Ginsenosides as Reversal Agents to Overcome Cross-Resistance to Anticancer Agents

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Background

Chemotherapeutics are the most effective and indispensable treatment for metastatic tumors. Unfortunately, about 50% of cancer cases do not respond to anticancer drugs in clinical practice duo to a phenomenon known as chemoresistance. To improve chemotherapy, the appearance of chemoresistance and invasion/metastasis, which are two representative malignant characteristics, should be overcome. There are two general classes of resistance to anticancer agents: those that arise from the body which impair delivery of anticancer agents to tumor cells, and those that arise in the cancer cell itself due to genetic and epigenetic alterations that affect drug sensitivity. Chemoresistance in cellular level --- a cross-resistance to structurally and functionally diverse chemotherapeutic agents is likely to be circumvented as experimental models can be easily generated by *in vitro* selection with cytotoxic agents. Many laboratories have developed cell lines *in vitro* to overcome cellular cross-resistance to drug candidates in anticancer drug discovery.

Ginseng has been used for traditional medicine in China, Korea, Japan and other Asian countries for the treatment of various diseases, including psychiatric and neuralgic diseases, as well as cancer (1-4). Ginsenosides (ginseng saponins) have been known as major efficacious components in ginseng (5). The naturally occurring ginsenosides, such as Rb1, Rb2, and Rc, have been reported to have anti-tumor effects, particularly on the inhibition of tumor-induced angiogenesis, tumor invasion and metastasis, and the control of phenotypic expression and differentiation of tumor cells by structurally transforming forms. Those ginsenosides, such as Rg3, Rh2, Rk1, Rg5, and compound K are minor or not naturally existed and might be obtained from natural-relatively abundant ginsenosides by transformation of acid, alkali hydrolysis or

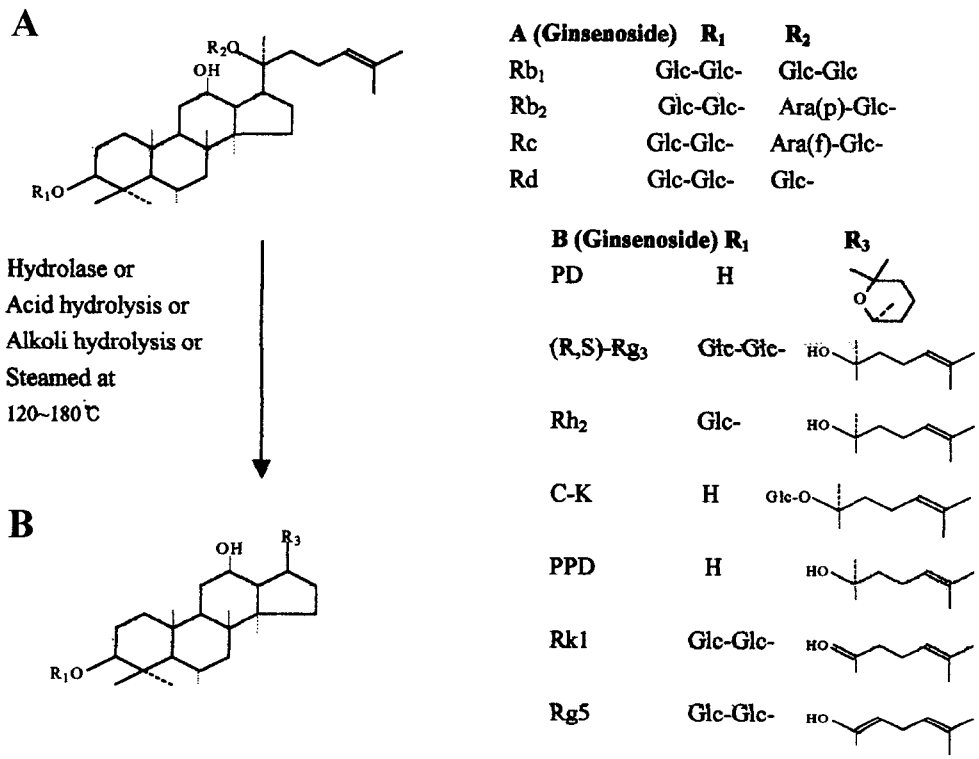

Fig. 1. Biological and Chemical Transformation of Ginsenosides.

various hydrolases (Fig 1). In this study, it was of interest to determine the effect of ginsenosides as MDR reversal agents and compare their structures with activities.

Abbreviation

C-K: Ginsenoside Compound K; PDG: Panaxadiol-type ginsenosides; Ppd: Protopanaxadiol; PT: Panaxatriol;

TGS: Total ginseng saponin;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoline bromide;

MDR: multidrug resistance; IC50: drug concentration that inhibits cell growth by 50%.

Experimental Procedures

Preparation of Ginsenosides

Naturally occurring ginsenosides were prepared from Ginseng roots power and Notoginseng saponin. The others were transformed as described in Fig 1. All ginsenoside were analyzed by HPTLC, HPLC and LC-MS.

Cell line and culture condition

Human adenocarcinoma cells MCF-7, human erythroleukemia cells K562 and their adriamycin-derived resistant cell lines MCF-7/ADM, and K562/ADM were cultured in RPMI1640 (SIGMA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Medium for resistant cell lines was further supplemented with 1.0 μg/ml ADM (PHARMACIA). Cells were continuously kept in logarithmic growth at 37°C in a humidified atmosphere of 5% CO_2. Before experiments, resistant cells were cultured in ADMfree medium for 2 weeks.

MTT assay for cytotoxic activity

Cells in log-phage, treated with 0.25% trypsin/EDTA, were collected and counted. Cell lines were seeded into 96-well plates at 2×10^4 viable cells per well with medium containing or lacking ADM or tested agents. After 48h incubation, additional medium containing 5.0 mg/ml MTT (AMRESCO) was given to each well in a volume of 10 μl and incubated for 4h. After centrifugation, the medium was then removed and 100 μl of dimethyl sulphoxide was added in each well for 15 min. The A_{570} value was determined in a 96-well microtitre plate reader (TECAN, AUSTRIA).

Data analysis

Inhibitory Rate (IR) Survival Rate (SR, %)

↓ $= (OD_t - OD_b) / (OD_c - OD_b) \times 100$

$IR (\%) = 100 - SR$

IC50 calculation *Bliss* method

↓

Statistical Analysis of IC50 and the curve slope Student's *t* test. $P<0.05$ was considered as statistically significance

↓

Curves of Inhibited Rate in Comparison EUKELIAN formula: $\Delta Y = [\Sigma (Y - Y_i)^2]^{1/2} / n$

↓

Calculation of Specific Factor (SF) or Resistant Index (RI) or Reversal Factor (RF) SF or RF or RI (%) = $\Delta Y_t / \Delta Y_b \times 100\%$ (ΔY_t states that value between reversal curve and resistant curve; ΔY_b states that value between sensitive and resistant curves)

Intracellular accumulation of ADM

K562/A cells (2×10^5/ml) were suspended in the medium containing ADM with or without panaxadiol (4 mg/ml), ginsenoside C-K (2 mg/ml). After 5h incubation, cells were centrifuged and washed with 0.1M PBS (4°C, pH 7.2), and then dissolved in 0.3N HCl 50% ethanol. The intracellular concentration of ADM was detected by the fluorescence spectrophotometer (TOSHIBA FL2500, JAPAN).

Results

1. MCF-7/R and K562/R cell line in this study were characterized by evaluating MDR's spectrum. Five known drugs are assayed and their IC_{50} against MCF-7 cell line pair (MCF-7/R and MCF-7/S) and K562 cell line pair (K562/S and K562/R) are respectively listed in Table 1. The cell exposed to adriamycin occur cross-resistance to all five drugs at different degree from high to low: in MCF-7 pair and VCR > ADR > VP16 > Cis-Pt > 5-FU in K562 pair.

2. In this study (see Table 2 and 3), RI of the sensitive cells (MCF-7/S, K562/S) was described

Table 1. Resistant spectrum of MCF-7 cell line and K562/ADM cell line

Drug	IC50 (uM) (MCF-7/A)	IC50 (uM) (MCF-7/S)	IC50 (uM) (K562/A)	IC50 (uM) (K562/S)
Adriamycin	42.3±6.8	1.2±0.2	118.1±22.3	2.3±0.8
Vincristine	403.2±67.9	17.4±2.4	579.24±38.3	3.8±0.6
Etopside	281±22.6	42.6±9.6	101.3±56.7	15.5±3.8
Cisplatin	38.7±8.9	8.63±1.5	43.9±12.4	18.6±6.6
5-Fluorouracil	>4000	573.5±80.2	273.3±78.9	281.4±69.5

Table 2. CV of RI value in cell line

Group	RI (%)	CV (%)
MCF-7/S	100	8.0
MCF-7/A + Tamoxifen	61.0	8.8
MCF-7/A + Verapamil	48.2	5.3
K562/S	100	5.9
K562/A + Tamoxifen	45.4	3.0
K562/A + Tamoxifen	57.3	5.8

(n = 6)

Table 3. CV of ADM IC_{50} value in cell lines

Group	CV of IC_{50} (%)
MCF-7	12.9
MCF-7/A	21.9
MCF-7/A + Tamoxifen	23.1
MCF-7/A + Verapamil	9.3
K562	15.3
K562/A	11.2
K562/A + Tamoxifen	20.0
K562/A + Verapamil	28.7

(n = 6)

as 100. Compared with the control, RI of tamoxifen and verapamil in MCF-7/A cells were 61.0, 48.2 respectively. RI of tamoxifen and verapamil in K562/A cells were 45.4, 57.3 respectively. The coefficients of variation (CV) of RI in each group was within 3.0~8.8. By contrast, CV of IC_{50} was within 9.3~28.7, which was much greater than that of RI. The above results indicate that RI calculation is an analytic way better than comparison with IC50 value for the resistance evaluation.

3. RI demonstrates the cytotoxic difference of assayed agents against MCF-7/S and MCF-7/R. SF=0 indicates no statistically significant difference between agent's cytotoxicity on MCF-7/S and MCF-7/R. As indicated in Table 4, 15 compounds are assayed and RI was calculated

Table 4. Cross resistance of doxorubicin- resistant MCF-7 cell line to ginsenosides

Compounds	RI*	Compounds	RI*
Panaxadiol	0	Rg5	0
Ppd	0	Rd	5.09
C-K	0	Rb1	5.64
Rh2	6.73	PT	6.89
Rk1	5.08	PDG	0
(R)Rg3	10.7	TGS	0
(S)Rg3	7.75	Tamoxifen	0
20-keto-Rg5	0		

Table 5. Comparison in cytostatic effect of ginsenosides on MCF-7 and HBL100 cell line

Compounds	IC_{50} (uM) (MCF-7)	Specific Factor	Compounds	IC_{50} (uM) (MCF-7)	Specific factor
Panaxadiol	203(±24)	0	Rg5	104(±11)	0
Ppd	420(±82)	0	Rd	325(±41)	0
C-K	121(±17)	0	Rb1	>500	0
Rh2	265(±25)	0	PT	312(±9)	8.72
Rk1	85(±8)	0	PDG	113(±22)	5.44
(R)Rg3	126(±31)	0	TGS	258(±57)	0
(S)Rg3	71 (±8)	0	Tamoxifen	54(±7)	0

respectively. Cross resistance of doxorubicin-resistance MCF-7 cell line occur to ginsenoside Rk1, Rd, Rb1, Rh2, PT, (S) Rg3, and (R) Rg3 as ordered from low to high according to RI.

4. Specific Factor (SF) demonstrates the cytotoxic difference of the assayed agent against MCF-7 and normal breast cell (HBL100). SF=0 indicates no statistically significant difference between agent's cytotoxicity on MCF-7/S and MCF-7/R. demonstrates the agent's cytotoxic difference between MCF-7 and the normal cell. As indicated in Table 5, 15 compounds are assayed and IC50 of Rk1 and (S) Rg3 are 85(±8) and 71 (±8) respectively. Ginsenosides, PDG and PT, are ordered from low to high according to SF, showing a statistical significance.

5. As indicated in Table 6, 15 compounds are assayed and 9 of them show different reversal

Table 6. Reversal effect of ginsenosides on doxorubicin resistance in MCF-7/R cell line

Compounds	IC_{50} (uM) (MCF-7)	RF* (%)	Compound	IC_{50} (uM) (MCF-7)	RF* (%)
Panaxadiol	13	15	Rg5	10	0
Ppd	20	0	Rd	32	12
C-K	10	20	Rb1	27	10
Rh2	16	0	PT	20	20
Rk1	13	20	PDG	10 (ug/ml)	0
(R)Rg3	10	15	TGS	10 (ug/ml)	0
(S)Rg3	5.1	14	Tamoxifen	5.4	64

*RF : Reversal factors(See experimental procedures)

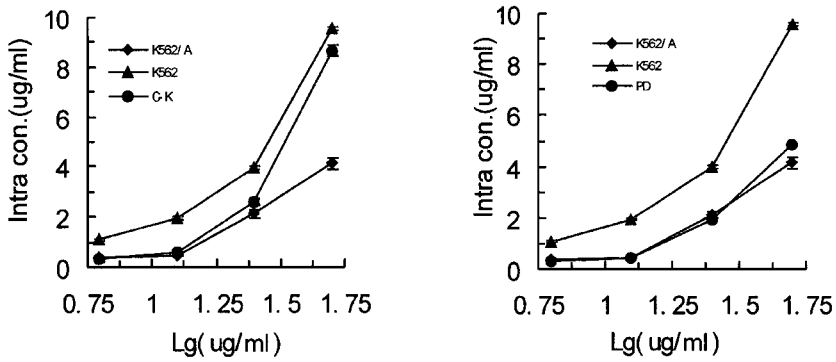

Fig. 2. Intracellular Accumulation of Adriamycin in K562/R Influenced by Ginsenosides.

activity against MCF-7/R cell line. According to RF, C-K, PT, and Rk1 are three compounds show higher activities against MDR.

6. To know what kinds of ginsenosides with reversal mechanism, comparison of C-K and panaxadiol in effects on intracellular accumulation of ADM into K562/R was observed. Figure 2 showed us that C-K could enhance the intracellular ADM concentration significantly. On contrast, panaxadiol showed little effect on it. The result indicated that panaxadiol and ginsenoside C-K might have different mechanism in reversing drug resistance.

Conclusion

1. Ginsenosides and their derivatives reverse multidrug-resistance (MDR) of tumor cells in vitro.
2. Ginsenosides as MDR reversal agents are classified into two classes: resistance-dependent and resistance-independent.
3. According to intracellular accumulation of adriamycin, ginsenosides as MDR reversal agents are also classified into two classes: intracellular uptake-dependent and uptake-independent.

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