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https://hdl.handle.net/2324/26045

出版情報：Fitoterapia. 81 (7), pp.902-905, 2010-10. Elsevier
バージョン：
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Abstract
Postate cancer is the fifth most common neoplasm worldwide, and the second most common cancer among men. Ginsenosides, the main component of ginseng, have been known for their medicinal effects such as anti-inflammatory and anti-proliferative activities. In this study, we investigated the inhibitory effects of ginsenosides (ginsenoside 20(R)-Rh2 and ginsenoside 20(S)-Rh2) on prostate cancer cells in vitro. Only ginsenoside 20(S)-Rh2 showed proliferation inhibition on androgen-dependent and –independent prostate cancer cells. These results implied that the stereochemistry of the hydroxyl group at C-20 may play an important role in antitumor activities.

Key words: ginseng, ginsenoside, prostate cancer cell
1. Introduction

Prostate cancer is the fifth most common neoplasm worldwide, and the second most common cancer among men [1]. Investigation of prostate cancer prevention is very attractive, because prostate cancer has a high incidence, long-term natural history, regional difference in incidence, and is affected by sex steroids. Since androgen-and androgen receptor-mediated signaling are essential for the initiation and progression of prostate cancer [2, 3], targeting the receptor and/or its signaling pathways represents a rational strategy for treating this disease [4]. Although anti-androgens provide effective treatment for androgen-dependent prostate cancers, these cancers often become androgen-independent and are, as a result, generally more aggressive, metastatic, and resistant to chemotherapeutic drugs[5]. Thus, this is an urgent need for novel therapeutic agents to improve both the androgen-dependent and androgen-independent prostate cancer.

Chemotherapeutic agents are defined as the use of specific agents to suppress or reverse carcinogenesis and to prevent the development of cancer [6]. The development of chemotherapeutic agent strategies against prostate cancer would be of medical and economic importance. Basic and clinical researchs of chemoprevention of prostate cancer are under active investigation. The compounds that inhibit cell-cycle progression, induce apoptosis, and target multiple aspects of androgen-dependent and androgen-independent signaling are especially desired.

Ginseng has been used for thousands of years in Asian countries, particularly in China, Korea and Japan, for its wide spectrum of medicinal effects such as immune function effects, anticancer effects, a sexual function-enhancing effect [7-9], and others. Many of the pharmacological effects of ginseng, including antitumor activities, are attributed to triterpene glycosides, known as ginsenosides. Ginsenosides share a similar basic structure, consisting of gonane steroid nucleus having 17 carbon atoms arranged in four rings. According to the existence of the hydroxyl group at C-6 or not, ginsenosides are divided into two main categories, the 20(S)-protopanaxatriol and 20(S)-protopanaxadiol family. Rh2 is a tri-terpenoid glycoside saponin with a chemical structure consisting of a steroid nucleus and a glucose sugar moiety. Of the antitumor aspect, it has been reported that some rare 20(S)-protopanaxadiol ginsenosides with one or two sugar moieties at C-3 position, including Rh2, inhibit the growth of both androgen-dependent and –independent prostate cancer cells [10, 11]. The biological activities of ginsenosides, which are still being defined, are related to their structures, especially to the type of aglycone and the number of sugars linked to the core structure.
For instance, as the number of sugar moieties increases, the cytotoxic and anti-cancer activity of the ginsenoside decreases [13]. However, an in-depth study of the structure-activity relationship (SAR) for ginsenosides has not been accomplished [14, 15].

We had found that the stereospecificity of the hydroxyl group at the carbon-20 of ginsenosides plays a differential role in pre-osteoclast proliferation by using RAW 264. More specifically, 20(R)-hydroxylation of ginsenoside Rh2 did not affect the pre-osteoclast proliferation. In contrast to 20(R)-Rh2, 20(S)-Rh2 showed strong cytotoxicity to pre-osteoclast proliferation [16]. We cannot confirm that these two compounds can work in the same for other kind cell line. As part of our continuing project to evaluate natural products for potential anti-prostate cancer activities, we have studied the structure-activity relationship of 20(R)-ginsenoside and 20(S)-ginsenoside on LNCaP cell, PC-3 and DU-145 cell. The efforts described in this report were directed toward comparison of two closely related structures, 20(R)-Rh2 and 20(S)-Rh2 (Fig. 1), to determine which would be the more promising as a potential treatment for both androgen-dependent and –independent prostate cancer. Our results show that a small difference in structure can relate to a large difference in biological activity, which is relevant in the identification of potent anti-cancer ginsenosides and in the exploration of novel molecular targets for prostate cancer prevention and treatment.

2. Experimental

2.1 Cell lines and cell culture

The androgen-dependent human prostate cancer LNCaP cells and androgen-independent cell PC-3 and DU-145 were obtained from the American Type Culture Collection. LNCaP cells were cultured in RPMI 1640 media containing 10% FBS. PC-3 and DU-145 cells were cultured in RPMI 1640 media containing 5% FBS. The purification of 20(R)-Rh2 and 20(S)-Rh2 are higher than 98% confirming by using HPLC. As the result of NMR analysis, the ratio of 20(R)-Rh2 and 20(S)-Rh2 in 20(R: S)-Rh2 is 2:1.

2.2 Cell Survival/Viability Assay

The effects of 20(R)-Rh2 and 20(S)-Rh2 on human cancer cell growth, expressed as the percentage of cell survival, were determined using 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 50% ethanol-aqueous solution (1% acetic acid). The culture medium were changed to NR solution and incubated for 3 hours at 37 °C, then the NR solution were aspirated and the cells were washed by PBS twice. Five hundred 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.

2.3 Cell proliferation assay

The LNCaP 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 2×10^5 cells/well density supplemented with 5% steroid-depleted (DCC-stripped) cFBS. PC-3 and DU-145 cells were plated into a 24-well plate with 5×10^4 cells/well density supplemented with 5% steroid-depleted (DCC-stripped) cFBS. Twenty-four hours later, the cells were incubated with various concentrations of test compounds (0-100 μM) for another three days.

3. Results and discussion

20(R)-Rh2, 20(S)-Rh2 and 20(R: S)-Rh2 were evaluated for their effects on prostate cancer cell growth in vitro by use of the NR assay. Following incubation with various concentrations of the compounds, amounts reducing growth by 20, 50, and 80% (IC_{20}, IC_{50} and IC_{80}) were calculated (Table). For all prostate cancer cells, 20(S)-Rh2 had the lowest IC_{50} values (in the low μM range) compared with 20(R)-Rh2 and 20(R: S)-Rh2. 20(R: S)-Rh2 also demonstrated weakly inhibitory effects. 20(R)-Rh2 did not significantly decrease cell viability even in the high-μM range. For all 20(R)-Rh2, 20(S)-Rh2 and 20(R: S)-Rh2, an anti-proliferative effect for 20(S)-Rh2 was observed (Fig. 2). LNCaP cells were generally more sensitive to the 20(S)-Rh2 than PC3 and DU145. At 25 μM, 20(S)-Rh2 inhibited LNCaP proliferation by 70%, PC3 cell proliferation by 40% and DU145 cell proliferation by 20% (Fig. 3, 4).

Of the three ginsenoside samples tested, only 20(S)-Rh2 displayed an anti-proliferative activity on three kinds of prostate cancer cells (Table). Untreated LNCaP cells appeared as spindle shape, attached smoothly on the culture surface, and some of the cells grouped together to form colonies (Fig. 5a). Following treatment with 20(S)-Rh2 for 72 h, the cells changed to round shape and made fewer cellular contacts.
(Fig. 5b), they lost their surface morphology and died at a concentration of 50 μM (Fig. 5c). The same situation was observed in PC3 and DU145 cells.

Prostate cancer poses a major public health problem worldwide. In the United States, it is the most frequently diagnosed cancer, and the third leading cause of cancer death in men. The enlargement of the prostate compresses the urethra, prevents the bladder from emptying and causes the subsequent micturition problem. Since androgen plays a critical role in regulating the growth and differentiation of prostate cells, hormone therapy becomes the standard treatment for primary prostate cancer. However, androgen-independent tumor normally develops in years when the patients no longer respond to hormone therapy. Androgen-independent prostate cancer is generally more aggressive, more resistant to the presently used chemotherapeutic agents, and more likely to metastasis. Agent capable of inhibiting cell proliferation or modulating signal transduction is currently used for the treatment of prostate cancer.

Ginseng is one of the commonly used herbal medicines whose underlying mechanism is not clear. Recent investigations have shown that ginseng extracts and its components could suppress tumor promoting activity, interfere with cell cycle progression, enhance immune activity and suppress tumor angiogenesis. Rh2 has been reported to inhibit the growth and induce anti-metastatic activity in B16 melanoma and ovarian cancer cells [17-19]. Recently, it is reported that Rh2 can inhibit the proliferation of LNCaP cell and PC-3 cell. The possible mechanisms of Rh2 against LNCaP androgen-sensitive and PC-3 androgen-insensitive prostate cancer cells responding for the anti-proliferation are pro-apoptotic and cell cycle arresting effects [20]. In these study, the 20(R: S)-Rh2 was used as the test sample. The present study has further demonstrated that out of 20(R: S)-Rh2, only 20(S)-Rh2 can inhibit the proliferation of prostate cancer cells.

In the present study, we found that the stereo-specificity of the hydroxyl group at the carbon-20 of ginsenosides plays a differential role in the proliferation of prostate cancer. More specifically, a slight difference in chemical structure between the ginsenoside Rh2 epimers produces a large difference in the proliferation of prostate cancer. More specifically, 20(R)-hydroxylation of ginsenoside Rh2 did not affect the proliferation of prostate cancer. In contrast to 20(R)-Rh2, 20(S)-Rh2 showed strong cytotoxicity to the proliferation of prostate cancer. These results indicate that the stereochemistry of hydroxylation at the C-20 of Rh2 is important for cell cytotoxicity.

In conclusion, first: it is the first time that the 5α-reductase inhibition of 20(S)-Rh2 was found; second: 20(R: S)-Rh2 showed weaker proliferate inhibition than 20(S)-Rh2; third: the inhibitory effect of ginsenoside Rh2 on prostate cancer cell proliferation is
stereoselective, and that the hydroxyl group at carbon-20 of Rh2 is important for the cytotoxicity. Although more studies are needed to determine the most effective dosing regimen, and to determine the molecular mechanism responsible for the anti-tumor activity of 20(S)-Rh2, the present results should aid in the development of an optimally active ginseng compound for both androgen dependent and independent prostate cancer.

**Acknowledgments**

We thank Ms. E. Yamauchi for technical assistance in cell culture.
References

Figure Legends

**Figure 1.** Structure of ginsenoside 20(R)-Rh2 and ginsenoside 20(S)-Rh2

**Figure 2.** The cell viability of ginsenoside on androgen dependent LNCaP cells.

■: 20(R)-Rh2; ◆: 20(S)-Rh2; ▲: 20(R: S)-Rh2; Results are given as the mean±S.D., n=3.

**Figure 3.** The cell viability of ginsenoside on androgen dependent PC-3 cells.

■: 20(R)-Rh2; ◆: 20(S)-Rh2; ▲: 20(R: S)-Rh2; Results are given as the mean±S.D., n=3.

**Figure 4.** The cell viability of ginsenoside on androgen dependent DU-145 cells.

■: 20(R)-Rh2; ◆: 20(S)-Rh2; ▲: 20(R: S)-Rh2; Results are given as the mean±S.D., n=3.

**Figure 5.** The morphological changes of human prostate cancer LNCaP cells treated with ginsenoside 20(S)-Rh2 for 72h. Normal LNCaP cells proliferated and formed colonies (a), but some of them lost their adherent activity in the present of 10 μM 20(S)-Rh2 (b), the cells subsequently died at 50 μM 20(S)-Rh2 (c).

**Table** Growth inhibitory activity of the ginseng compounds
Figure 1

<table>
<thead>
<tr>
<th>Ginsenoside</th>
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<td>20(S)-Rh2</td>
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<tr>
<td>20(R)-Rh2</td>
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Figure 2

The diagram illustrates the relationship between cell viability (vs control) and concentration (μM) for various experimental conditions. The graph shows a clear trend where cell viability decreases as concentration increases. The data points are marked with statistical significance indicators, indicating a significant difference from the control group.
Figure 3

![Graph showing cell viability vs concentration](image.png)

- **Cell viability (vs control)**
- **Concentration (μM)**

The graph illustrates the relationship between cell viability and concentration, with significant differences indicated by stars (*).
Figure 4

![Graph showing cell viability (vs control) against concentration (μM).]
Figure 5
<table>
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<th>Cell line</th>
<th>Inhibitory concentration</th>
<th>20(S)-Rh2 (μM)</th>
<th>20(R)-Rh2 (μM)</th>
<th>20(R:S)-Rh2 (μM)</th>
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