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Reversal of P-glycoprotein-mediated multidrug resistance by ginsenoside Rg_3

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Abstract

Multidrug resistance has been a major problem in cancer chemotherapy. In this study, *in vitro* and *in vivo* modulations of MDR by ginsenoside Rg_3, a red ginseng saponin, were investigated. In flow cytometric analysis using rhodamine 123 as an artificial substrate, Rg_3 promoted accumulation of rhodamine 123 in drug-resistant KBV20C cells in a dose-dependent manner, but it had no effect on parental KB cells. Additionally Rg_3 inhibited [^{3}H]vinblastine efflux and reversed MDR to doxorubicin, COL, VCR, and VP-16 in KBV20C cells. Reverse transcriptase-polymerase chain reaction and immuno-blot analysis after exposure of KBV20C cells to Rg_3 showed that inhibition of drug efflux by Rg_3 was due to neither repression of *MDR1* gene expression nor Pgp level. Photo-affinity labeling study with [^{3}H]azidopine, however, revealed that Rg_3 competed with [^{3}H]azidopine for binding to the Pgp demonstrating that Rg_3 competed with anticancer drug for binding to Pgp thereby blocking drug efflux. Furthermore, Rg_3 increased life span in mice implanted with DOX-resistant murine leukemia P388 cells *in vivo* and inhibited body weight increase significantly.

Keywords: *Panax ginseng*; 20(*S*)-Ginsenoside Rg_3; Multidrug resistance (MDR); P-glycoprotein (Pgp)

1. Introduction

The exposure of cancer cells to a single hydrophobic cytotoxic agent including the vinka alkaloids, epipodophylotoxins, anthracyclines, COL or taxol frequently results in a specific resistance not only to the cytotoxic agent but also to other structurally and mechanically unrelated anticancer drugs. MDR of cancer cells is a major obstacle for cancer chemotherapy. It is correlated with the overexpression of Pgp in the plasma membrane of resistance cells, where the Pgp acts as an ATP-dependent efflux pump by extruding the anticancer drugs and decreasing their intracellular accumulation [1]. Pgp is a 170-kDa protein encoded by the human *MDR1* gene, and has been implicated in intrinsic and acquired drug resistance in a number of human tumors [2–6]. Therefore, inhibition of Pgp transporter by pharmacological agents should improve the activity of existing chemotherapy against human cancer [7]. Numerous agents interfering with the activity of Pgp have been described [8–14], however, clinical application has been limited by the deleterious toxicities and/or the low efficacy of the Pgp inhibitors in animal studies.

Panax ginseng has been used in the Far East without any significant toxicity [15,16] for a long time and has gained popularity in the West during the last decade. Its major active components are the ginsenosides, which are mainly triterpenoid dammarane derivatives [17,18]. Ginsenoside Rh_2, found only in red ginseng, exhibited potent cytotoxicities against several cancer cells [19]. In addition, human hepatoma cells treated with ginsenoside Rh_2 induced apoptosis by a mechanism that involves the activation of cyclin A/Cdk2 by caspase 3-mediated cleavage of p21$^{(WAF1/CIP1)}$ [20]. Recently, ginsenosides Rg_1, Re, Rc, and Rd were found to have a moderate inhibitory effect on the drug efflux pump

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Abbreviations: COL, colchicine; DOX, adriamycin; IC_{50}, dose causing 50% growth inhibition; MDR, multidrug resistance; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; VCR, vincristine; VP-16, etoposide.

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in multidrug resistant mouse lymphoma, and increased drug accumulation and tumor antigen expression at 2.0–20.0 μg/mL [21]. Although ginsenosides had a chemical structure-dependent immunomodulating effect by enhancing the activity of natural killer cells and antibody-dependent cellular cytotoxicity activities [21], the mechanisms of ginsenoside actions on MDR cells remain unclear. This led us to determine effects of ginsenosides on MDR. Of several ginseng components 20(*S*)-ginsenoside Rg_3, found only in red ginseng, was shown to have the most potent inhibitory activity on multidrug resistant human fibroblast carcinoma KBV20C, capable of inhibiting 50% growth at a concentration of 82 μM. Rg_3, however, did not reverse MDR due to overexpression of multidrug related protein [22]. The aim of this study was to elucidate the mechanism of Pgp inhibition by ginsenoside Rg_3, and determine *in vivo* efficacy.

2. Materials and methods

2.1. Chemical and drugs

VCR, COL, DOX, VP-16, verapamil, and rhodamine 123 were purchased from Sigma. [^{3}H]vinblastine (specific activity, 23 Ci/mmol) and [^{3}H]azidopine (specific activity, 49 Ci/mmol) were purchased from Amersham. 20(*S*)-Ginsenoside Rg_3 was prepared as described previously (Fig. 1) [22]. C494 mouse anti-Pgp monoclonal antibody was from Signet Laboratories.

2.2. Cell lines and animals

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° in 5% CO_2. The mouse leukemia P388 cells obtained from 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°. VCR-resistant KBV20C cells (generous gift of Dr. Yung-Chi Cheng at Yale University School of Medicine) and multidrug resistant P388/DOX cells (generous gift of Dr. H.-M. Kim at Korea Research Institute of Bioscience and Biotechnology) were developed from the parental KB and P388 cells by stepwise selection for resistance with increasing concentration of VCR and DOX, respectively, and cultured in the presence of 20 nM concentration of VCR and 172 nM concentration of DOX, respectively. These cells have been shown to overexpress Pgp by Western blot [23] and were used for Pgp MDR inhibition study.

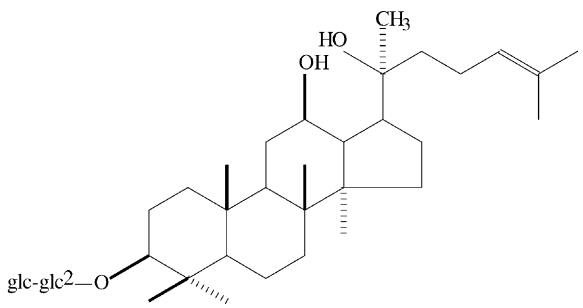

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Fig. 1. Chemical structure of ginsenoside Rg_3.

For *in vivo* studies, female BDF1 mice, 6–8 weeks of age, were obtained from Charles River Laboratories, and 1×10^6 cells of P388 or P388/DOX cells were implanted into the peritoneum of BDF1 mice and passed twice weekly.

2.3. Rhodamine 123 retention assay

A total of 2×10^5 cells of KBV20C or KB in 1 mL of culture media were inoculated in 24-well plates and incubated at 37° for 24 hr, and 1 mL of fresh media containing appropriate concentrations of Rg_3 or verapamil was added and incubated further at 37° for 20 min. Subsequently, 5 μg of rhodamine 123 was added, and the cells were further incubated at 37° for 30 min. The cells were then rinsed once with media to remove rhodamine 123 and incubated again in fresh media containing Rg_3, or verapamil at the indicated concentrations at 37° for 30 min. Cells were then removed from the wells after trypsin-EDTA treatment and collected by centrifugation (500 *g*). The cells were resuspended in phosphate buffered saline (PBS; NaCl 8 g, KCl 0.2 g, Na_2HPO_4 0.24 g, pH 7.4 per 1 L) and green fluorescence of rhodamine 123 was analyzed by flow cytometry with a Becton Dickinson FACScan at 488 nm and 300 mW. The median fluorescence was used as a quantitative measure of intracellular fluorochrome accumulation and used as an indicator for Pgp inhibition [24].

2.4. Plasma membrane preparation and photo-affinity labeling

Plasma membranes from KB or KBV20C cells and subsequent photo-labeling with [^{3}H]azidopine in the presence or absence of Rg_3 or verapamil were performed as described previously [25]. Briefly, the cells were incubated with Rg_3 at the indicated concentrations at 37° for 24 hr. Cells were harvested, washed and disrupted by homogenization with a glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min. To prepare a membrane-enriched fraction, the supernatant was overlayed on a 35% sucrose solution and centrifuged at 18,000 *g* for 60 min. The membrane fraction at the interface was centrifuged for another 60 min at 100,000 *g*. Pellets were resuspended in 10 mM Tris–HCl (pH 7.4), 125 mM sucrose, and stored at −70° until their use. The protein concentration was determined by the method of Bradford [26]. Plasma membranes (50 μg protein) were photo-labeled with 200 nM [^{3}H]azidopine in the absence or presence of the indicated modulator. Twenty-five microgram of photo-labeled membrane proteins were then analyzed using 7% sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS–PAGE; [27]). Subsequently, the gel was dried and exposed to a radioactive sensitive imaging plate for several days to obtain the images. The quantitative radiographic imaging data was analyzed by an image analysis system (Fujix Bio-imaging Analyzer BAAS2500).

2.5. Determination of MDR1 mRNA and Pgp level

Total RNA was isolated from KB and KBV20C cells by Trizol reagent (Gibco BRL) as suggested by the manufacturer. Total RNA was reverse transcribed in a reaction mixture containing random hexamers, each deoxynucleoside triphosphate (dNTP), and Moloney murine leukemia virus reverse transcriptase (Promega) as described by the standard methods [28]. The *MDR1* gene expression was determined [29] by reverse transcriptase-polymerase chain reaction (RT-PCR; *MDR1* forward primer, 5′-CCC ATC ATT GCA ATA GCA GG-3′; reverse primer, 5′-GTT CAA ACT TCT GCT CCT GA-3′) with β_2-microglobulin as internal control (forward primer, 5′-ACC CCC ACT GAA AAA GAT GA-3′; reverse primer, 5′-ATC TTC AAA CCT CCA TGA TG-3′).

For determination of Pgp level, membrane proteins (100 μg) separated on a 6% SDS–polyacrylamide gel were electroeluted onto polyvinylidine difluoride membrane (Millipore Immobilon P) and developed. The membranes were exposed to C494 anti-Pgp monoclonal antibody and then Pgp recognized by antibody was visualized using a horseradish peroxidase assay kit (BioRad). Visualized bands were photographed and intensity was quantified with densitometry.

2.6. In vitro drug sensitivity

Cells were maintained in drug free media 3 days before determination of the concentration capable of inhibiting 50% growth (IC_{50}). 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 different concentrations of anticancer drugs in the absence or presence of MDR modulator with 5% CO_2 at 37° for 72 hr. Subsequently, the Sulforhodamine B (SRB) cell staining method [30] was used to measure the cytotoxic effect. Optical density was read by ELISA reader (Titertek Multiskan MCC/340).

2.7. Drug accumulation

For the study of drug accumulation, KBV20C cells in drug free medium with 5% fetal bovine serum were plated (2×10^5 cells/well) in 24-well plates. The next day, the growth medium was replaced with 0.5 mL of fresh medium per well. After incubation at 37° for 10 min, the medium was removed and replaced with 0.5 mL of assay medium containing [^{3}H]vinblastine (0.1 μCi, 13 pmol) in the presence of either verapamil or Rg_3. After the indicated time, the cells were washed with ice-cold PBS twice, solubilized in 0.2% Triton X-100 in 10 mM phosphate buffer (pH 7.4), harvested, and then counted.

2.8. In vivo evaluation

Multidrug resistant 2×10^6 P388/DOX cells washed with serum-free medium twice were implanted by intraperitoneal injection into BDF1 female mice (10 mice). All drug treatments (16 mice for DOX only group, 12 mice for DOX + Rg_3 10 mg/kg group, 15 mice for DOX + Rg_3 40 mg/kg group) started on the same day as tumor implantation. DOX (stock concentration was 2 mg/mL in PBS) and Rg_3 (stock solution was 25 mg/mL in 10% dimethyl sulfoxide) were freshly prepared immediately prior to administration. Mice received drug of choice by intraperitoneal administration and continued to be treated every 3 days until death. The survival of mice in each group was examined daily. Mean body weights were recorded every 3 days.

2.9. Statistics

Data was expressed as the mean ± SD, and analyzed by the Student's *t*-test. *P*-values below 0.05 were regarded as statistically significant.

3. Results

3.1. Modulation of drug resistance

Since the 20(*S*)-ginsenoside Rg_3 enhanced cytotoxicity of anticancer drugs to multidrug resistant KBV20 cells in the presence of 20 nM VCR but not to the parental drug sensitive KB cells [22], we further examined Rg_3's efficacy on various drugs in MDR reversal activity. As shown in Table 1, 5 μM Rg_3 moderately reversed the resistance to COL, DOX, VCR and VP-16 in KBV20C cells. In the presence of 20 μM of Rg_3, the IC_{50}s of anticancer drugs such as COL, DOX, VCR and VP-16 to KBV20C cells were reduced about 30-, >126-, 8.5- and 2.1-fold, respectively. A higher concentration of Rg_3 (40 μM) reduced the IC_{50}s of COL, VCR and VP-16 to KBV20C cells 107-, 101-, and 5.9-fold, respectively. These results demonstrated that Rg_3 is an effective modulator in restoring the sensitivity of resistant cells to the various anticancer drugs at concentrations from 5 to 40 μM in human Pgp MDR cells.

3.2. Inhibition of rhodamine 123 efflux by Rg_3

Rhodamine 123 acts as a good substrate for MDR-associated Pgp [31] and agents that block Pgp have been found to increase the retention of rhodamine 123 in MDR cells [24]. Therefore, drug sensitive and resistant cells were

Table 1
Modulation of resistance to various chemotherapeutic agents in KB and KBV20C cells by Rg_3[a]

	VCR (nM)	DOX (nM)	VP-16 (μM)	COL (nM)
KB	3.2 ± 0.26 (1)[b]	59 ± 4.1 (1)	0.92 ± 0.14 (1)	7.0 ± 0.52 (1)
KBV20C	5210 ± 417 (1628)	>2586 (>43)	>300 (>326)	>1500 (>214)
KBV20C + Rg_3 (5 μM)	2806 ± 192 (876)	1920 ± 210 (32)	267 ± 20 (290)	951 ± 96 (135)
KBV20C + Rg_3 (20 μM)	612 ± 58 (191)	20 ± 1.6 (0.34)	143 ± 11 (155)	50 ± 7.1 (7.1)
KBV20C + Rg_3 (40 μM)	52 ± 4.1 (16)	ND	51 ± 3.2 (55)	14 ± 1.3 (2.0)

ND: not determined.
[a] IC_{50} values were determined by SRB assay in duplicate in two independent experiments and results were presented as means ± SD of three independent experiments.
[b] The numbers in parenthesis are the relative resistance of cells to the drug sensitive parental cells: IC_{50} of KBV20C/IC_{50} of KB.

exposed to rhodamine 123 at the various concentrations of verapamil or Rg_3 for 30 min and retention of rhodamine 123 was determined. Treatment of KB cells with Rg_3 did not affect retention of rhodamine 123, but treatment of KBV20C cells significantly increased the accumulation of rhodamine 123 in a dose-dependent manner (Fig. 2A). Rg_3 did not increase efflux of rhodamine 123 effectively at lower doses, however Rg_3 was more potent than verapamil in modulating MDR function at the higher concentrations than 200 μM of Rg_3, as reflected by higher rhodamine 123 retention in the resistant cells. Since incubation of 320 μM of Rg_3 for 30 min with KBV20C cells significantly increased efflux of rhodamine 123 without losing cells integrity in PBS buffer, subsequent time course experiment used 320 μM of Rg_3. The time course experiment revealed that Rg_3 increased rhodamine 123 retention in KBV20C cells 19-fold than that of the untreated cells after 15 min incubation of KBV20C cells with Rg_3. The maximum rhodamine 123 retention was attained after 30 min incubation of KBV20C cells with Rg_3 but thereafter retention of rhodamine 123 decreased slowly due to the instability of the cells in PBS buffer (Fig. 2B). These results clearly demonstrated that Rg_3 reversed MDR in KBV20C cells specifically and effectively in time- and dose-dependent manner.

3.3. Inhibition of [^{3}H]vinblastine efflux by Rg_3

To ascertain further that Rg_3 can enhance the accumulation of chemotherapeutic agents, KB or KBV20C cells

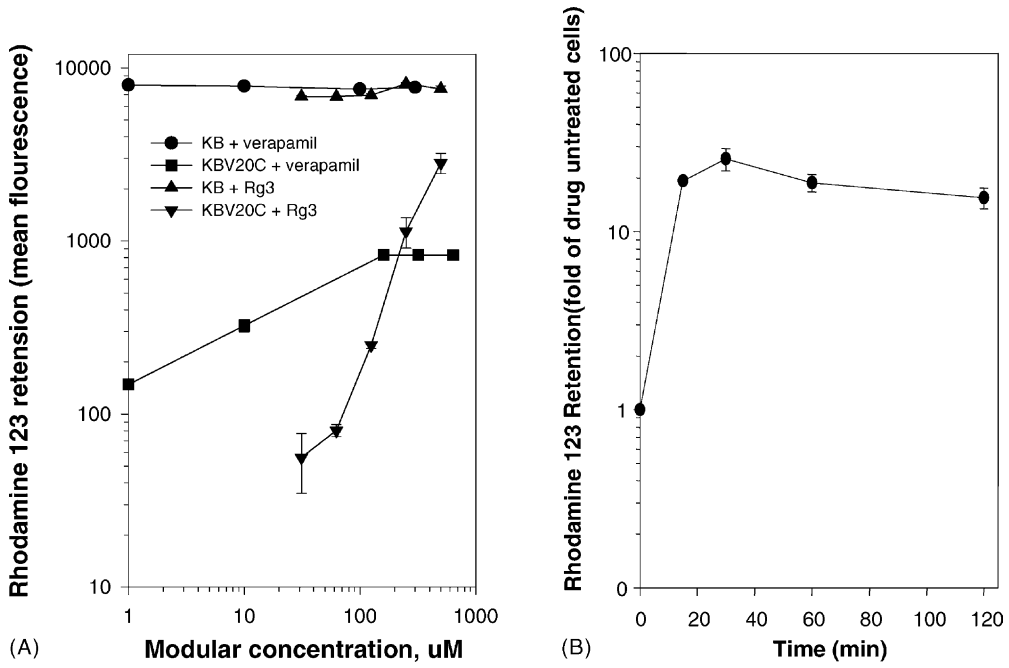

Fig. 2. Increase of rhodamine 123 retention by Rg_3. (A) Rhodamine 123 retention in resistant KBV20C but not in parent KB cells. KB or KBV20C cells were incubated at 37° for 24 hr, and were resuspended in fresh media containing rhodamine 123, and further incubated at 37° for 30 min. After washing, the cells were incubated again in fresh media containing Rg_3, or verapamil at the indicated concentrations at 37° for 30 min. Cells were then removed and re-suspended in PBS, and analyzed by flow cytometry. The median fluorescence was used as a quantitative measure of intracellular fluorochrome accumulation and hence an indicator for Pgp inhibition. The vertical bar represents mean ± SD of the two experiments in triplicate. If vertical bars are not apparent, the size of the SD was close to zero. The average fluorescence values in KB and KBV20C without Rg_3 or verapamil were 6777 and 44.5, respectively. (B) Time-dependent retention of rhodamine 123. KBV20C cells were incubated with 320 μM of Rg_3 for the indicated time and changes in fluorescence were monitored over time.

were incubated with [^{3}H]vinblastine for the indicated time in the presence of either verapamil or Rg_3, and the accumulated [^{3}H]vinblastine in KB or KBV20C cells was subsequently counted. In KB cells, Rg_3 or verapamil did not exhibit any difference from the group without any treatment in [^{3}H]vinblastine accumulation (data not shown). In KBV20C cells, 80 μM of Rg_3 had a marginal effect in reversing MDR, however at 320 μM [^{3}H]vinblastine accumulation was enhanced by approximately 4-fold after 30 min incubation at 37°. In comparison, verapamil at 100 μM augmented drug accumulation by 2-fold demonstrating that 320 μM of Rg_3 inhibited drug efflux more potently than 100 μM of verapamil (Fig. 3). This result confirmed that Rg_3 is an effective MDR modulator. Also potentiation of the accumulation of drug in MDR cells, but not in drug-sensitive cells, indicate that the MDR reversing effects of Rg_3 were not due to the toxicity of Rg_3 itself.

3.4. Effect of Rg_3 on the MDR1 mRNA and Pgp level

To determine Rg_3's MDR modulation is due to alteration of the *MDR1* mRNA or Pgp protein levels, KBV20C cells were treated with various concentrations of Rg_3 (40–320 μM) for 30 min, and total cellular RNA and plasma membrane were prepared. Subsequent determination of *MDR1* mRNA by RT-PCR revealed that the levels of *MDR1* mRNA in KBV20C cells in the presence or absence of Rg_3 did not exhibit any difference (data not shown). Visualization of Pgp protein by immunoblot analysis using C494 monoclonal antibody raised against Pgp revealed that Pgp levels in plasma membranes from KBV20C cells were not changed by Rg_3 treatment (data not shown), demonstrating that Rg_3 does not affect expression of *MDR1* gene nor Pgp protein level in KBV20C cells.

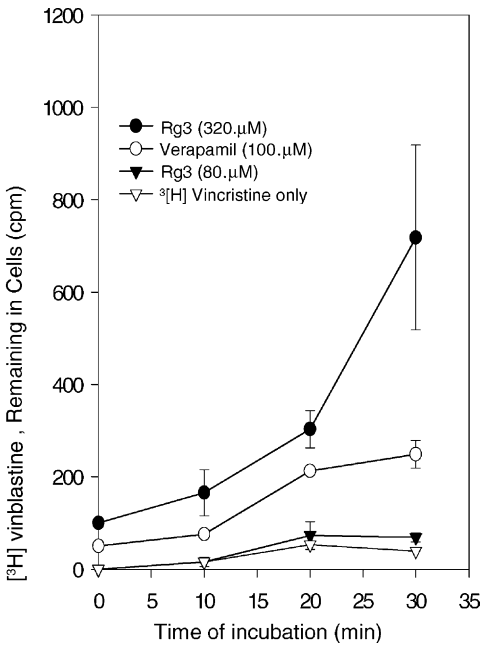

Fig. 3. Increase of [^{3}H]vinblastine accumulation by Rg_3. [^{3}H]vinblastine accumulation in KBV20C cells was determined after incubation with 26 μM [^{3}H]vinblastine and the appropriate modulator for the indicated time. After the indicated time, the cells were washed with ice-cold PBS twice, solubilized in 0.2% Triton X-100 in 10 mM phosphate buffer (pH 7.4), harvested, and then counted. The vertical bar represents mean ± SD of triplicate determinations.

3.5. Inhibition of [^{3}H]azidopine binding to Pgp by Rg_3

Since Rg_3 did not affect *MDR1* gene expression, MDR reversal by Rg_3 might be due to inhibition of drug binding to Pgp by Rg_3. To demonstrate competitive inhibition of drug binding by Rg_3, photo-affinity labeling of Pgp with [^{3}H]azidopine was used. Treatment of 100 μM of Rg_3 completely inhibited binding of [^{3}H]azidopine to Pgp demonstrating that Rg_3 competes with [^{3}H]azidopine for binding to Pgp (Fig. 4).

3.6. In vivo efficacy

For *in vivo* evaluation of Rg_3, a standard P388 murine leukemia model was used. In a preliminary experiment, DOX alone or a combination of a wide range of doses (2.5, 10, 40, 80 mg/kg) of the Rg_3 with 4 mg/kg of DOX was administered to mice (6 per group) implanted with P388/DOX tumors. The result demonstrated that administration of a combination of 2.5 mg/kg of Rg_3 and 4 mg/kg of DOX did not result in significant increase in life span of mice or mice weight, and administration of 80 mg/kg of Rg_3, by itself, did not produce any significant difference in life span compared with the vehicle control. In addition, combination treatment with 4 mg/kg DOX and 80 mg/kg Rg_3 produced no significant increase in life span of mice than

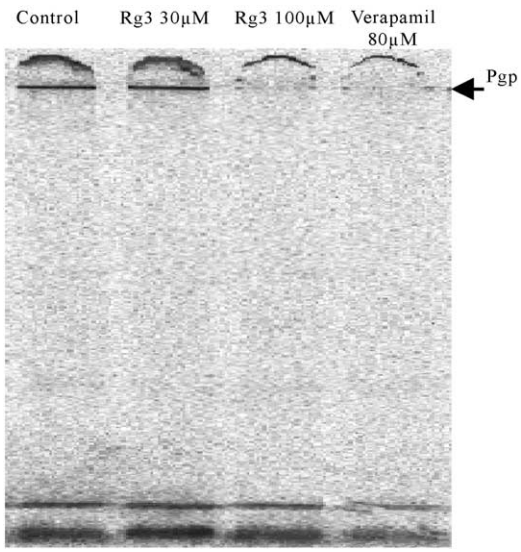

Fig. 4. Inhibition of [^{3}H]azidopine labeling of Pgp by Rg_3. To determine inhibition of [^{3}H]azidopine binding to Pgp, resistant KBV20C plasma membrane proteins were incubated with 0.2 μM [^{3}H]azidopine in the absence or presence of Rg_3 or verapamil at the indicated concentrations and cross-linked by UV irradiation. Photo-labeled membrane proteins were analyzed by SDS–PAGE and visualized by autoradiography.

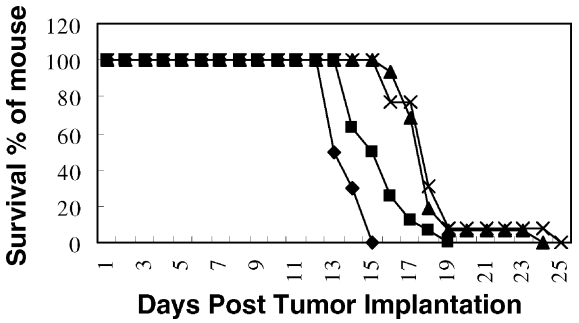

Fig. 5. Increase of the survival of mice implanted intraperitoneally into P388/DOX murine leukemia tumor cells by Rg_3 in combination with DOX. Multidrug resistant P388/DOX cells (2×10^6 cells/0.1 mL) were inoculated intraperitoneally into BDF1 female mice. Compounds were administered intraperitoneally at every 3 days after tumor implantation. The results represent the survival percentage vs. days after tumor implantation. Control (◆); DOX 4 mg/kg (■); DOX 4 mg/kg + Rg_3 10 mg/kg (▲); DOX 4 mg/kg + Rg_3 40 mg/kg (×).

combination treatment with 4 mg/kg DOX and 40 mg/kg Rg_3 (data not shown). Therefore, mice were treated with a combination of 4 mg/kg DOX plus 10 or 40 mg/kg Rg_3. When mice were treated with a combination of 4 mg/kg DOX plus 10 mg/kg of the Rg_3, a significant increase in life span ($P < 0.01$) was observed. Increasing the dose of Rg_3 upto 40 mg/kg did not extend life span any further (Fig. 5). In addition, increase of weight due to tumor growth was significantly suppressed in both groups treated with DOX plus two doses (10 or 40 mg/kg) of the Rg_3 compared with DOX alone (Fig. 6). On the basis of weight and mortality,

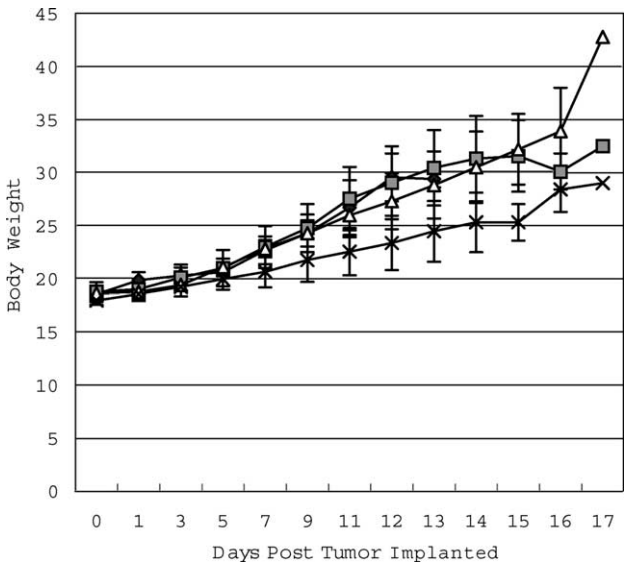

Fig. 6. Inhibition of body weight gain after intraperitoneal implantation with P388/DOX murine leukemia tumor cells by Rg_3 in combination with DOX. Mice were implanted with P388/DOX tumors and treated with Rg_3 every 3 days. Body weight gain was measured over time. The data is expressed as the mean ± SD. DOX 4 mg/kg + Rg_3 40 mg/kg treated group was significantly different from the group treated with DOX only at day 6 ($P < 0.05$) and 12 ($P < 0.001$), and the control group at day 12 ($P < 0.05$). Control (◆); DOX 4 mg/kg (■); DOX 4 mg/kg + Rg_3 10 mg/kg (△); DOX 4 mg/kg + Rg_3 40 mg/kg (×).

these results suggest that the increase of DOX cytotoxicity by Rg_3 *in vivo* is modulated through the interaction of the Rg_3 with Pgp.

4. Discussion

Our results indicate that Rg_3, one of the panaxadiol saponins present only in trace amounts, was a highly effective and potent modulating agent for MDR cells *in vitro* although it has no effect on either transcription or translation of *MDR1* gene. The rhodamine 123 retention (Fig. 2) and cytotoxicity studies (Table 1) showed that Rg_3 promotes drug accumulation and potentiates the toxicity of VCR in MDR cells, but not in a drug-sensitive cells. Furthermore, photo-affinity labeling of Pgp with [^{3}H]azidopine demonstrated that Rg_3 competitively inhibits binding of anticancer-drugs to Pgp (Fig. 4). Thus, the rhodamine 123 retention and inhibition of [^{3}H]vinblastine efflux by Rg_3 were at least partly due to a pronounced inhibition of Pgp function. Consistent with these data, coinjection of DOX and Rg_3 into mice carrying a multidrug resistant P388 transplantable tumor significantly increased the survival time of the mice compared with DOX treatment alone (Fig. 5) and decreased body weight gain by tumor growth (Fig. 6).

In vitro, a wide range of Rg_3 concentrations were used from 5 to 320 μM. To validate concentration effects of Rg_3, we compared the effect of 80 μM of Rg_3 on rhodamine 123 retention, [^{3}H]vinblastine accumulation, and [^{3}H]azidopine binding. Although 80 μM of Rg_3 increased rhodamine 123 accumulation almost 2-fold in 30 min in PBS buffer (Fig. 2), the same concentration of Rg_3 in RPMI culture media marginally increased [^{3}H]vinblastine accumulation in the same period (Fig. 3) suggesting that some components in the culture media, especially serum proteins, might interfere Rg_3's modulating effect temporarily. Nevertheless, 100 μM of Rg_3 was shown to almost completely inhibit binding of [^{3}H]azidopine to Pgp after 24 hr incubation of MDR cells in the same culture media (Fig. 4), eventually leading to cell death. Consistent with this, 72 hr incubation of MDR cells in 82 μM concentration of Rg_3 resulted in inhibition of growth of half the MDR cells [22]. Thus, at a specific concentration of Rg_3, time-dependent reversal of MDR was demonstrated. Furthermore, Rg_3 inhibited Pgp dose-dependently in both IC_{50} (Table 1) and rhodamne 123 accumulation (Fig. 2).

In contrast to dose- and time-dependent mode of Rg_3 results *in vitro*, *in vivo* results exhibited no dose–response relationship. Administration of 10 mg/kg of Rg_3 and 4 mg/kg of DOX at every 3 days increased life span almost the same as that of 40 or 80 mg/kg of Rg_3 and 4 mg/kg of DOX (Fig. 5) suggesting that at this dose Pgp may be saturated, or Rg_3's toxic effects might limit the modulating effect of Rg_3 on Pgp in murine MDR model. To compare cell culture experiment with *in vivo* result, pharmacokinetic data on Rg_3 is required. However, pharmacokinetics on a single ginsenoside component has only been determined with

Rg_1. Ginsenoside Rg_1 (molecular weight 801 Da) has the same steroidal skeleton as Rg_3 except for a sugar moiety at carbon number 6 and 20. Rg_1 was rapidly absorbed, and the highest serum concentration (0.9 μg/mL serum) was attained 30 min after oral administration of 100 mg/kg into rat. The level of Rg_1 dropped off thereafter, and was not detectable 6 hr after oral administration, although the sensitivity of Rg_1 detection was relatively low (0.2 μg Rg_1/mL serum) [32]. Furthermore, Rg_1 was excreted via urine, bile and feces without undergoing significant metabolization in the liver. In the case of intravenous administration of Rg_1, the level of Rg_1 in rat serum declined more rapidly with a half life of 6.3 min, and was not detected after 1 hr [32]. If Rg_3 is assumed to have same pharmacokinetics as Rg_1, Rg_3 would be rapidly excreted after several hours of Rg_3 administration and its plasma level would be very low. Nevertheless, injection of Rg_3 every 3 days produced significant life span extension demonstrating that low level of Rg_3 might be enough to inhibit Pgp *in vivo*. Furthermore, the effect of Rg_3 on rhodamine 123 uptake *in vitro* is very clearly competitive with a Hill number far higher than unity. In contrast, verapamil has a far smaller Hill number, indicating the possibility that Rg_3 could bind to Pgp with high affinity to inhibit anticancer drug efflux. Further study will be needed to clarify the exact mechanism of binding of Rg_3 with both high affinity and stability to Pgp.

Ginsenoside Rg_3 possesses a four trans-ring rigid steroid skeleton with a modified glucopyranoside chain at carbon number 3 [33]. Structural similarity of Rg_3 to cholesterol and steroids prompted us to compare the action mechanism of Rg_3 to that of cholesterol and steroids. Steroid hormones are lipophilic and may intercalate into the bilayer of target cell plasma membranes, potentially altering the fluidity and function of the membrane. However, the steroids do not always affect phospholipid fluidity; progesterone was found to decrease the lipid fluidity, and testosterone had no effect on lipid movement, whereas estrogen, 17 β-estradiol, increased lipid mobility [34]. Progesterone and deoxycorticosterone, but not estradiol, caused an increase in [^{3}H]vinblastine accumulation [35]. Also progesterone caused a rapid stimulation of efflux of rhodamine 123 from KBV20C cells but did not stimulate efflux from drug-sensitive KB cells, indicating that rhodamine 123 is removed from KBV20C cells by the MDR pump [36]. Since Rg_3 was shown to modulate the fluidity of the plasma membrane [37], it seemed to have similar mode of action to that of progesterone although it is more closely related to cholesterol than progesterone in chemical structure.

Ginseng has been known to have diverse pharmacological effects. In addition to inducing MDR reversal, ginsenoside Rg_3 inhibits both cell growth in human prostate carcinoma cells [38] and metastasis of murine intestinal adenocarcinomas [39] besides from MDR reversal. For the modulation of receptors and channels, it also significantly inhibits the secretion of catecholamines from bovine adrenal chromaffin cells stimulated by acetylcholine [37,40], and modulates Ca^{2+} channel currents in rat sensory neurons [41]. Rg_3 has also been shown to be one of the major active components for inhibiting glutamate-induced neuronal cell death and Ca^{2+} influx through glutamate receptors [42] and inhibits vascular smooth muscle tone via activation of K^+ channels [43]. These properties of Rg_3 would help to understand pharmacological effects of ginseng.

Taken together, the present results demonstrate for the first time that a ginseng component, Rg_3, can specifically inhibit Pgp-mediated drug accumulation in KBV20C, but not in drug-sensitive cells. Thus, Rg_3 could be a highly feasible candidate for the development of MDR modulators with higher potency and specificity.

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