Bioorganic & Medicinal Chemistry Letters 22 (2012) 1082–1085

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Structural modification of ginsenoside Rh_2 by fatty acid esterification and its detoxification property in antitumor

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ARTICLE INFO

Article history:
Received 16 October 2011
Revised 23 November 2011
Accepted 28 November 2011
Available online 7 December 2011

Keywords:
12,6′-Dioctanoyl ginsenoside Rh_2
Antitumor
Structural modification
Detoxification

ABSTRACT

Ginsenoside Rh_2, one of the most important ginsenosides with anticancer properties in red ginseng, has been developed as principal antitumor ingredient for clinical use. However, the cytotoxicity test in human hepatocyte cell line QSG-7701 (IC_{50} 37.3 μM) indicated that Rh_2 might show strong cytotoxic side-effect on the normal liver cells. For blunting the toxicity, Rh_2 was structurally modified by reacting with octanoyl chloride to give a dioctanoyl ester of Rh_2 (D-Rh_2) in the present study. MTT assay in QSG-7701 cell line in vitro showed that the cytotoxicity of D-Rh_2 on human hepatocyte cells (IC_{50} 80.5 μM) was significantly lower than that of Rh_2. While antitumor xenograft assay in mice bearing H22 liver cancer cells in vivo showed that the antitumor activity of D-Rh_2 retained to be strong as that of Rh_2. According to previous pharmacokinetic studies, the fatty acid esterification of Rh_2 might be of detoxification reaction to cells. Additionally, D-Rh_2 showed significant enhancement on increasing thymus index at the dose of 10 mg/kg compared with vehicle treated control group. Thus, D-Rh_2 might indirectly affect tumor growth by stimulating lymphocytes to become cytotoxic to tumor cells. Finally, our findings suggested that D-Rh_2, the fatty acid ester of Rh_2, might attenuate the side-effect by detoxification to human normal cell and could be a more potential candidate for developing as an antitumor drug.

Ginsenoside Rh_2 (Rh_2) is a protopanaxadiol type saponin firstly isolated from red ginseng which has been used as traditional medicine and natural tonic in oriental countries for thousands of years.[1] This metabolite shows various biological effects such as reducing blood glucose,[2] ameliorating ischemic brain injury,[3] and inhibiting allergy.[4] In particular, Rh_2 exhibits beneficial impacts on anticancer. It has been previously reported to have a growth suppressive effect on various cancer cells such as lung adenocarcinoma, neuroblastoma, breast cancer, prostate cancer, colorectal cancer, glioma, malignant melanoma, and hepatocarcinoma cells.[5–19] In addition, Rh_2 inhibits tumor growth in mice bearing human cancer cells,[20,21] and exhibits some synergetic effects with chemotherapeutic agents both in vitro and in vivo studies.[22,23]

Currently, Rh_2 has been developed as a principal antitumor ingredient of 'Jinxing capsule' (Authentication Code: GSJZ-G20060157) for clinical use in China. However, Rh_2 might exhibit high toxicity to normal cells since being cytotoxic to the human hepatocyte cell line QSG-7701 with an IC_{50} value 37.3 μM. In addition, previous studies showed that little Rh_2 could be absorbed into plasma from the rat gastrointestinal tract.[24] Collective evidences indicated that the oral drug bioavailability of Rh_2 is low.[25,26] Thus, it is essential to design and optimize the chemical structure of Rh_2 by structural modification.

Previous pharmacokinetic studies have demonstrated that oral main ginsenosides accounting for 90% (w/w) of the total saponins in ginseng were cleaved the terminal sugar stepwise by colonic bacteria to afford the major protopanaxadiol monoglucoside (M1 or Rh_2) and protopanaxatriol (M4) metabolites. These metabolites were then further esterified by fatty acids as ginsenoside fatty acid esters which could be sustained longer in the body.[27–29] Pharmacokinetic studies also revealed that ginsenoside fatty acid esters might be the real antitumor active species in vivo.[30,31] More importantly, the fatty acid esterification of ginsenoside possibly represented a detoxification reaction in the body.[28] Accordingly, fatty acid esterified ginsenoside could be pharmaceutically active and might be excellent potential drug candidate. In our previous research, several fatty acid esters of ginsenoside M_1 and Rh_1 had been chemically synthesized for improving their anticancer properties.[32,33] In the present research, we aimed to optimize Rh_2 by structural modification with esterification for lowering its possible side-effect.

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doi:10.1016/j.bmcl.2011.11.104

Esterification of Rh_2 was carried out using triethylamine-catalysed synthesis of ester, which is a typical esterification procedure. An octanoyl chloride reagent was chosen because of being sufficient in industrial supply and cheap. The esterification process is quite simple (Scheme 1). Briefly, Rh_2 (2 g) was dissolved in 500 mL of $CHCl_3$ and was slowly added the octanoyl chloride (1.2 mL) and Et_3N (1.2 mL). The mixture was reacted under stirring at room temperature for 15 min. Then the reactant was washed by 1.0 L distilled water and concentrated in vacuo. The concentrate was dissolved in MeOH, and filtered through a 0.22 μm membrane. Finally, the filtrate was condensed and subjected to silica gel column chromatography, eluted with $CHCl_3$–MeOH = 15:1 to give a pure compound **1** (1.38 g). The purification of **1** analyzed by HPLC was more than 98%. The percentage yield of esterification reaction from Rh_2 to **1** was 48.1%.

The chemical structure of **1** was characterized by 1D and 2D NMR analysis. At first, the ^1H and ^{13}C NMR data of **1** were similar to those of Rh_2, except for the obvious differences due to the presence of two additional fatty acyl signals (Table 1 and Supplementary data). In the ^{13}C NMR spectra, carbon signals of δ_C 173.03, 35.24, 25.29, 29.38, 29.51, 30.08, 22.92, 14.36 (C-1″∼8″), and δ_C 173.79, 34.59, 25.39, 29.38, 29.08, 29.70, 22.98, 14.29 (C-1‴∼8‴) could be identified as two separate octanoyl groups. In addition, an upfield shift δ_C 45.82 and a downfield shift signal δ_C 75.21 compared with δ_C 48.71 (C-13) and δ_C 71.11 (C-12) in Rh_2 were observed in the ^{13}C NMR spectra. The observation indicated that one of the octanoyl groups might combine to the hydroxyl group at C-12 of Rh_2 and change the original chemical surroundings of C-12 and C-13 in Rh_2. The conclusion could be confirmed by the analysis of HMQC and HMBC spectra (Fig. 1). Firstly, δ_H 2.08 (dd) and δ_H 5.13 (ddd) respectively correlated to δ_C 45.82 and δ_C 75.21 could be observed in HMQC spectra of **1**. HMBC correlations between δ_H 2.08 and δ_C 73.55, 75.21, 52.70, 53.39 indicated that δ_C 45.82 belongs to C-13, while δ_C 75.21 belongs to C-12. Secondly, δ_H 5.13 (H-12) showed correlationship with δ_C 173.03, as well as C-11 and C-13. Thus, one octanoyl group should be connected to the hydroxyl group of C-12 in Rh_2. By 2D NMR spectra, another octanoyl group could be deduced to connect with the hydroxyl group on C-6′ of the glucose moiety due to the correlations between H-6′ (δ_H 4.74 and 4.95) and δ_C 173.79. Thus, **1** was finally elucidated as 12, 6′-dioctanoyl ginsenoside Rh_2 (D-Rh_2).

D-Rh_2 was firstly tested for its cytotoxicity against human hepatocyte cell line QSG-7701 to evaluate the detoxification effect on human normal cells comparing with Rh_2. For the purpose, MTT assay for cell viability as previously described was used.[34] QSG-7701 cells were seeded at 5×10^3 cells/mL in 96 well plates (180 μL per well). The test samples were dissolved in DMSO and adjusted to the final test concentration range by diluting with RPMI 1640 medium. The final DMSO concentration was adjusted to <0.1%. Each sample was prepared to triplicate, and added 20 μL to each well. These cells were incubated for 48 h. Then 20 μL MTT (Amresco, OH, USA, 5 mg/mL in PBS) were added to each well and incubated for 2 h. Microplates were centrifuged for 5 min (25 °C, 1500 rpm).

Table 1
^{13}C NMR spectroscopic data of compound **1** (^{13}C NMR, 400 MHz, C_5D_5N)

No.	Rh_2	D-Rh_2(**1**)	No.	Rh_2	D-Rh_2(**1**)
	δ_C	δ_C		δ_C	δ_C
1	39.25	39.24	27	17.79	17.87
2	27.22	27.16	28	28.28	28.21
3	88.89	89.25	29	15.95	15.79
4	40.13	40.04	30	17.15	17.70
5	56.49	56.53	1′	107.11	107.14
6	18.57	18.53	2′	75.94	75.73
7	36.01	37.13	3′	78.90	78.65
8	39.81	39.78	4′	72.01	71.78
9	50.51	50.42	5′	78.52	75.21
10	37.09	37.54	6′	63.21	64.71
11	32.20	31.99	1″		173.03
12	71.11	75.21	2″		35.24
13	48.71	45.82	3″		25.29
14	51.83	52.70	4″		29.38[a]
15	31.46	31.57	5″		29.51[a]
16	26.85	26.76	6″		30.08[a]
17	54.95	53.39	7″		22.92[b]
18	16.91	16.82	8″		14.36[c]
19	16.48	16.35	1‴		173.79
20	73.05	73.55	2‴		34.59
21	26.97	26.94	3‴		25.39
22	35.27	34.94	4‴		29.38[a]
23	23.12	23.23	5‴		29.08[a]
24	126.45	126.45	6‴		29.70[a]
25	130.86	130.87	7‴		22.98[b]
26	25.92	25.96	8‴		14.29[c]

[a,b,c] Values may be interchangeable in each column.

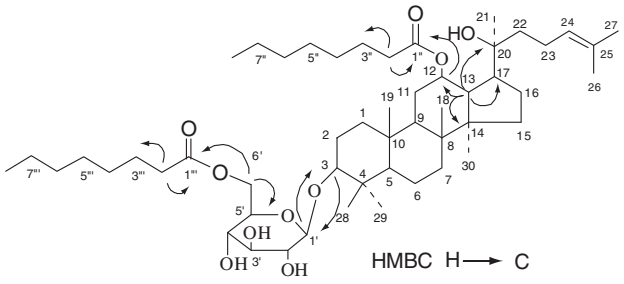
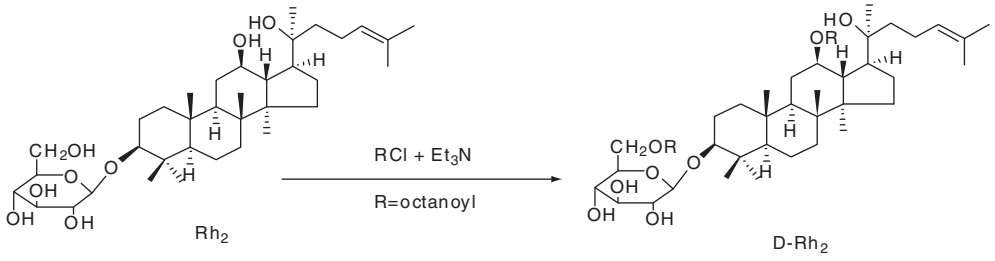


Figure 1. Selected HMBC correlations of **1**.

The supernate was removed and formed formazan crystals were dissolved with 150 μL DMSO. Each plate was shaken for 20 min and the OD values were read at 570 nm (450 nm as a reference) on the microplate reader within 30 min. The IC_{50} value was defined as the concentration of sample needed to reduce 50% of absorbance relative to the vehicle-treated control.

As shown in Fig. 2, Rh_2 (50 μM) inhibited 83% of the cell growth, while treating with D-Rh_2 (50 μM) inhibited 18%. The IC_{50} values of Rh_2 and D-Rh_2 for inhibiting cell growth were 37.3 and 80.5 μM, respectively. D-Rh_2 might largely reduce the cytotoxicity for hu-



Scheme 1. Fatty acid esterified reaction of ginsenoside Rh_2.

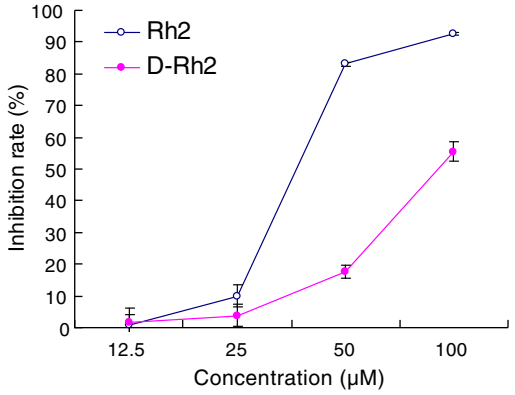


Figure 2. The effects of Rh_2 and D-Rh_2 on inhibiting QSG-7701 cells growth.

man normal cells. The results indicated that the fatty acid esterification of Rh_2 might represent a detoxification reaction. This was well consistent with the previous speculation by Hasegawa et al.[28]

We then examined D-Rh_2 for its antitumor activity on Kunming mice bearing mouse hepatoma (H22 cell line) using Xenograft assay. Kunming mice were provided by the Laboratory Animal Center of Jilin University (Changchun, China) and housed at 23 ± 0.5 °C, 60% humidity in a 12 h light–dark cycle. All animals were acclimated for seven days before the experiment began. Then the mice were injected subcutaneously on right flanks above the hind limb with 0.1 mL H22 cells (1×10^6) and divided into groups of 10 mice at random. After 7 days of the tumor cell implantation, mice were gavaged orally with cytoxan (CTX) (20 mg/kg), Rh_2 (10 mg/kg), Rh_2 (5 mg/kg), D-Rh_2 (10 mg/kg), and D-Rh_2 (5 mg/kg) in 0.5 mL normal saline solution with 1% Tween-80 once per day for 10 days. The control mice were gavaged orally with 0.5 mL vehicle with a similar dosing schedule. Body weights of mice were recorded throughout the experiment. At the termination of the experiment, the tumor tissues were harvested.

As shown in Fig. 3, CTX, Rh_2, and D-Rh_2 treated groups were all significantly inhibited the tumor growth compared with the negative control group. Among them, CTX reduced ~72% of tumor growth in mice at oral treatment dose 20 mg/kg (body weight of mouse). D-Rh_2 exhibited ~52–73% of tumor inhibition at dose 5 and 10 mg/kg (5.7 and 11.4 μmol/kg), while the parental Rh_2 showed ~53–75% inhibitory rate at dose 5 and 10 mg/kg (8.0 and 16.0 μmol/kg). The tumor inhibitory activity of D-Rh_2 at dose

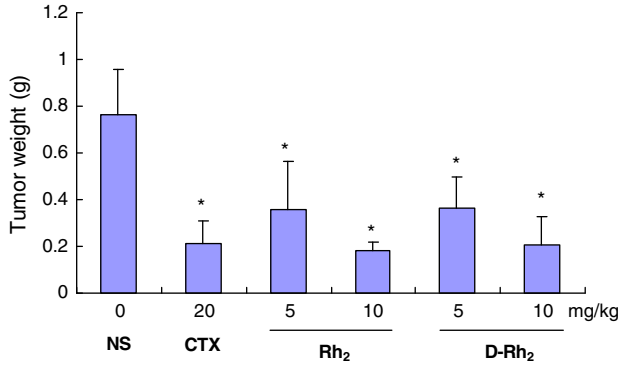


Figure 3. Inhibition of tumor growth in animals. Tumors were dissected and measured weights. Normal saline (NS) treated control group was considered as no tumor inhibition. Data were presented as the means ± standard deviation (SD). Statistical analysis was performed using the SPSS 13.0 statistical software (SPSS ins., Chicago, IL, USA). ANOVA procedures followed by the Dunnett's Multiple Comparison test were used to analyze variance. Differences with *p*-value less than 0.05 were considered to be statistically significant.

11.4 μmol/kg was observed almost as strong as that of Rh_2 at dose 16 μmol/kg or CTX at dose 76.6 μmol/kg. In addition, the average body weights of normal mice and CTX, Rh_2 (dose 20 mg/kg), and D-Rh_2 (dose 20 mg/kg) treated mice changed from 23.0 ± 1.8 to 24.6 ± 2.2 g, 25.3 ± 1.5 to 24.0 ± 1.3 g, 23.4 ± 2.8 to 23.2 ± 1.2 g, and 24.8 ± 1.1 to 27.1 ± 1.5 g, respectively, after 10 days of treatment. D-Rh_2 (dose 20 mg/kg) showed increasing the mice body weight similar to those of control group which grew up normally; while Rh_2 (dose 20 mg/kg) and CTX could not increase or even block the increase of body weight.

Intriguingly, the above results indicated that D-Rh_2 could significantly reduce the toxicity to human hepatocyte cell line QSG-7701 in vitro without blunting the antitumor activity in vivo. According to the previous pharmacokinetic study,[31] it is possible that part of oral Rh_2 were esterified by some fatty acids in the body after digesting. The transformed Rh_2 fatty acid esters might be sustained longer in the body and served as antitumor species. However, the remained Rh_2 could be harmful to the human normal tissue due to its considerable cytotoxicity. After oral intake, the absorbed D-Rh_2 might possess similar antitumor function in vivo to those of transformed Rh_2 fatty acid esters in the body. Compared with Rh_2, D-Rh_2 which showed lower cytotoxicity would largely attenuate the injury for normal tissue in the body. Thus, our findings suggested that D-Rh_2, the fatty acid ester of Rh_2, might depress the side-effect by detoxification to human normal cell and could be an attractive candidate for antitumor. However, the molecular acting mechanism and pharmacokinetics of D-Rh_2 need to be further studied.

Ginseng has been treasured in Asian countries, especially in China, Korea and Japan due to its tonic property. There were many evidences that ginseng and its active component ginsenosides enhance immune activity.[35,36] Jinxing Ginseng Rh_2 Capsule, which was declared to improve immune function and increase resistance, was marketed as a health food for the patients in the treatment and rehabilitation period with cancer. We therefore compared the effects of spleen and thymus indexes in the tumor bearing mice with control and D-Rh_2 treatment. Mice were inoculated tumor cells and divided into groups (10 mice per group). Additionally, 10 normal mice were grouped as blank control. After 7 days of the tumor cell implantation, mice were orally administrated once a day with D-Rh_2, Rh_2, positive control CTX and negative control normal saline (NS) for 10 days following above Xenograft assay. At the termination of the experiment, thymus gland, and spleen were harvested and weighted, respectively.

As showed in Table 2, the CTX group significantly decreased spleen and thymus indexes (P <0.05) compared with NS group. D-Rh_2 at the dose of 10 mg/kg significantly increased the thymus index (P <0.05). No significant difference of spleen and thymus index was observed at 5 mg/ kg dose of the D-Rh_2 while compared with negative control. The above results indicated that cytoxan could significantly (P <0.05) lead to atrophy of the spleen and thy-

Table 2
Effects of D-Rh_2 on spleen and thymus index in Kunming mice

Group	Dose (mg/kg)	Spleen index (mg/g)	Thymus index (mg/g)
Blank	—	6.64 ± 1.04	3.35 ± 0.89
NS	—	6.61 ± 1.84	1.83 ± 0.85*
CTX	20	4.58 ± 1.11*,#	0.74 ± 0.23*,#
Rh_2	5	5.48 ± 1.32	2.25 ± 0.61*
Rh_2	10	5.35 ± 0.83	2.09 ± 0.78*
D-Rh_2	5	5.27 ± 1.21	2.15 ± 1.10*
D-Rh_2	10	6.31 ± 1.44	3.42 ± 0.82#

The data were presented as means ± SD (n = 10).
* P <0.05 significantly different from the blank group.
# P <0.05, significantly different from the NS treated group.

mus in tumor-bearing mice (Table 2), which were in a good agreement with the previous data on immunostimulatory activities.[37] While in D-Rh_2 treated groups, there was significant enhancement of thymus index at the dose of 10 mg/kg. The results indicated that the antitumor effect of D-Rh_2 might in part attribute to the immunostimulatory function.

In summary, for the purpose of reducing side-effect, Rh_2 was structurally modified as a dioctanoyl ester of Rh_2 (D-Rh_2) by reacted with octanoyl chloride. Compared with the parental Rh_2, D-Rh_2 could significantly decrease toxicity to the human hepatocyte cell line QSG-7701 in vitro but not attenuate the antitumor activity in vivo. In addition, the enhanced effects of D-Rh_2 on thymus index indicated that D-Rh_2 might indirectly affect tumor growth by stimulating lymphocytes to become cytotoxic to tumor cells.[30] D-Rh_2 could be a more potential candidate for using as an antitumor drug due to the possible lower side-effect. The results obtained in the study will provide basic data for further development and utilization of D-Rh_2 in tumor prevention and therapy.

Acknowledgments

This research was financially supported by the Science and Technology Development Program from Jilin Provincial Science & Technology Department (yyzx 201135), and in part by Grant 2010J05085 from Fujian Provincial Department of Science & Technology. We are grateful to the Laboratory Animal Center of Jilin University (Changchun, China) for providing experimental animals.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.104.

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