Effect of Ginsenosides from *Panax ginseng* on Proliferation of Human Osteosarcoma Cell U_2OS

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Abstract

Object To find out which of the 27 ginsenosides isolated from Panax ginseng C.A. Mey that may inhibit the proliferation of human osteosaocoma cell line U_2OS. **Methods** Effects of each individual ginsenoside on the proliferation of U_2OS cell were studied by determining the viability of cancer cells during culture with or without the presence of the test compound. DNA assay was determined by flow cytometry. **Results** Ginsonosides -Ro, -Rh_1, -Rh_2, -F_1 and -L_8 at concentrations of 5 μmol/L could obviously suppress the proliferation of U_2OS cells while ginsenosides -Rg_1, -F_3, -Rf, PPT and PT significantly inhibited the cancer cells. Flow cytometry revealed that ginsenosides -Ro,-Rg_1,-Rf,-F_1,-Rh_2,PPT and PT induced cell cycle arrest at G_0/G_1 phase with obvious decrease of cell count at S and G_2+M phase, Moreover, ginsenosides -Rf_1, -Rg_1, -F_1 and PPT induced significantly high rates of cell death as compared with the control. **Conclusion** These data suggested that ginsenosides inhibited U_2OS proliferation Via cell cycle arrest or induction of cell death.

Key words: ginsenosides, osteosarcoma U_2OS, cell proliferation, cell cycles, cell death

Introduction

Many experiments demonstrated that ginsenosides have stronger anticancer and antitumour activity,[1-3] and it does not only have effects on some specific organ.[4] The main active constituents of *Panax ginseng* are ginsenosides, their function of inhibiting cancer cells have largely been reported ,especially about ginsenoside-Rh_2, it can not only inhibite the proliferation of the cancer cells[5,6], but also induce their differentiation[7,8]. In addition, the inhibiting effect of ginsenoside-Rh_1[8], Rb_1[9], Rb_2 and -Rg_3[10] on cancer cells have also been covered. In this paper, by way of counting cell number, flow cytometry and fluorescence staining, The effects of 27 ginse-

nosides and monomeric compounds were examined on the proliferation, cell cycle and cell death of culture human osteosarcoma cell line U_2OS.

Materials and Methods

***Experimental materials*:** PI and Hoechst 33342 were purchased from Sigma company; DMEM medium was purchased from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from JRM Bioscience (Lenexa,USA).

***Compounds*:** 27 individual ginsenosides isolated from Panax ginseng C.A.Mey were resolved in DMSO at the concentration of 50 mmol/L, and were deposited at -20°C. Their used concentration was 5 μmol/L.The concentration of DMSO in the cell cultured supernatant was 0.01%, a concentration that did not interfere with the test system.

***Cell Culture*:** human osteosarcoma cell U_2OS was purchased from ATCC corporation, USA. U_2OS cells were cultured in DMEM medium supplemented with 10% heat-inactivated (56°C, 30 min.)FBS at 37°C in the presence of 5%CO_2.

***Analysis of Proliferation of Tumor Cells*:** The proliferation of U_2OS cells was tested by counting the cell number. 3.6×10^4cells were seeds in 24-well plate at day 1. On the second day monomeric compounds were added to the culture medium respectively to meet with the final concentration of 5 $\mu mol\cdot L^{-1}$. The cell numbers were counted at the day 3nd, 5th and 7th day .

***Analysis of Cell Cycle*:** The shifts of cell cycle were indicated by the content of DNA determined with flow cytometry. 4×10^5 cells were seeded into 60 mm dishes. Then on the second day the monomeric compounds were added to the culture medium to meet with the final concentration of 5 $\mu mol\cdot L^{-1}$. The medium was removed on the seventh culture day. After rinsing with PBS twice, cells were harvested for 5 minutes by using 0.25% at 37°C trypsin and then centrifuged at 500 rpm .After removing the supernatant, the residues were washed with PBS twice again. After incubating with 50 μg/ml PI staining solution (containing 0.112% sodium citrate and 0.15% Triton x-100 in PBS) in room temperature for 1 min, the cells were separated by centrifugation. Followed by being washed with PBS twice, the cells were suspended with 50 $\mu g\cdot ml^{-1}$ PI solution (not containing Triton x-100) 300 μl. The cell numbers of different phased were carried out by fluorescence-activated cell sorting. The cell number in every analysis is 10000.

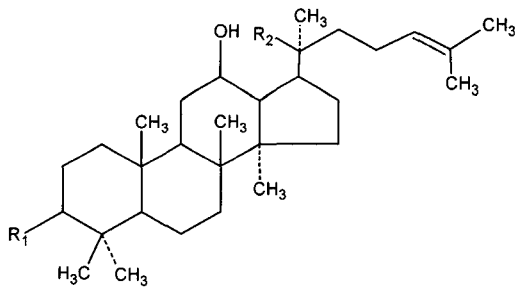
***Analysis of Cell Death*:** 5×10^4 U_2OS cells were seeded in 35 mm glass-bottom dishes. On the second test day, the monometric compounds were added to the culture medium to meet the

resultant concentration of 5 μmol·L^{-1}. At the seventh cultured day the medium was removed and the cells were rinsed with PBS twice. Then the cells were incubated with staining solution (containing 20 μg·ml^{-1} PI and 6 μg·ml^{-1} Hoechst 33342 in PBS) at twice and observed by using a fluorescence microscope. The cell death rate was calculated by cell death numbers statistic.

Results

Effects of Ginsenosides on Proliferation of Human Ostesarcoma Cells

The twenty -seven monomeric ginsenosides are divided into three groups according to the structure of aglycone, i.e. The protopanaxadial(PPD)-type compounds are from 1-12, protopanaxatrial(PPT) -type compounds are from 13-25, and the rest two compounds are oleanolic acid type (Fig. 1). The results in Table 1 showed the effects of Ginsenosides on Proliferation of



Human Ostesarcoma Cells:

1-Ginsenoside-Ra$_1$ R_1=OGlc(2-1)Glc; R_2=OGlc(6-1)Ara(p)(4-1)Xyl

1-Notoginsenoside-R_4 R_1=OGlc(2-1)Glc; R_2=OGlc(6-1)Glc(6-1)Xyl

2-Ginsenoside-Rb$_1$ R_1=OGlc(2-1)Glc; R_2=OGlc(6-1)Glc

3-Rb$_2$ R_1=OGlc(2-1)Glc;R_2=OGlc(6-1)Ara(p)

4-Rb$_3$ R_1=OGlc(2-1)Glc; R_2=OGlc(6-1) Xyl

5-Rc R_1=OGlc(2-1)Glc; R_2=OGlc(6-1)Ara(f)

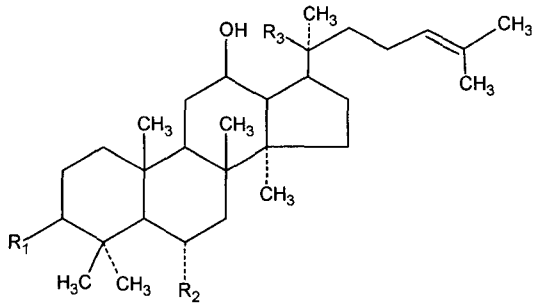
6-Rd R_1=OGlc(2-1)Glc; R_2=OGlc

7-Rg$_3$ R_1=OGlc(2-1)Glc; R_2=OH(20R)

8-Rd R_1=OGlc; R_2=OGlc(6-1)Ara(p)

10- Notoginsenoside-Fe R_1=OGlc; R_2=OGlc(6-1)Ara(f)

11- Ginsenoside-Rh$_2$ R_1=OGlc;R_2=OH



13-Ginsenoside-Re R_1=OH; R_2=OGlc(2-1)Rh_a R_3=OGlc

14-Rf R_1=OH; R_2=OGlc(2-1)Rh_a R_3=OGlc(2-1)Glc;R_3=OH

15- Rg_1 R_1=OH; R_2=OGlc;R_3=OGlc

16-20(R)-ginsenoside-Rg_2 R_1=OH; R_2=OGlc(2-1)Rh_a ;R_3=OH(20R)

17-Ginsenoside-Rg_2 R_1=OH; R_2=OGlc(2-1)Rh_a ;R_3=OH(20S)

18-F_3 R_1=OH; R_2=OH;R_3=OGlc(6-1)Ara(p)

19-Chikusetsaponin-L_8 R_1=OH; R_2=OH;R_3=OGlc(6-1)Ara(f)

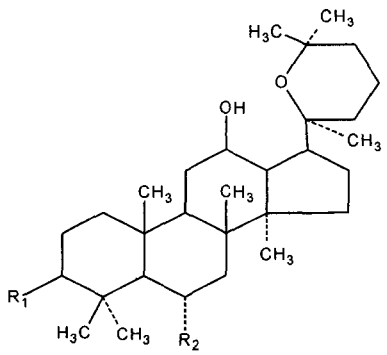
20-Ginsenoside-Ia R_1=OGlc; R_2=OH; R_3=OGlc

21-20(R)-ginsenoside-Rh_1 R_1=OH; R_2=OGlc ;R_3=OH(20R)

22-Rh_1 R_1=OH; R_2=OGlc ;R_3=OH(20S)

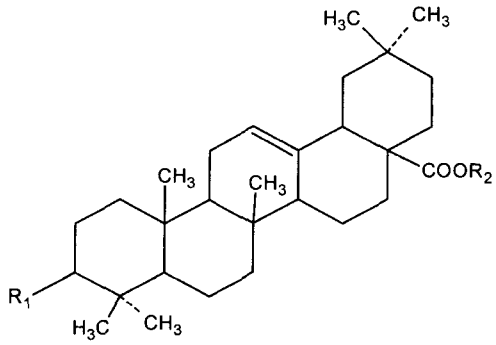
23-Ginsenoside-F_1 R_1=OH; R_2=OH;R_3=OGlc

24-Protopanaxatriol (PPT) R_1=OH; R_2=OH ;R_3=OH



12-Panaxadial(PD) R_1=OH; R_2=H

25-Panaxatrial(PT) R_1=OH; R_2=OH



26-Oleanolic R_1=OH; R_2=H

27-Ginsenoside-Ro R_1=OGlucA(2-1)Glc;R_2=Glc

Fig. 1. The structure of ginsenoside and aglycone.

The results in Table 1 showed that ginsenoside-Ra_1(1), notoginsenoside-R_4(2), ginsenosede-Rb_1(3), -Rb_2(4), -Rb_3(5), -Rc(6), Rg_3(8), -Rd_2(9), -Fe(10), 20(R)-Rg_2(16) and oleanolic acid (26) showed almost no influence on the proliferation of tumor cell during one week .While PD (12). -Re(13) and Ia(20) exhibited obvious inhibition on proliferation of tumor cell cultured at early time (3rd day), but rapid decreasing as time went on .However, ginsenoside-Rh_2(11) merely disclosed definite inhibition on tumor cell even cultured in a long time (7 days). Ginsenoside-F_1(23), -Rg_1(15), PPT(24) and PT(25) had a similar manners as Rh_2 on proliferation of tumor cell, but they are stronger and showed significant inhibition action on tumor cell as compared with control group. Ginsenoside-Rd(7), Rg_2(17), -L_8(19), 20(R)-Rh_1(21), -Rh_1(22) and Ro(27) show obvious suppression on tumor cell. The rest two compounds, ginsenoside-F_3(18) and-Rf(14), especially the latter, completely inhibited the proliferation of tumor cells during the whole culture time .

Effects of Ginsenosides on the Cell Cycle of Osteosarcoma Cell U_2OS

Based on the results obtained in table 1, Ginsenosides-Ro(27), -Rf(14), -Rg_1(15), -F_3(18), -L_8(19), -Rh_2(11), -Rh_1(22), 20(R)-Rh_1(21), F_1(23), PPT(24) and PT(25) were chosen to test their effects on cell cycle of tumor cells. As illustrated in chart 1. U_2OS cells chiefly existed in the S- and G_2+M-phases, tumor cells in G_0/G_1 phase account for 43% .Ginsenoside-Rh_1 and 20(R)-Rh_1 showed no influence on the cell cycle. Ginsenoside-Rh_2-F_3,-L_8, PPT and PT obviously increased

Table 1. Effects of ginsenoside on the proliferation of human osteosarcoma cell line U_2OS

Sample No.	Day 1	Day 3		Day 5		Day 7	
Control	36000	44740± 4449		169374± 11239		355751± 30337	
1	36000	55468± 1072		162770± 11078		338045± 35068	
2	36000	43346± 4999		137939± 3236		349125± 28760	
3	36000	45136± 3894		155404± 21265		322722± 21368	
4	36000	44845± 3868		146602± 18145		338988± 33534	
5	36000	44502± 4616		134052± 15028		368084± 44148	
6	36000	46105± 2134		168834± 12273		348036± 20486	
7	36000	41304± 3668		132302± 17271	***	326841± 22775	
8	36000	40212± 2577		168380± 20326		375142± 41464	
9	36000	45135± 1838		150183± 9777		330623± 27663	
10	36000	52463± 3992		166563± 11658		362932± 23884	
11	36000	44525± 4424		152867± 10694		319911± 21961	
12	36000	30051± 4979	***	151958± 13395		344136± 23466	
13	36000	33920± 3874	***	150197± 20149		349714± 12336	
14	36000	21510± 3883	***	117693± 16214	***	258446± 6913	***
15	36000	43098± 3915		121347± 5571	***	277895± 26539	***
16	36000	44114± 3461		158530± 17507		333542± 21357	
17	36000	38277± 5747	*	142891± 14378	**	327839± 29512	
18	36000	32433± 4658	***	149928± 8648		269733± 20207	***
19	36000	31475± 1842	***	144871± 9951	**	318382± 28626	
20	36000	34830± 4680	***	147614± 24793		335508± 23957	
21	36000	37918± 5870	*	153739± 16570		315886± 25871	*
22	36000	34923± 5525	***	157282± 10704		312721± 37272	*
23	36000	40250± 3841		141454± 10278	***	295662± 21092	***
24	36000	43957± 2870		148651± 9985	*	277940± 23583	***
25	36000	42311± 8458		156894± 16323		289047± 13607	***
26	36000	43516± 2070		141965± 22171		326452± 20002	
27	36000	40030± 4224	**	150263± 22344		310204± 14675	**

The data were expressed as mean ± SD. From three independent experiments. The P value were obtained by comparing with the control at the same day. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the quantity of cells at G_0/G_1 phase, suggesting that they definitely induced cell cycle arrest at G_0/G_1 phase. However Ginsenoside-Ro, -Rf, -F_1 and -Rg_1 significantly induced the increment of cells at G_0/G_1 phase, indicating their inhibitory effect on U_2OS cell cycle.

Effects of Ginsenosides on Death of Osteosarcoma Cells U_2OS

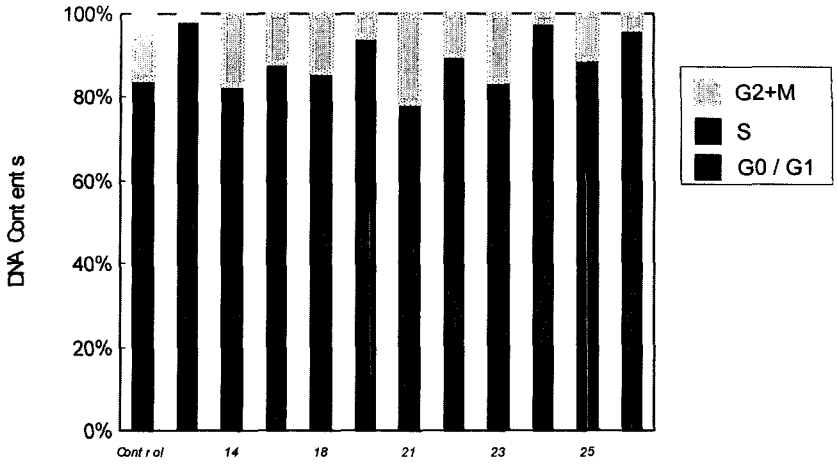


Chart 1. Effects of Ginsenosides on the Cell Cycle of U_2OS.

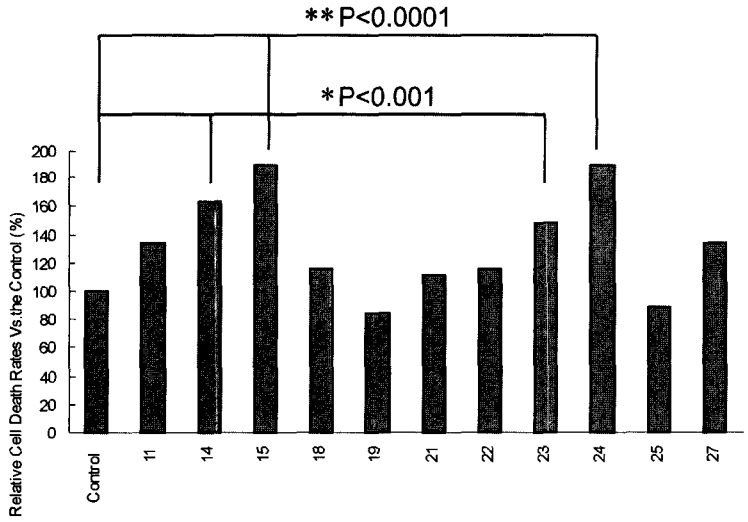


Chart 2. Effects of Ginsenosides on Cells Death U_2OS.

The results shown in chart 2 indicated that except PT and -L_8, Other ginsenosides by and large exhibited activities to induce the death of tumor cells. Ginsenoside-Rf, -Rg_1, -F_1 and PPT significantly induced cell death of tumor cells as compared with control group.

Discussion

The anticancer activities of Ginsenosides have been highly thinking for a few years. The results

of the experiments in vivo and in vitro indicated that ginsenosides had inhibitory action on many tumors such as melanoma[7], abnormality cancer[11], carcinoma uteri[5], rectum cancer[10], lung cancer[12], carcinoma hepatic and cholangiolitis cancer[12], breast cancer[13], carcinoma ventriculi[12] and leukaemia[8] ect. But the inhibitory action on Osteosarcoma Cells U_2OS was the first reported. Therefore, in the experiment the influence of the monomeric compounds from 27 ginseng on the proliferation of cultured in vitro Osteosarcoma Cells U_2OS was tested. The results showed that almost half of ginsenosides (compound 1-6, 8-10, 16) at the concentration of 5 μmol/L had no inhibitory action on the proliferation of U_2OS. Generally, there are two factors affects the proliferation of cells. One is the cell cycle arrest. Another one is the induction of the cell death. So we tested that 11 compounds having inhibitory activity influenced on cell cycle and cell death of U_2OS. Conclusions in chart 2 revealed that ginsenoside-Ro, -Rh_2, -F_1, -Rf, Rg_1, PPT and PT significantly arrested the cell cycle. Ginsenoside-F_3 also obviously inhibited the cell cycle. But ginsenoside-Rh_1 almost had no effects.When observing cell death, we found that true cell death rates were all quite low, the largest value was less than 5%. But results in chart 3 exhibited -Rf, -Rg_1, -F_1 and PPT significantly induced the high rates of cell death. -Ro and -Rh_2 showed only obviously inductions to the cell death ($p<0.01$), However -Rh_1, -F_3 -L_8 and PT presented almost no effect on cell death. In short, these facts coincides with effects of the compounds except -F_3 on proliferation of tumor cells. That is to say, the compounds blocked the proliferation of U_2OS cells either through cell cycle arresting or inducing cell death or both ways in common. The mechanism of -F_3 on tumor cells suggested to be further studied .

The results in the paper are not completely consistent with the reported documents. For example, in reported documents, ginsenoside-Rh_2 exhibited very strong anti-tumor activity,but in the experiment, its activity was weak. Moreover, Anticancer activities of ginsenoside -Rb_1, -Rb_2, -Rb_3 has also been reported, but in the experiment their action on U_2OS can't been observed. Maybe there are two reason: one is the difference of the species and genus of tumor cell used, another is the difference of detector method. Ginsenoside- Rh_1 and Rg_1 showed anti-tumor activity, which is consistent with the documents. The research results indicated that ginsenoside-Rf had the strongest inhibitory activity on the proliferation of U_2OS.

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