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Effects of Ginseng Saponin on Modulation of Multidrug Resistance

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Multidrug resistance (MDR) has been a major problem in cancer chemotherapy. To overcome this problem, we prepared minor ginsenosides stereoselectively from ginseng saponins and searched for a ginseng component which is effective for inhibition of MDR. MDR inhibition activity was determined by measuring cytotoxicity to MDR cells using multidrug resistant human fibrocarcinoma KB V20C, which is resistant to 20 nM vincristine and expresses high level of *mdr1* gene. Of several ginseng components, 20(S)-ginsenoside Rg_3, a red ginseng saponin, was found to have the most potent inhibitory activity on MDR and it's concentration capable of inhibiting 50% growth was 82 μM.

Keywords : *Panax ginseng*, Ginsenosides, 20(S)-ginsenoside Rg_3, Multidrug resistance

INTRODUCTION

Chemotherapy involving regimens of anticancer drug combinations continues to play a key role in the treatment of a varitety of human neoplasms. However, despite initial responses, many tumors eventually fail chemotherapy, because standard chemotherapy is inadequate to deal with a large tumor burden in many kinds of cancer and dose intensification, which might lead to better results, is often hindered by inherent side effects of present chemotherapeutic agents.

One of the major side effects to the effective treatment of human malignancies is the acquisition of broad based anticancer drug resistance by tumor cells. The exposure of malignant cells or tumor cell lines to a single hydrophobic cytotoxic agent of natural origin including the *vinka* alkaloids (vincristine and vinblastine), anthracyclines, epipodophyllotoxins (VP-16 and VM-26), actinomycin D, colchicine or taxol frequently results in the emergence of cell populations exhibiting resistance to the selecting agent as well as to the remaining pleiotropic drugs that are structurally and mechanically unrelated lipophilic antibiotics. This phenomenon has been termed multidrug resistance (Beck, 1987; Fojo *et. al.*, 1985; Gottesman & Pastan, 1988, 1993; Ueda *et. al.*, 1987).

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A common form of MDR in human cancer results from expression of the *mdr1* gene, which encodes a 1280 amino acids transmembrane phosphoglycoprotein, termed P-glycoprotein. P-glycoprotein acts as a plasma membrane energy-dependent multidrug efflux pump. Mammalian cells that exhibit the MDR produce high levels of the P-glycoprotein that removes a number of cancer chemotherapeutic agents from the cell (Gottesman & Pastan, 1993). This phenotype, Pgp-MDR, has been implicated in intrinsic and acquired drug resistance in a number of human tumors (Endicott & Ling, 1989; Gros *et. al.*, 1986; Herzog *et. al.*, 1993; Mirski *et. al.*, 1987; Pastan *et. al.*, 1988).

A second type of pleiotropic resistance does not overexpress *mdr1* (Mirski *et. al.*, 1987; Taylor *et. al*, 1991; Grant *et. al*, 1994) and poorly reversed by modulators that are effective in cells overexpressing *mdr1* (Cole *et. al.*, 1989, 1991; Cole, 1992). Protein responsible for this type of multidrug resistance is named as multidrug resistance associated protein (MRP), and it's nucleotide sequence encoding 1531 amino acids was determined (Cole & Deelay, 1993; Krishinamachary & Center, 1993). Multidrug resistance due to MRP is known as MRP-MDR.

Pharmacological intervention aimed at inhibiting Pgp-MDR transporter should improve the activity of existing chemotherapy against human cancer. Numerous agents which interfere with the activity of P-glycoprotein, such as verapamil, have been des-

cribed, and the majority of these appear to be substrates for the transporter, which compete with anticancer drugs for their transport. However, inherent and potentially toxic side effects of prototypical resistance-modifiers limit the clinical usefulness of these drugs (Dalton *et. al.*, 1989; Ford & Hait, 1990; Markus & Werner, 1993; Teeter, 1989; Twentyman *et. al.*, 1987). Therefore development of new modulators for inhibition of efflux transporter is required.

Ginseng saponins isolated from the root of *Panax ginseng* C. A. Meyer have been regarded as the main effective ingredients responsible for the pharmacological activities. A great deal of investigations on the pharmacology and biochemistry of ginseng, so far, have been made especially on the major saponins. Since ginsenoside Rh_2, found in minor amount only in red ginseng, has been recently reported to exhibit potent cytotoxicities against cancer cells such as Lewis lung, Morris hepatoma B16, and HeLa cells (Kitagawa *et al*, 1983; Kitagawa, 1984), many researchers focused on the minor ginsenosides and recognized them as biologically active compounds. This result led us to separate each minor ginsenosides stereo- selectively and determine anticancer effects (Baek *et. al.* 1995; Im *et. al.*, 1995; Kim *et. al.*, 1991a, 1991b, 1995). Here we examined the effects of ginseng saponins and their degradation products on inhibition of multidrug resistance. The results demonstrated that ginseng saponin 20(S)-ginsenoside Rg_3 is effective in purging MDR cells from multidrug resistant human fibroblast carcinoma KBV 20C *in vitro*.

MATERIALS AND METHODS

Chemicals and drugs

Vincristine (VCR) and other chemicals were obtained from Sigma Chemical Co.

Tissue culture

The human fibroblast carcinoma KB cells obtained from ATCC were grown in RPMI 1640 media with 5% fetal bovine serum, and 0.1 mg/ml Kanamycin at 37°C in 5% CO_2. VCR-resistant KB V20C cells (generous gift of Dr. Cheng at Yale University School of Medicine) were developed from the parental KB cells by stepwise selection for resistance with increasing concentration of VCR and cultured in the presence of 20 nM concentration of VCR. These cells have been shown to overexpress P-glycoprotein by Western blot (Chen *et al.*, 1993) and used for Pgp-MDR inhibition study. KB 7D cells (generous gift of Dr. Cheng at Yale University School of Medicine), which overexpress the mrp gene and display MRP-MDR phenotype (Gaj *et. al.*, 1995), were cultured in the presence of 7 μM concentration of etoposide.

Cytotoxicity assay

The KB V20C cells were maintained in drug free media 3 days before determination of the concentration capable of inhibiting 50% growth. Cytotoxicity assays were performed in triplicate by plating 1×10^4 cells in each well of a 96-well plate. The cells were incubated with 20 nM concentration of VCR in the absence or presence of ginseng components at 5% CO_2 and 37°C for 72 hr. Subsequentally, the methylene blue cell staining method described by Graeme *et. al.* (1984) was used to measure the cytotoxic effect.

Stereoselective preparation of minor ginsenosides from major saponin

As described in the previous report (Kim *et. al.*, 1991), 20(R)-ginsenoside Rg_2 (**1**, 42 mg) and its 20(S) epimer (**2**, 46 mg), 20(R)-ginsenoside Rh1 (**3**, 74 mg) and its 20(S) epimer (**4**, 33 mg) were stereoselectively prepared from the protopanaxatriol saponin(3 g), and also 20(R)-ginsenoside Rg_3 (**5**, 84.2 mg) and its 20(S) epimer (**6**, 80.8 mg), 20(R)-ginsenoside Rh_2 (**7**, 41.6 mg) and its 20(S) epimer (**8**, 21.6 mg) were prepared from ginsenoside Re (6 g) by treating the compound at two different deacetylation temperatures, respectively. Acidic and alkaline hydrolysis of protopanaxadiol saponin gave ginsenoside Rh_3 (**9**), which was further hydrolyzed to give its aglycone, quasiprotopanxadiol (**10**) and they were identified by comparing physical and spectral data with the data reported previously (Kim *et. al.*, 1995). Ginsenoside Rh_4 (**11**) was isolated from EtOH extract of red ginseng and was hydrolysed with 9% HCl/MeOH to provide its aglycone, quasiprotopanaxatriol (**12**) (Kim *et. al.*, 1995)

Acetylation of 20(S)-ginsenoside Rg_3 (6)

A solution of 20(S)-ginsenoside Rg_3 (10 mg) in Ac $_2$O-pyridine (1 : 1, 2 ml) was allowed to stand at room temperature overnight, then poured into ice water (20 ml), and the reaction mixture was extractd with $CHCl_3$, 5% HCl and saturated $NaHCO_3$ successively, and dried over anhydrous sodium sulfate. The product was purified by silica gel column chromatography with CH_2Cl_2-EtOAc (6 : 1) to provide heptaacetate (6 mg): white powder, ^{1}H-NMR (400 MHz, $CDCl_3$)δ 0.82-1.68 (each 3H, s, -$CH_3\times8$), 1.98, 2.00, 2.02, 2.04, 2.06, 2.07, 2.10 (each 3H, s, -OAc$\times$7), 4.98 (1H, d, J=7.6 Hz, 1''-H), 5.44 (1H, d, J=7.0 Hz, 1'-H), 5.32 (1H, t-like, 24-H).

Acetylation of 20(R)-ginsenoside Rg_3 (5)

A solution of 20(R)-ginsenoside Rg_3 (10 mg) in Ac

$_2$O-Pyridine (1 : 1, 2 ml) was acetylated in the same way as described above to give heptaacetate (7.2 mg): white powder, ^{1}H-NMR (400 MHz, $CDCl_3$) 0.84-1.72 (each 3H, s, $-CH_3\times 8$)δ, 1.99, 2.00, 2.01, 2.03, 2.04, 2.07, 2.10 (each 3H, s, -OAc×7), 4.96 (1H, d, J=7.6 Hz, 1''-H), 5.42 (1H, d, J=7.2 Hz, 1'-H), 5.30 (1H, t-like, 24-H).

Selective acetylation of 20(R)-ginsenose Rg_3 (5)

A solution of 20(R)-ginsenoside Rg_3 (10 mg) in Ac $_2$O-Pyridine (1 : 1, 2 ml) was acetylated at 4°C overnight. The reaction mixture was examined in the same way as described above to give diacetate (3.2 mg): white powder, ^{1}H-NMR (400 MHz, $CDCl_3$)δ 0.86-1.64 (each 3H, s, $-CH_3\times 8$), 1.90, 1.94 (each 3H, s, -OAc×2), 4.90 (1H, d, J=7.4 Hz, 1''-H), 5.28 (1H, d, J=7.0 Hz, 1'-H), 5.22 (1H, t-like, 24-H).

Preparation of ginsenoside Rg_{31} (13)

Ginsenoside Rb1 (0.4 g) was treated with 50% acetic acid at 70°C for 3 hr. The reaction mixture was left to stand overnight at 4°C, followed by filtration and extraction with n-BuOH. After removing the solvent from the filtrate, the product was purified by silica gel column chromatography with $CHCl_3$/MeOH/H $_2$O (9 : 3 : 1) to provide ginsenoside Rg_{31} (8.4 mg): white powder, mp 188-192°C, ^{1}H-NMR(400 MHz, $CDCl_3$)δ 0.85-1.86 (each 3H, s, $-CH_3\times 8$), 2.77 (2H, dd, J=6.9, 7.0 Hz, 23-H), 4.90 (1H, d, J=7.4 Hz, 1''-H), 5.21 (1H, t, J=6.9 Hz, 24-H), 5.33 (1H, d, J=7.6 Hz, 1''-H), 5.49 (1H, t, J=7.0 Hz, 22-H).

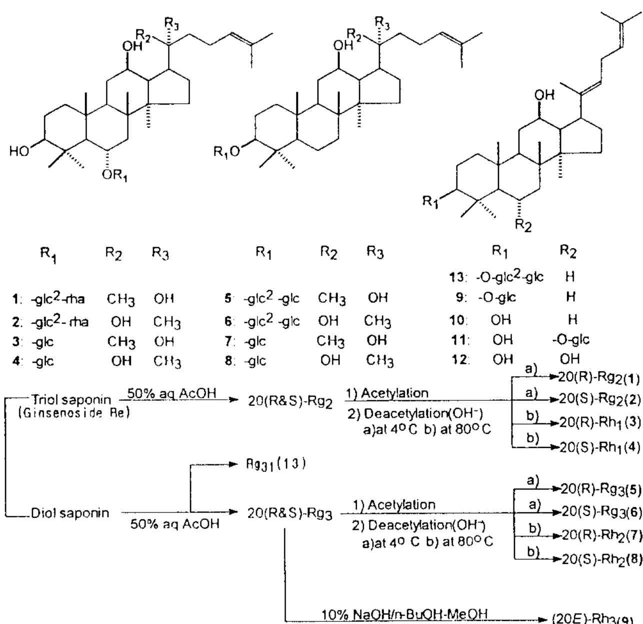

Fig. 1. Stereoselective preparation of several minor ginsenosides from ginseng saponins

RESULTS AND DISCUSSION

Pgp-MDR inhibition by red ginseng extract and ginsenosides

We seperated each minor ginsenoside stereoselectively in good yields from major saponins (Fig. 1) and its absolute chemical structure was determined on the basis of chemical and spectral evidences, together with the application of modern 2D-NMR techniques. As a part of our search for new MDR reversal agents from natural products, these minor ginsenosides and their degradation products were evaluated for MDR reversal activites using KB V20 cells and KB 7D cells.

Water extract fraction of the red ginseng did not inhibit propagation of Pgp-MDR cells, nor n-butanol fraction of the red ginseng fraction showed significant Pgp-MDR inhibition, i.e., IC_{50} value for Pgp-MDR inhibition was 852 μg/ml. But purified ginseng saponin containing the major ginseng saponin was shown to inhibit Pgp-MDR (IC_{50} value=185 μg/ml) although each component of the major ginseng saponins did not inhibit Pgp-MDR (Table 1). These results suggest that the minor ginsenosides might be responsible for Pgp-MDR reversing activity.

Table I. IC_{50} Values of P-glycoprotein-mediated MDR reversal activity and cytotoxicity of Red ginseng saponin and major ginsenosides to cancer cells[1)]

Samples	MDR inhibition (KB V20C cells)	Cytotoxicity (KB cells)
H2O Ext.2[2)]	>1000 μg/ml	NT
Saponin fr.[3)]	852 μg/ml	980 μg/ml
P.Saponin(I)[4)] (81.6%)	315 μg/ml	385 μg/ml
P.Saponin(II) (87.5%)	185 μg/ml	290 μg/ml
Ginsenoside		
$-Rb_1$	>1000 μM	NT
$-Rb_2$	>1000 μM	NT
-Rc	>1000 μM	NT
-Rd	>1000 μM	NT
-Re	>1000 μM	NT
-Rf	>1000 μM	NT
$-Rg_1$	>1000 μM	NT

[1)]IC_{50} Value represents the concentration M) of a compound required for 50% inhibition of cell growth. Each compound was examined at least at three concentrations in duplicate. NT: Not Tested
[2)]Water extract of red ginseng
[3)]n-BuOH soluble fraction of red ginseng
[4)]Major ginsenoside composition of purified saponin (P. Saponin)

(unit: %)

Sample	$-Rb_1$	$-Rb_2$	-Rc	-Rd	-Re	$-Rg_1$	Total
P. Saponin (I)	10.8	7.2	18.4	5.8	24.6	14.8	81.6
P. Saponin (II)	12.1	9.8	19.1	8.7	21.0	16.8	87.5

Table II. IC_{50} Values of Pgp(P-glycoprotein)-mediated MDR reversal activity and cytotoxicity of minor ginsenosides[1)]

(Unit: M)

Samples	MDR inhibition (KB V20C cells)	Cytotoxicity (KB cells)	Rf[2)]
Panaxadiol	254	408	0.62
Protopanaxadiol	202	332	0.61
Panaxatriol	258	707	0.36
Protopanxatriol	292	806	0.36
Ginsenoside-			
20(R)-Rg_2(**1**)	〉250	94	-
20(S)-Rg_2(**2**)	〉250	NT	-
20(R)-Rg_3(**5**)	〉250	NT	-
20(S)-Rg_3(**6**)	82	254	0.32
20(R)-Rh_1(**3**)	〉250	78	-
20(S)-Rh_1(**4**)	〉250	92	-
20(R)-Rh_2(**7**)	〉250	〉200	-
20(S)-Rh_2(**8**)	〉250	67	-
-Rh_3(**9**)	302	250	1.20
Qppd[3)](**10**)	〉250	〉200	-
-Rh_4(**11**)	216	92	2.34
Qppt[4)](**12**)	〉250	〉200	-

[1)] IC_{50} Value represents the concentration (μM) of a compound required for 50% inhibition of cell growth. Each compound was examined at least at three concentrations in duplicate.
NT: Not Tested
[2)] Rf(Resistance factor): IC_{50} of resistant cells/IC_{50} of sensitive cells
[3)] The aglycone moiety of Ginsenoside Rh_3
[4)] The aglycone moiety of Ginsenoside Rh_4

Table 2 exhibits that panaxadiol and protopanaxadiol, which were prepared during the course of preparation of minor ginsenosides, had IC_{50} value of 254 μM, and both of them also showed similar Pgp-MDR inhibition activity at the same concentration. But most of ginsenosides including Rg_2, Rg_3, Rh_1, Rh_2, Rh_3 except 20(S)-Rg_3 (6) and Rh_4 (11) showed higher IC_{50} value for Pgp-MDR inhibition than those of panaxadiol and panaxatriol. 20(S)-ginsenoside Rg_3, which was contained only in red ginseng, had such potent Pgp-MDR inhibition that only 82 μM was required to reduce multidrug resistant population into half, but cytotoxicity to KB cells was 254 μM, demonstrating that it works preferably for Pgp-MDR inhibition rather than cytotoxicity to cancer cells. Also removal of a glucoside portion of the ginsenosides Rh_3 (**9**) and Rh4 (**11**) failed to inhibit Pgp-MDR (Table 2).

MDR inhibition by ginsenoside Rg_3 and its derivatives

In order to clarify the correlation between chemical structures of ginsenoside Rg_3 and it's MDR modulator activity, several peracetate, diacetate forms of the ginsenoside Rg_3 were prepared and used for determination of Pgp-MDR and MRP-MDR inhibition (Table 3). Only 20(S)-ginsenoside Rg_3 (**6**) and Rg_{31} (**13**), in which C-20 hydroxyl group of ginsenoside Rg_3 was lost and a double band was introduced, inhibited Pgp-MDR at 82 μM and 72 μM concentration, respectively. MRP-MDR was inhibited only by Rg_{31} (**13**) at 68 μM concentration but it might be due to strong cytotoxicity to KB cancer cells itself because IC_{50} values for cytotoxicity to KB cells as well as inhibition of both multidrug resistant cells showing Pgp-MDR and MRP-MDR phenotypes were same.

Table. III. IC_{50} Values of Pgp and multidrug resistance associated protein mediated MDR reversal activities and cytotoxicities of Gisenoside Rg_3 and its derivatives[1)]

(Unit: M)

Ginsenosides	Pgp-MDR inhibition (KB V20C cells)	MRP-MDR inhibition (KB 7D cells)	Cytotoxicity (KB cells)
20(R)-Rg_3(**5**)	〉250[2)]	〉1000	NT
20(S)-Rg_3(**6**)	82	〈 500	254
20(R)-Rg_3(Ac)7	〉500	〉500	〉500
20(S)-Rg_3(Ac)7	〉500	〉500	〉500
20(R)-Rg_3(Ac)2	〉500	〉500	〉500
-Rg_{31}(**13**)	72	68	74

[1)] IC_{50} Value represents the concentration (μM) of a compound required for 50% inhibition of cell growth. Each compound was examined at least at three concentrations in duplicate.
NT: Not Tested
[2)] Each compound was examined at least at three concentrations in duplicate.

Agents that reverse MDR have been extensively reviewed (Ford & Hait, 1990; Beck, 1990). The great impetus for identifying MDR modulators came after the observations that the calcium channel antagonist verapamil reversed resistance (Tsuruo *et. al.*, 1981). Since then there are a great number of diverse chemical structures that can reverse MDR, but of some interest are the structural similarities of ginsenosides to steroid hormones. Based upon the observation that a pregnant uterus expressed high levels of mdr1 transcripts, it has been postulated that certain steroids may be natural substrates for P-glycoprotein (Arceci *et. al.*, 1988). Yang *et. al.* (1989) found that progesterone and deoxycorticosterone, but not estradiol, caused an increase in [3H]vinblastine accumulation and slight reversal of resistance in MDR J7 V1-1 murine macrophage-derived cell. Progesterone was the most potent of a series of steroids tested for inhibition of [^{3}H]azidopine affinity labeling of P-glycoprotein. Further studies on ginsenoside Rg_{31} (**13**) would elucidate it's MDR reversal mechanism.

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