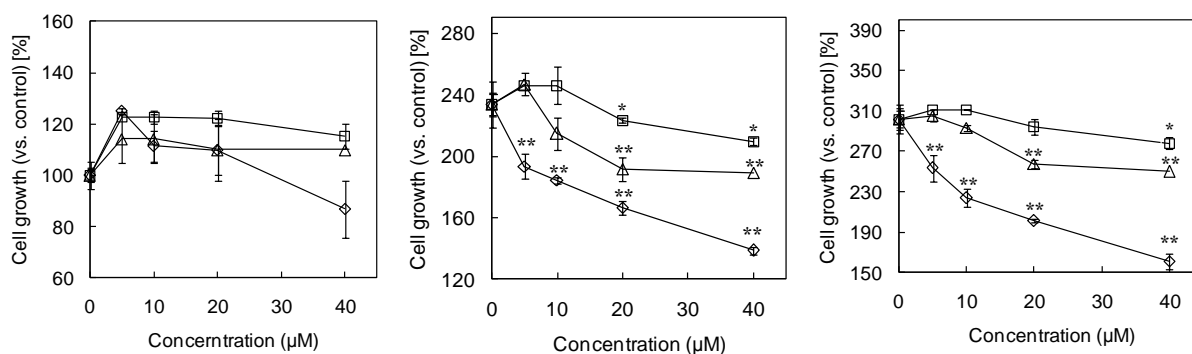


Fig. 2 The effects of ganoderol B (**1**), ganoderatriol (**2**) and ganoderiol F (**3**) on the proliferation of LNCaP cell. (\diamond : ganoderol B (**1**), \square : ganoderatriol (**2**), \triangle : ganoderiol F (**3**)) Results are given as the mean \pm S.D., n=4. * $P < 0.05$ vs vehicle control; ** $P < 0.01$ vs vehicle control. (left: without androgen, middle: added testosterone, right: added DHT)

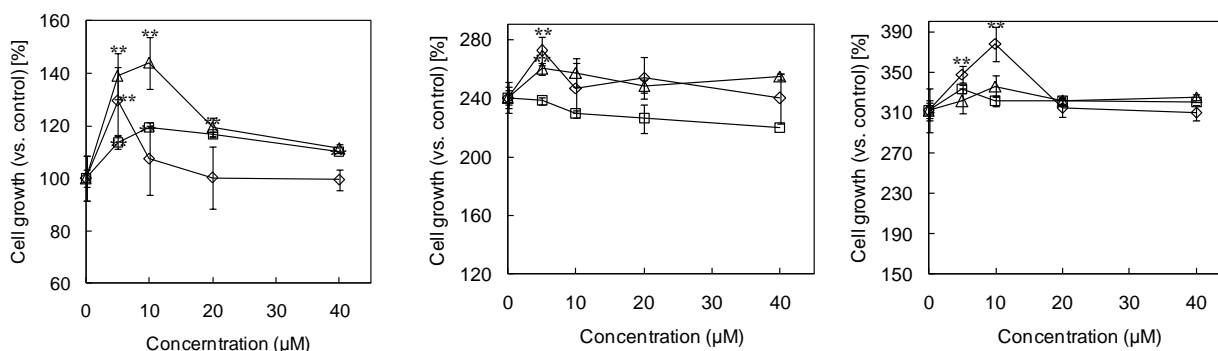


Fig. 3 The effects of lucidumol B (**4**), ganodermanontriol (**5**) and ganoderiol A (**6**) on the proliferation of LNCaP cell. (\diamond : lucidumol B (**4**), \square : ganodermanontriol (**5**), \triangle : ganoderiol A (**6**)) Results are given as the mean \pm S.D., n=4. ** $P < 0.01$ vs vehicle control. (left: without androgen, middle: added testosterone, right: added DHT)

Table 2 The effect of ganoderma alcohol on LNCaP cell at 40 μ M

Compounds	Cell viability (vs Testosterone)	Cell viability (vs DHT)
Ganoderol B (1)	0.59**	0.53**
Ganodermatriol (2)	0.90*	0.92*
Ganoderiol F (3)	0.81**	0.83**
Lucidumol B (4)	1.03	1.03
Ganodermanontriol (5)	0.94	1.06
Ganoderiol A (6)	1.09	1.08

* $P < 0.05$ vs vehicle control ; ** $P < 0.01$ vs vehicle control

Treating LNCaP cells with compound **4**, **5** and **6** resulted in no inhibition on cell growth. If anything, these compounds showed proliferation effect to LNCaP cell growth when cultured without testosterone or DHT (Fig. 3). Compound **4** and **6** showed proliferation effect to LNCaP cell growth when cultured with testosterone or DHT at low concentration (5 μ M to 20 μ M). Compound **5** showed proliferation effect to LNCaP cell growth when cultured without testosterone or DHT only at low concentration (5 μ M to 10 μ M). This proliferation effect cannot be observed when compound **5** cultured with testosterone or DHT.

3.4. Discussion

Since an essential step in the action of androgens in target cells is binding to the androgen receptor, a logical approach for neutralizing the androgens is the use of anti-androgens or compounds which prevent the interaction of testosterone and DHT with the androgen receptor. Since prostate cancer is so highly sensitive to androgens, the anti-androgen used should be a compound having high specificity and affinity for the androgen receptor while not possessing any androgenic activity. Up to now, over 120 kinds of triterpenoids have been isolated from *G. lucidum* and the genus *Ganoderma*. Triterpenoids from the delta7, delta 9(11) series are rather unstable. This time we used the androgen receptor binding affinity assay to measure the correlation between the compound structure and the androgen receptor. *G. lucidum* has been reported to produce many bioactive oxygenated triterpenoids. Six triterpenoids were used and divided to two groups (I and II). The triterpenoids (**1**, **2**, **3**) belong to group I have the unsaturated C-24 in 17 β -side chain, while group II (**4**, **5**, **6**) have the saturated 17 β -side chain. The relative binding activities for the androgen receptor of these triterpenoids are, in decreasing order, **1**>**3**>**4**>**2**>**5**>**6**. Compound **1** has one hydroxyl group in 17 β -side chain, compound **2**, **3** and **4** has two hydroxyl groups, and compound **5** and **6** has three hydroxyl groups. This result suggested that the androgen receptor binding affinity of triterpenoids depends on the hydrophobic 17 β -side chain. Compound **2** and **3** has the same 17 β -side chain, but they have different C-3. This result lead us to conclude that when the compound has the same number hydroxyl groups in 17 β -side chain, the one which has C-3 carbonyl group showed better binding activity to androgen receptor than that with C-3 hydroxyl group. The same tendency was also observed between compound **5** and **6**. These

results were also similar with those reported with Singh, who reported that a "17 β -hydroxy-3-one" structure is needed for effective binding to AR and removal of the 3-keto group results in the significant loss of binding affinity. (Fig. 4) [24].

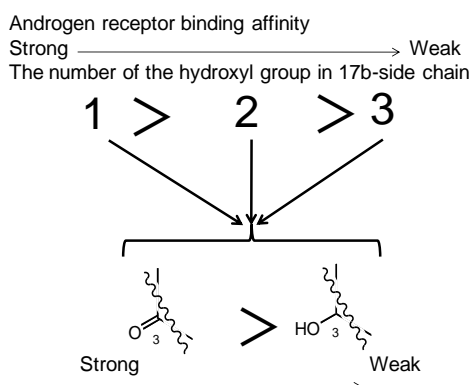


Fig. 4 The relative binding activities for the androgen receptor of ganoderma alcohol

In general, most compounds which display affinity for the androgen receptor were androgen receptor agonists. In androgen receptor binding affinity assay, we found that all compounds showed androgen receptor binding affinity. One significant deficiency of this measurement of receptor binding affinity is that this assay does not distinguish between agonists and antagonists. So we used the LNCaP cell to clarify whether these triterpenoids act as agonist or antagonist by measuring the effect of them on cell growth. Interestingly, all of these triterpenoids showed proliferation effect to LNCaP cell growth when cultured without testosterone or DHT at low concentration. These compounds acted as partial agonist to androgen receptor. Compounds **1**, **2** and **3** showed the proliferation inhibition on cell growth in the presence of testosterone or DHT. In our previous paper, we reported that compound **1**, with androgen receptor binding activity, and androgen-induced growth suppression of LNCaP cells, exhibits its anti-androgenic effects on androgen action in prostate cancer cells by inhibiting AR and PSA expressions. Considering the similar behavior of these three compounds, the similar mechanism can be hypothesized. Further biological evaluation of these triterpenoids is needed to find the mechanism of their anti-androgen effects. Comparing with compounds in group I, which showed the proliferation inhibition on prostate cancer cell in the presence of testosterone or DHT, compounds in group II showed no inhibition on the proliferation of prostate cancer cell, which showed that the unsaturated 17 β -side chain is needed for the cell proliferation of androgen-induced LNCaP cells growth. The unsaturated 17 β -side chain may be involved in the delivery of the compound into the cell and binding to the AR. The delivery of compound relies on general molecular parameters such as the molecule's size, its ability to partition into hydrophobic solvents and its capacity to participate in hydrogen bonding to ensure bulk delivery to the systemic circulation. This delivery may cause that the compound **4** showed stronger AR binding activity than compound **3**, but this compound could not inhibit the cell proliferation.

The partial agonistic activity of compound **1**, **2** and **3** was suggested to have beneficial implications, (e.g., differences in tissue selective actions could be enhanced because of distinct receptor reserve contents in androgen-responsive tissues). Furthermore, in tissues with high natural androgen content, such as the prostate,

a partial AR agonist could act as a functional antagonist of androgen action by effectively competing with both T and DHT. Compound **4**, **5** and **6** showed agonistic activities to androgen receptor in low concentration (lower than 20 μM). These compounds act as a functional agonist with both T and DHT (lower than 20 μM). These compounds can be developed for the treatment of different kind of androgen-related disorder, for example, male hypogonadism, muscle wasting at the low concentration, and for benign prostate hyperplasia, and prostate cancer at the high concentration.

It has been estimated that approximately 65% of clinically used anti-cancer agents have a root of natural products and therefore, there is an increasing interest in searching for novel natural product with anti-cancer effects which showed less side effects. From our results, some estimates of the potential of a particular compound to inhibit the proliferation of prostate cancer cell were provided *in vitro*. This study identified several natural products that inhibited the androgen-induced proliferation of prostate cancer and bound to androgen receptor. For AR binding, ganoderma alcohols with less than two hydroxyl groups in 17 β -side chain are preferred for binding to androgen receptor. In the case of the ganoderma alcohols with the same number of hydroxyl groups in 17 β -side chain, the one which has C-3 carbonyl group showed better binding activity to androgen receptor than that with C-3 hydroxyl group. For the cell proliferation of androgen-induced LNCaP cells growth, the unsaturated 17 β -side chain is needed. Without this unsaturated 17 β -side chain, the LNCaP cells growth is not be affected even the added compound showed the AR binding activity. *In vivo* experimentation will be needed to confirm the effects of these compounds and to make sure those active levels of these compounds are supplied to target tissues. If some of these compounds are active *in vivo*, they may be important candidates for prostate cancer therapy.

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