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Effects of Non-Saponin Red Ginseng Components on Multi-drug Resistance

Eun-Hye Kim*, Jong-Dae Park¶, Suhk-Neung Pyo*, and Dong-Kwon Rhee*#

**College of Pharmacy, SungKyunKwan University, Su-Won 440-746*
¶Ginseng Science Research Group, KT & G Central Research Institute, Daejeon 305-805, Korea
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Abstract : Multi-drug resistance (MDR) is a major problem in cancer chemotherapy and has often ended up with termination of the therapy. The aim of this study was to identify any fractions of Korean red ginseng that would be effective in modulating for MDR. Although ginsenosides have been reviewed as possible MDR modulators, the MDR modulation activity of the other component is unknown. Therefore, a red ginseng was extracted with methanol, ether, ethylacetate, and n-butanol, followed by several fractionations by silica gel chromatography. And the activity of MDR modulating for these fractions was examined via sulforrhodamine B assay. We have found that several ether fractions, as nonsaponin components are effective on MDR modulation. We have expect that these results helpful to improvement of cancer chemotherapy.

Key words : Red ginseng, multi-drug resistance, multi-drug related protein, MDR, MRP.

INTRODUCTION

Recently, the number of cancer patients has increased worldwide as a result of environmental pollution, food additives, processed food, and several stresses. Several effective treatments have been used through surgery, radiation therapy, photo therapy, and chemotherapy. However, world wide people die even after these treatments every year.

Among the many types of drug resistance, multi-drug resistance (MDR) phenotypes are usually resistant to vinca alkaloids (vincristine and vinblastine), anthracyclines, colchicines, actinomycin D, epipodophyllotoxins, and macrolides (taxol, epothilones) as well as cross resistance to anticancer drugs that are different from the chemical construction and effect of the particular anticancer medicine.

The MDR gene encodes a 1280-amino acid transmembrane phosphoglycoprotein, known as the P-glycoprotein (P-gp). Tumor cells that show high levels of the P-gp can remove a number of cancer chemotherapeutic agents from the cell via an energy-dependent efflux mechanism. This phenotype, P-gp MDR, has been implicated *mdr1* (ABCB1) in intrinsic and acquired drug resistance in a number of human tumors[1-6]. As a consequence, the death rate is up to 60 %[7,8]. Therefore, pharmacological agents that can inhibit the P-gp transporter are being developed[3,9,10]. The multi-drug resistance-associated protein (MRP2, ABCC2) of 190 kDa membrane protein is found in head, neck, lung cancer that discharges the drug dependent on energy [11-13].

Several agents that interfere with the activity of P-gp, such as verapamil, have been reported[8]. The clinical tests with verapamil and dexverapamil were discontinued due to the high condiovascular toxicity when administered with doxorubicin. Moreover, dexverapamil, which induces verapamil, was also discontinued due to cardiac toxicity. The clinical tests are being carried out with cyclosporin A as well as substance, that can induce cyclosporin to inhibit MDR when used with etoposide, doxorubicin, or paclitaxel. Although, several drugs can affect the function of Pgp *in vitro,* they cannot be used for anticancer therapy *in vivo* because of cytotoxicity. In 1993, the third generation of MDR inhibitor, GF120918, was generated[15]. In 1999, XR9576 was clinically tested, but it was too toxic for the use in cancer treatment.[16]. Several studies have been carried out to determine the anticancer effect of ginseng[17-21] but there is limited knowledge of the multi-drug resistance effect of ginsenoside[22, 23]. Moreover, there are no reports on the effect of other fractions except for ginsenoside. Therefore, our research examined the inhibitory activity of the fractions for red ginseng on MDR cells.

#To whom correspondence should be addressed.
(Tel) +82-31-290-7707; (Fax) +82-31-292-8800
(E-mail) dkrhee@skku.ac.kr

MATERIALS AND METHODS

1. Materials

(1) Red ginseng

Six-year Korean red ginseng was obtained from the Korean Society of Ginseng.

(2) Cell lines

The human 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. The mouse leukemia P388 cells obtained from the ATCC were grown in RPMI 1640 media with 10 % fetal bovine serum and 0.1 mg/ml kanamycin, and incubated under humidified air with 5 % CO_2 at 37 °C. VCR-resistant KBV20C cells (a generous gift from Dr. Yung-Chi Cheng at Yale University, School of Medicine) and related MRP KB7D cells (a generous gift from Dr. Y. C. Cheng at Yale University, School of Medicine), and P388/ADR cells, which over express Pgp, were developed from P388/D1 cells by stepwise selection for their resistance to increasing concentration of adriamycin (ADR) (a generous gift from Dr. H-M. Kim at Korea Research Institute of Bioscience and Biotechnology, Daejon, Korea).

(3) Drugs

Vincristine (VCR), adriamycin (ADR), etoposide (VP-16), the media, and other chemicals were purchased from Sigma.

2. Methods

(1) Making the extract from red ginseng

100 g of 6-year red ginseng was extracted three times with 1000 ml of MeOH for 4 hours under reflux. The resulting extract, 13.5 g, was subjected to filtration and concentration. 10 g of this was dissolved in 100 ml of water and was extracted 3 times with 100 ml ether. The supernatant was dehydrated by Na_2SO_4, and was decompressed for concentration within 40°C yielding 830 mg of the powder (Fig. 1). The water saturated fraction was extracted three times with 100 ml ethylacetate and was separated with ethylacetate (EtOAc fr.) and water. The water fraction was extracted three more times with 100 ml n-BuOH, filtered and concentrated yielding a 1.4 g saponin fraction. Finally, 10.2 g of the H_2O fraction was obtained by the decompression followed by the condensation of the remaining supernatants (Fig. 1).

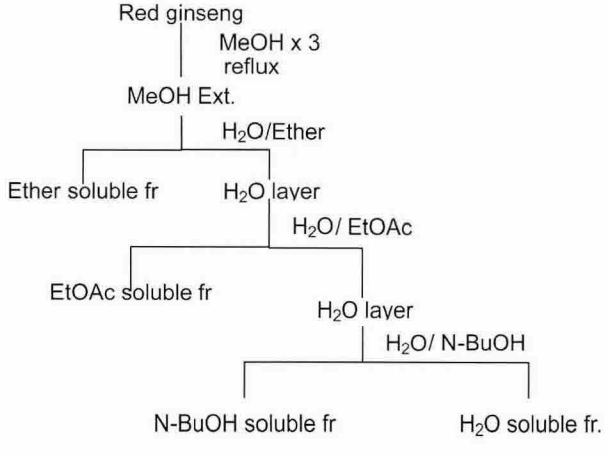

Fig. 1. Various solvent fraction of red ginseng.

(2) Tissue culture

The human fibroblast carcinoma KB, KBV20C, and KB7D cells were grown in RPMI 1640 media containing 5 % of fetal bovine serum and 100 μg/ml kanamycin at 37 °C in 5 % CO_2. The multi-drug resistant KBV20C, KB7D cells were cultured in the presence of vincristine 20 nM and etoposide 7 μM, respectively.

Mouse leukemia P388 cells were grown in the RPMI 1640 media containing 10 % of fetal bovine serum and 200 units/ml penicillin G, 200 μg/ml streptomycin sulfate and were incubated under humidified air containing 5 % of CO_2 at 37 °C. The P388/ADR cells were grown in the RPMI 1640 media containing 0.1 mg/ml adriamycin under the same conditions used for the other cell cultures.

(3) Inhibition of MDR by red ginseng fraction.

The extracted sample, 10 mg, was dissolved in 100 μl DMSO and was diluted to a final concentration of 4 mg/ml. The resulting solution was then diluted 2-fold to 2 mg/ml by being mixed with the same amount of the media containing 20 nM vincristine and 7 μM etoposide with the non-drug media.

(a) Anti-cancer reagent preparation

One mg of vincristine was dissolved in 1 ml of H_2O and was filtered. After filtration, the optical density was determined at 259 nm. In order to determine molarity, vincristine was diluted to 1 mM with H_2O and then stored at -20 °C before use.

The etoposide was dissolved in DMSO up to 7 mM, then stored at -20 °C, and finally was diluted to 20 μM immediately before use.

(b) Cytotoxicity assay

For the preliminary experiment, a methylene blue cell

staining method was used and the effect of red ginseng fraction was examined using the sulforrhodamine B (SRB) assay [24] to check the amount of cellular protein.

(c) Measurement of inhibition of MDR relating to the P-glycoprotein

The anti-cancer drug resistant KBV20C cells were inoculated 2×10^4 per well and the cytotoxicity of the cells were exposed to 1 mg/ml, 0.25 mg/ml, 0.5 mg/ml of the red ginseng fraction was checked cytotoxicity after adding 20 nM vincristine. BIBW22 solution (1 μM, 0.3 μM, 0.1 μM) and DMSO (1 %, 0.5 %, 0.25 %(v/v)) was used as the positive and negative controls, respectively.

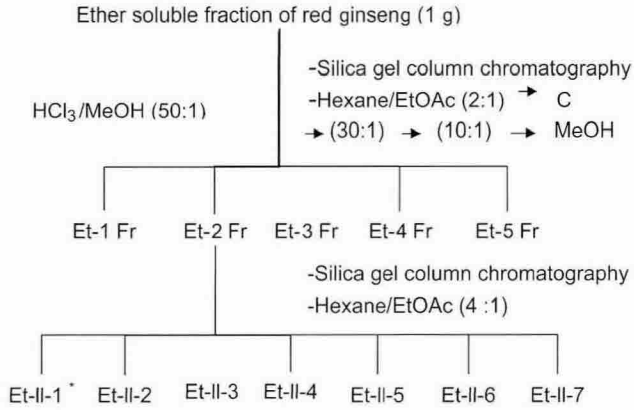

Fig. 2. Fractionation of active Ether-soluble fraction by silica gel colmn chromatography *Fraction showing MDR reversal activity.

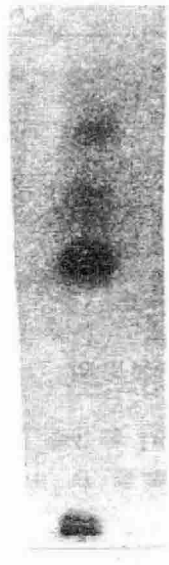

Fig. 3. TLC chromatogram of ether fraction II-1 of red ginseng. Solvent system, hexane/EtOAc (5 : 1); plate, precoated silica gel $60F_{254}$ (0.25 mm, Merck); detection, 10% sulfuric acid. Compound 1 = Rf 0.82, compound 2 = Rf 0.67, compound 3 = Rf 0.52.

(d) Measurement of inhibition of MDR relating to the MRP

The level of inhibition of the multidrug related-MDR cells was checked in the process of seeding the KB7D cells with 2×10^4 cells/ml and adding 7 μM etoposide (VP-16). The cytotoxicity of the cells that contained 1 mg/ml, 0.2 mg/ml, 0.04 mg/ml of the red ginseng fraction was then checked.

RESULT AND DISCUSSION

1. Inhibition effect of ginseng fraction on Pgp MDR cells

After separating the hydrophobic or lipophilic compounds from red ginseng, inhibition of multidrug related-MDR cells was tested *in vitro*. The results showed that the MDR cells had no cytotoxicity when we used the extracted from butanol and methanol but the compounds extracted from ethylacetate and H_2O slightly inhibited the MDR cells, and the compound that were extracted from ether strongly inhibited the MDR cells (Fig. 1). The level of inhibition of the cell growth was up to 76 % when 20 μg/ml of the compound extracted from ether was added. Moreover, 100 and 500 μg/ml of the compound was found to inhibit the cell growth by 62 % and 98 %, respectively.

The fractions from the ether extraction were examined by silica gel column chromatography. Five fractions of the compound were obtained (Fig. 2). Among these, the second fraction (Fraction -II) was found to inhibit the growth of MDR cells. The 5 fractions on spot from silica gel chromatography were confirmed (Fig. 3). Besides, silica gel column chromatography was performed using the

Table 1. Cytotoxicity of the solvent fractionated extract on multidrug resistant KBV20C cells.

Fraction	Dose, ug/ml@		
	20	100	500
Methanol	0.79	0.76	0.69
Ether	0.76	0.38	0.02
Ethylacetate	1.12	1.19	0.44
Butanol	0.93	0.85	0.88
H_2O	1.35	0.84	0.55

@ Relative ratio of KBV20C cells with solvent fractionated extract/KBV20C cells only with DMSO.

Table 2. IC_{50} Values of the Red Ginseng Fractions Extracted with Ether on KB V20C.

Fraction	IC_{50} (mg/ml)
II-1	0.06
II-2	0.21
II-3-1	0.21
II-3-2	0.1
II-4	0.3
II-5	-
II-6	0.27
II-7	-
III	0.21

Table 3. IC_{50} Values of the Red Ginseng Fractions Extracted with Ether on KB7D cells.

Fraction	IC_{50} (mg/ml)
II-1	0.0265
II-2	>0.5
II-3-1	>0.5
II-3-2	0.35
II-4	>0.5
II-5	>0.5
II-6	>0.5
II-7	0.175
III	>0.5

second and third fraction with different solvent conditions. In addition, the effect of ginseng on inhibition of MDR was screened using two kinds of cells. When the cytotoxicity of P-gp phenotype cells (KB V20C) we rechecked, fractions II-1 and II-3-2 were found to be effective with IC50 of 60 μg/ml and 100 μg/ml, respectively. However, the other fraction did not inhibit the growth of the MDR cells (Table 2)

2. Inhibition effect of ginseng fraction on MRP-MDR cells.

KB 7D cells that over express the multi-drug related protein (MRP) showed IC50 values of 26 μg/ml and 175 μg/ml after being exposed to the compounds from fractions II-1 and II-7, respectively. However, the inhibition of these MDR cells with the other fraction showed no effect on these MDR cells (Fig. 3).

One of the major side effects of the treatment of human malignancies is the acquisition of broad based anticancer drug resistance by tumor cells. Therefore, a potent MDR inhibitor for cancer therapy is essential. Accordingly, a development of an inhibitor of multi-drug resistance that suppresses P-gp activation is vital[16]. Therefore, this report of the inhibitory effect of the fractions from red ginseng on the multi-drug resistance is expected to be very important for the future clinical experiments and the efficacy of Korea red ginseng.

This study discovered that several non-saponin red ginseng fractions can inhibit multi-drug resistance. Therefore, future studies will aim at determining the effects of a modification of the structure of these compounds that might help in the development of a drug that can effectively inhibit multi-drug resistance.

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