

Reversion of P-Glycoprotein-Mediated Multidrug Resistance in Human Leukemic Cell Line by Carnosic Acid

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Abstract

One of the common hindrances to successful chemotherapy is the development of multidrug resistance (MDR) by tumor cells to multiple chemotherapeutic agents. In this regard, P-glycoprotein (P-gp) acts as an energized drug pump that reduces the intracellular concentration of drugs, even of structurally unrelated ones. The modulators of P-gp function can restore the sensitivity of MDR cells to anticancer drugs. Therefore, to develop effective drug-resistance-reversing agents, we evaluated the P-gp modulating potential of carnosic acid (CA) in multidrug-resistant K562/AO2 cells in the present study. The reversing effect of CA was evaluated by determining the inhibition rates of cell viability with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assays. The intracellular adriamycin fluorescence intensity and the expression of P-gp were measured by flow cytometry (FCM). Meanwhile, the subcellular distribution of adriamycin was detected *via* Laser Scanning Confocal Microscopy (LSCM). The mRNA expression of *mdr1* was then detected *via* semiquantitative reverse transcription polymerase chain reaction (RT-PCR). The findings showed that CA decreased apparently the Inhibition Concentration 50% (IC₅₀) of adriamycin by increasing its intracellular concentration and thus enhancing the sensitivity of K562/AO2 cells. Adriamycin was distributed evenly in the cytoplasm when the cells were treated with CA. The expression of *mdr1* was decreased. Overall, the results indicated that CA can serve as a novel, non-toxic modulator of MDR, and it can reverse the MDR of K562/AO2 cells *in vitro* by increasing intracellular adriamycin concentration, down-regulating the expression of *mdr1*, and inhibiting the function of P-gp.

Key Words: carnosic acid, multidrug resistance, P-glycoprotein, reverse

Introduction

The most common form of multidrug-resistance (MDR) is the result of over-production in the cancer cell membrane of P-glycoprotein (P-gp), a protein that pumps the anti-cancer drug out of the cell. The MDR phenotype mediated by P-gp results in chemotherapy

failure in acute leukemia (35). In relation to this, the number of research had shown that P-gp, the product of the *mdr1* gene, plays a very significant role in MDR. It is an ideal way to identify novel compounds that may specifically reverse P-gp-mediated MDR in cancer cells. To overcome MDR, enormous efforts have been exerted to find an inhibitor of the drug-efflux pump,

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and various compounds such as verapamil, cyclosporin, quinidine, tamoxifen, progesterone, reserpine, and others have been reported to overcome MDR *in vitro* (15). Significant improvement has been made since the discovery that first-generation inhibitors, originally designed for targets other than P-gp, had unacceptable toxicities in clinical studies. More recently, designs of molecules for the specific interaction with P-gp, including Tariquidar (XR9576) (27, 30) and LY335979 (28), have advanced in clinical applications. Although hundreds of compounds have been found to be able to modulate the MDR phenotype, their clinical application was limited due to their unacceptable side effects or toxicity at the doses required for effectiveness (8). The key to the clinical use of reversal agents therefore lies in searching for agents with low toxicity and high reversal activity. Many researchers have been working on screening natural product drugs to reverse MDR. Particularly, there are many plant-derived drugs or herbal formulations, such as gomisin A and Schisandrol A, which were identified from Chinese herbs (*Schisandra Chinensis*) and which might reverse P-gp-MDR *in vitro* (7, 32). These findings provide evidence for the identification of plant-derived reversal agents, which have been receiving increasing scientific attention recently.

Carnosic acid (CA) is an antioxidant phenolic diterpenes and is the major component of *Rosmarinus officinalis* L (20). It has many pharmacological actions such as antioxidation, anti-inflammatory (24), antiplatelet (17), and anti-tumor properties (25, 31). Recent studies show that CA-combining 1,25(OH)₂D₃ or all-trans-retinoic acid (ATRA) can promote monocytic differentiation of human leukemia cells (4, 5, 29, 33). In our previous study, we also identified CA-enhanced differentiation and apoptosis effects of ATRA or Arsenic Trioxide (18). It is very interesting to note that CA can enhance the activity of antibiotics against the resistant efflux strains of *Staphylococcus aureus* (26).

To test the hypothesis that CA can reverse MDR for human leukemic cells, we conducted this study.

Materials and Methods

Drugs and Reagents

CA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). We dissolved CA in dehydrated alcohol, with the stock solution concentration at 1 g/l. Meanwhile, adriamycin was obtained from ZheJiang Hisun Chemical Co. (Zhejiang, PRC). We dissolved it into 0.9% Sodium Chloride solution, and the final concentration was 1 g/l. Verapamil was purchased from ShangHai Harvest Pharmaceutical Co. Ltd. (Shanghai, PRC). Finally, the primers were synthesized by Shanghai Sangon Biological Engineering & Tech-

nology and Service Co. Ltd. All drugs were stored at -20°C.

Cell Lines and Cell Culture

The human myelogenous leukemia K562 and K562/AO2 cell lines were provided by Professor Ling Zhang, the Institute of Basic Medicine of Shandong Academy of Medical Sciences, PRC. They were both cultured in RPMI1640 medium (Gibco, Los Angeles, CA, USA) supplemented with 10% (v/v) heat-inactivated new-born calf serum (HangZhou Sijiqing Biological Engineering Materials Co. Ltd. Hongzhou, People's Republic of China). Furthermore, both cell lines were grown in a humidified incubator at 37°C and 5% CO₂. In particular, the K562/AO2 cell line was maintained in a 1 µg/ml adriamycin-containing medium and was incubated in adriamycin-free medium for 2 weeks before the experiments.

Assay of In Vitro Drug Sensitivity

K562/AO2 cells were seeded in 24-well plates at a density of 1×10^5 cells/well. After treatment with CA for different periods (24 h, 48 h, 72 h) with different concentrations (0-50 µM), the viability of the cells was determined by trypan blue exclusion. Cell suspension (0.9 ml) was mixed with the 0.4% trypan blue solution (0.1 ml) and cells were counted using light microscopy. Cell Viability (%) = Total number of viable cells / Total number of viable and nonviable cells \times 100%. The experiments were repeated in triplicate.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (22) was used to compare the multidrug resistance to Adriamycin of K562 and K562/AO2 cells. Briefly, K562 and K562/AO2 cells were grown on a 96-well plate at 1×10^5 cells/ml in a complete RPMI 1640 medium. Adriamycin was added at various different drug concentrations. K562 cells and K562/AO2 cells without drugs in medium were used as the blank controls. The experimental group was treated with CA at a concentration of 25 µM, and verapamil (5 mg/l) treatment served as positive control. After the drug treatment for 44 h at 37°C, 20 µl MTT was added into each well, and further incubated for 4 h. Then the medium was removed, and 150 µl dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The absorbance value was measured with a spectrophotometer at the wavelength of 570 nm. IC₅₀ to the drugs was calculated on the basis of the MTT assay.

To determine whether CA can sensitize MDR cells to the cytotoxicity of adriamycin, K562/AO2 cells were seeded into 96-well culture plates at a density of 1×10^5 cells/ml. Adriamycin was added with varying concentrations of CA in a final volume

of 200 μ l per well. K562/AO2 cells with adriamycin and verapamil were used as the positive control. The cells were analyzed using the MTT method. The inhibition rate of cell viability was calculated using the following formula: Inhibition of cell viability = $(1 - \text{average A value of experimental group} / \text{average A value of control group}) \times 100\%$.

Detection of Intracellular Adriamycin Concentration by FCM and LSCM.

K562/AO2 cells were treated with 25 μ M CA and 5 mg/l verapamil for 48 h (11). Then adriamycin was added to each sample to a final concentration of 5 mg/l. After coincubating for a further 2 h (3), the cells which were harvested by centrifugation were washed twice with ice-cold phosphate buffered solution (PBS), and were then resuspended in PBS. Adriamycin was excited effectively at a single wavelength (488 nm), and the emitted light was collected in the fluorescence-3 (FL3) channel. Events were gated on FSC vs. SCC dot plot to exclude the influences of cell debris and aggregates. A total of 10,000 gated cells were detected for each sample, which were analyzed by Modfit LT software.

Adriamycin was dissolved in PBS to different concentrations (Y mg/l), and then its fluorescence intensity (X) was detected by LSCM. A linear correlation was obtained between the concentration of adriamycin and its fluorescence intensity (2). Intracellular adriamycin concentration could be obtained using the linear regression equation. K562 and K562/AO2 cells were treated with CA for 48 h, and then they were co-cultured with adriamycin with a final concentration of 5 mg/l for 2 h (15, 36). Intracellular adriamycin fluorescence intensity was detected under LSCM. The excitation wavelength was 488 nm, and the receiving wavelength was 575 nm.

Subcellular Distribution of Adriamycin

K562 and K562/AO2 cells were treated with CA for 48 h, and then they were cocultured with adriamycin for two more hours, washed twice by PBS, and then resuspended in PBS. Subcellular distribution was detected under LSCM. The excitation wavelength was 488 nm, and the receiving wavelength was 575 nm.

Expression of P-gp by FCM

The expression of P-gp on the membrane surface of K562/AO2 cells was determined by a direct immunofluorescence staining technique. K562/AO2 cells were cocultured with CA for 48 h. They were washed twice with PBS and then were suspended in PBS. The cell suspension was incubated with

phycoerythrin-conjugated UIC2, a mouse antihuman P-gp monoclonal antibody (P-gp-PE), and the homotype control IgG2a-PE, was reacted at room temperature and kept away from light for 30 min, washed twice, and then detected by FCM. The specific P-gp antibody UIC2 was detected in the FL2 channel, and thus P-gp expression can be assessed on the cell surface. The expression of protein was analyzed using Cell Quest software.

Semiquantitative RT-PCR Assay

In vitro total mRNA was extracted from the cells with Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by reverse transcription from 1 μ g of the total RNA using Reverse Transcriptase RNase M-MLV (Invitrogen Co., Carlsbad, CA, USA) and oligo-dT. The amplification was performed in a final volume of 50 μ l, containing 5 μ l cDNA, 0.5 μ l of each oligonucleotide primer, 1 μ l of each dNTP, and 1 unit of Taq DNA polymerase. Amplification was carried out in a Thermal Cycler. The PCR primers and expected product size were as follows: mdrl, forward: 5'-cccatcattgcaatagcagg -3' and reverse: 3'-actctctgtcttcaaaacttg- 5' (157 bp); β -actin, forward: 5'-gtggggcgccccaggcacca-3 reverse: 5'-ctccttaatgtcacgcacgatttc-3' (539bp). Circulating condition: 94°C one min, 58°C one min, 72°C one min, 30 cycles, then 72°C prolong 7 min. The end products were identified by electrophoresis using 1.5% agarose gel.

Statistical Analyses

Statistical calculations were carried out with SPSS 13.0 for Windows software package. The results are expressed as means \pm standard deviation of three independent experiments. Student's *t*-test was used for the statistical analyses, and *P* values < 0.05 were considered to be significant.

Results

Drug Sensitivity

The trypan blue exclusion test showed that more than 90% of CA-treated K562/AO2 cells was alive at each experiment. The results indicated that CA had no toxic effect on K562/AO2 cells. The IC₁₀, the highest non-proliferate concentration of CA, was used in all further experiments. We determined the reversal effect of CA at the concentration not affecting cell proliferation.

The MTT assay was used to study the cytotoxicity of adriamycin, and the ability of CA at 25 μ M to en-

Table 1. IC₅₀ of adriamycin in K562 and K562/AO2 cells

Cell Line	Adriamycin IC ₅₀ (mg/l)	Drug Resistance (fold)	Reverse Fold
K562	0.4 ± 0.1		
K562/AO2	16.3 ± 1.6	40.8 ^a	
K562/AO2+CA	1.4 ± 0.2		12.1 ^b
K562/AO2+verapamil	0.9 ± 0.0		19.0 ^c

The IC₅₀ of adriamycin in K562 cells and K562/AO2 cells was 0.4 mg/l and 16.3 mg/l, respectively, indicating a resistance of 40.8-fold. After treatment with CA or verapamil, the IC₅₀ of K562/AO2 cells decreased to 1.4 mg/l and 0.9 mg/l. The reverse multiples were 12.1, 19.0. ^a*P* < 0.01 vs. K562; ^b*P* < 0.01, ^c*P* < 0.01 vs. K562/AO2

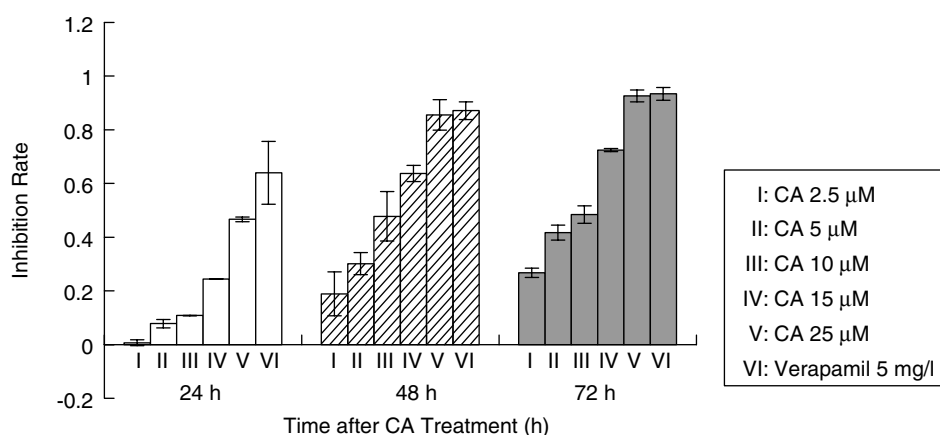


Fig. 1. The inhibition rates of cell viability of treated K562/AO2 cells at different time periods. K562/AO2 cells were treated with adriamycin for varying time intervals in the presence of 2.5–25 μM CA, and cytotoxicity was determined by the MTT assay. The inhibition rates of CA-treated K562/AO2 cells showed that they were both concentration-dependent and time-dependent. This indicated that when the concentration ranged from 2.5 to 25 μM, the reversal effect became obvious.

hance the cytotoxicity of adriamycin in K562/AO2 was examined. The results are summarized in Table 1. The IC₅₀ value of adriamycin for K562/AO2 decreased after treatment with CA. There were likewise significant differences between the CA-treated groups and untreated groups (*P* < 0.01). The time- and concentration-dependent reversal effect of CA was observed on K562/AO2 cells for 24, 48, and 72 h. The data of the three experiments are shown in Fig. 1. The higher the concentration, the better the effect.

Detection of Intracellular Adriamycin Concentration

The effect of CA on the intracellular accumulation of adriamycin was examined by FCM. The intracellular concentration of adriamycin in K562/AO2 cells was reduced as compared to that in K562 cells. The fluorescence intensity of adriamycin in K562 cells was 27.5, while it was 17.1 in K562/AO2 cells. After treatment with CA or verapamil, adriamycin fluorescence intensity increased to 60.5 and 46.2, respectively. Therefore, there was a significant difference between K562/AO2

cells and treated K562/AO2 cells (*P* < 0.05, Fig. 2).

We measured the fluorescence intensity of adriamycin in K562/AO2 cells *via* LSCM, and the results are shown in Table 2. After treatment with CA or verapamil, the fluorescence intensity of adriamycin increased from 732.5 to 1968.2 and 1440.0, respectively. With different concentrations of adriamycin (*Y* mg/l) and their fluorescence intensity (*X*) detected by LSCM, the following linear regression equation was established: $Y = 8.5 \times 10^{-3}X - 1.3$. The *X*-axis represents the fluorescence emission intensity of adriamycin in arbitrary units, while the *Y* axis represents the concentration of adriamycin. With this formula, we could calculate the intracellular concentration of adriamycin as shown in Table 2. After treatment with CA or verapamil, the concentration of adriamycin increased (*P* < 0.05).

Alteration of the Subcellular Distribution of Adriamycin

In K562 cells, adriamycin fluorescence was mainly located in the nucleus and was diffusely present in the

Table 2. Concentration of adriamycin in K562/AO2 cells

Groups	Fluorescence Intensity of Adriamycin (Mean \pm Standard Deviation)	Corresponding Concentration of Adriamycin (mg/l)
K562/AO2	732.5 \pm 483.6	4.9
K562/AO2+CA	1968.2 \pm 484.6 ^a	15.3
K562/AO2+verapamil	1440.0 \pm 383.9 ^b	10.9

The intracellular adriamycin concentration of K562/AO2 cells was 4.9 mg/l. After treatment with CA or verapamil, the concentration was increased to 15.3 mg/l, 10.9 mg/l.

^a $P < 0.05$, ^b $P < 0.05$ vs. K562/AO2

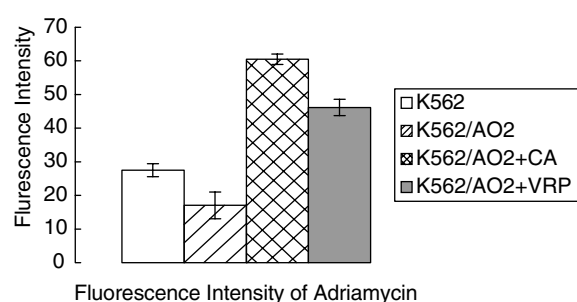


Fig. 2. The fluorescence intensity of adriamycin. K562/AO2 cells were treated with CA for 48 h, then adriamycin was added and coincubated for a further 2 h. The mean adriamycin fluorescence intensity in K562/AO2 cells was increased 3.55 times that of the untreated K562/AO2 cells.

cytoplasm, whereas in K562/AO2 cells, it was distributed primarily in the perinuclear and perimembrane regions and was lacking in cytoplasm. After treatment with CA, the subcellular distribution of adriamycin in K562/AO2 cells was similar to that in K562 cells with an even distribution of intracellular adriamycin in the nucleus and cytoplasm. Intracellular adriamycin aggregation increased significantly (Fig. 3).

Alteration of P-gp Expression

The expression of P-gp in K562 cells was lower than that in K562/AO2 cells. After treatment with CA or verapamil, the expression of P-gp in K562/AO2 cells decreased to the level shown in K562 cells ($P < 0.05$, Fig. 4).

Detection of *mdr1* Gene Expression

As demonstrated by Semiquantitative RT-PCR, over-expression of *mdr1* mRNA was detected in K562/AO2 cells, but low-level expression of *mdr1* in K562 cells was detected. After treatment with reversal

agents for 48 h, the expression of *mdr1* in CA-treated K562/AO2 cells decreased as it did in verapamil-treated cells. The mean PCR value of the ratio relative to the β -actin gene of *mdr1* was 0.7 in K562/AO2, while the ratio was 0.5 in CA-treated K562/AO2 cells.

Discussion

A major mechanism of multidrug resistance is the enhanced efflux of a wide variety of structurally distinct classes of chemotherapeutic agents due to the overexpression of P-gp (10). P-gp is able to pump various anticancer drugs out of cells, thus resulting in a low intracellular drug concentration that is insufficient to kill tumor cells (19). To use compounds with low toxicity or with none at all, binding P-gp and blocking its transport function is one of the most common methods of reversing MDR (9). In the present study, we demonstrated the effect of CA on reversing MDR in the K562/AO2 cell line.

The MDR leukemia cell line K562/AO2 was established through *in vitro* selection of K562 cells with an increasing concentration of adriamycin (21), overexpressing the *mdr1* gene and P-gp. K562/AO2 cell line is not only resistant to adriamycin but also to other antitumor drugs, and it is extensively used in the research of MDR (23).

Using MTT assays, we showed that CA enhanced the toxicity of adriamycin in K562/AO2. The data indicate that CA could decrease the IC₅₀ of adriamycin against K562/AO2 cells as well as increase its chemosensitivity.

The fluorescence intensity value of adriamycin measured *via* FCM reinforced the opinion that the reduction of intracellular drug concentration results in the increase of drug resistance in cells. Particularly, the intracellular adriamycin concentration in CA-treated K562/AO2 cells was 3.8-fold higher than that of the untreated K562/AO2 cells. This result indicates that CA could inhibit the function of P-gp in K562/AO2 cells. We also found that after treatment with CA, the P-gp expression decreased significantly as

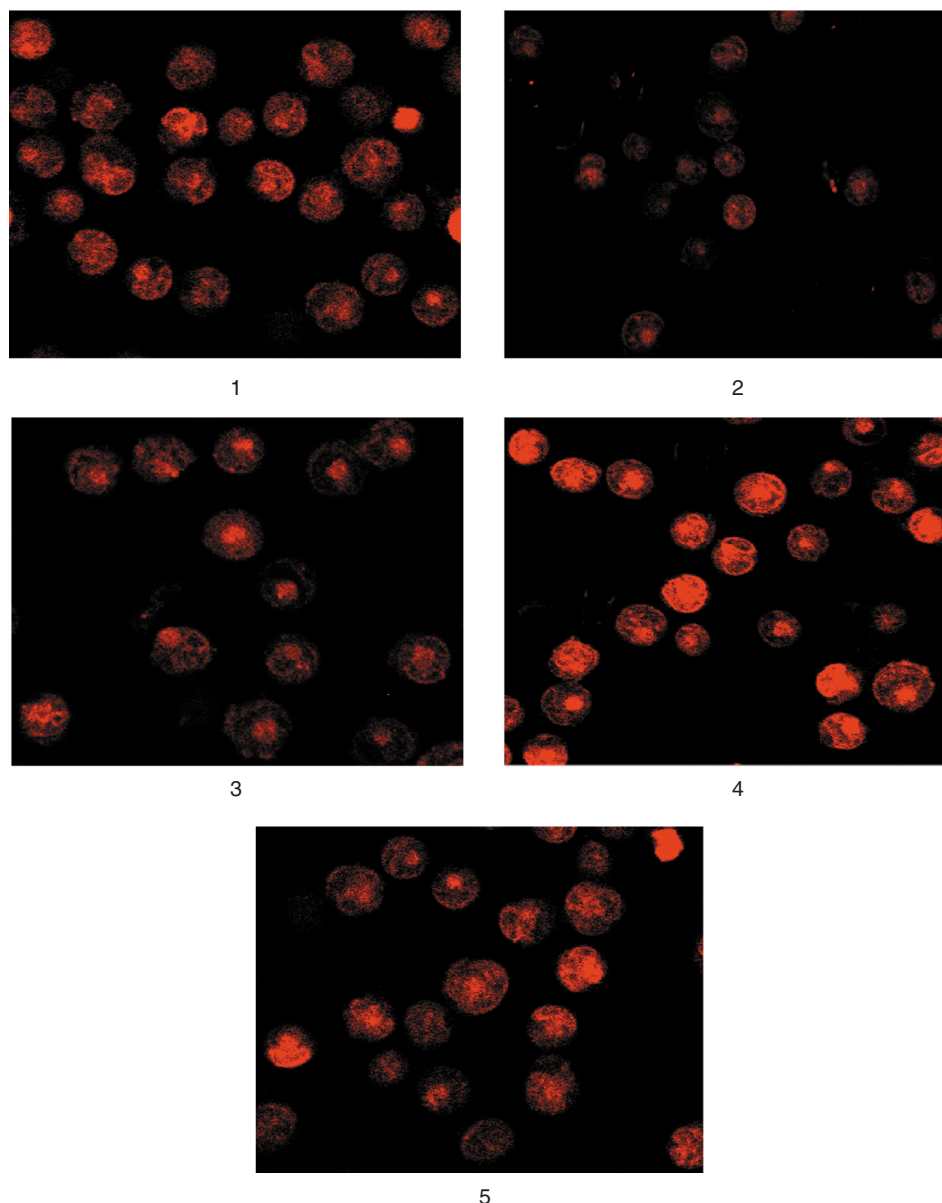


Fig. 3. Concentration and subdistribution of adriamycin in living K562/AO2 cells by LSCM. K562/AO2 cells were treated with CA and verapamil for 48 h, and then adriamycin was added to each sample to a final concentration of 5 mg/l after coincubating for a further 2 h. The distribution of adriamycin in K562 cells (1) was even. The fluorescence of vacant K562/AO2 cells (2) was ignored, and adriamycin in K562/AO2 cells (3) was distributed to the perinuclear and perimembrane regions, while it was lacking in cytoplasm. After treatment with CA (4), verapamil (5), the diffused distribution of intracellular adriamycin was restored in the nucleus and cytoplasm, and the aggregation of intracellular adriamycin was increased greatly. Compared with untreated cells, this shows a significant difference.

measured by FCM, which demonstrated that CA could reverse MDR by inhibiting P-gp overexpression. Hence, CA could inhibit P-gp's function and suppress its expression. The decreased expression of P-gp was consistent with the down-regulation of *mdr1* gene.

However, we found that even though the intracellular drug concentration in CA-treated K562/AO2 cells was 2.4-fold higher than that in K562 cells, the drug resistance of K562/AO2 cells to adriamycin

was still higher by 3.4 times, as compared to that of the K562 cells. This indicates that some other factors must play an important role in MDR mechanism besides the reduction of intracellular drug accumulation. The mechanism of modifying drug localization (16) was explored by LSCM in this study.

We also observed that adriamycin fluorescence appeared diffused in the nucleus and cytoplasm in K562 cells, while K562/AO2 cells showed a bright

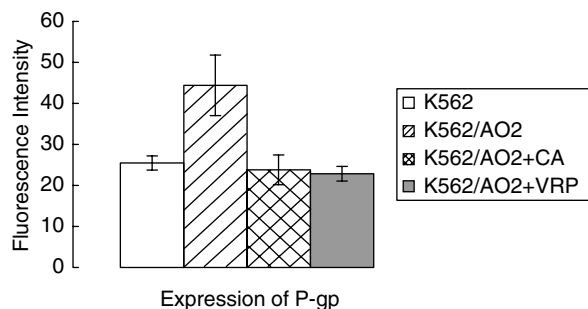


Fig. 4. The expression of P-gp. CA-treated K562/AO2 cells were incubated with phycoerythrin-conjugated UIC2, and then were detected by FCM. The expression of P-gp in K562 cells was lower than that in K562/AO2 cells. After treatment with CA or verapamil in K562/AO2 cells, the P-gp expression was decreased to the level of K562 cells. There was a significant difference between K562/AO2 cells and treated K562/AO2 cells ($P < 0.05$).

fluorescent signal located in the peripheral cytoplasm and perinuclear regions; the nucleus and other cytoplasmic regions showed little signal, and the same result was found in a previous study (12). Chemotherapeutic drugs target nuclear DNA to cause DNA damages and thereby induce apoptosis. The abnormal distribution of adriamycin in K562/AO2 cells keeps the adriamycin away from the nucleus and results in the MDR of the cells. When K562/AO2 cells were treated with CA, the intensity of the intracellular fluorescence increased, and normal subcellular distribution was restored. This observation indicates that P-gp not only pumps adriamycin out of the cells but also transports adriamycin from the cytoplasm to the perinuclear and perimembrane areas. Since P-gp is located in the cell membrane, the actions of the protein may cause the reduction of intracellular drug concentration, which correlates well with drug sensitivity results as previously reported (6). Another research (34) indicates that P-gp is concentrated in the plasma membrane with only very small amounts detected in the internal organelles membrane. Further research suggests that multidrug-resistant cells retain daunorubicin in the Golgi apparatus (1), lysosomes (14), and mitochondria (13). Since P-gp is also located in the organelles membrane of these resistant cells, it is capable of transferring the drugs into the organelles, resulting in the reduction of intracellular adriamycin in the cytoplasm and also promoting MDR in these K562/AO2 cells. Since the classical P-gp inhibitor verapamil could restore the diffused cytoplasmic and nuclear fluorescence distribution in K562/AO2 cells, it indicates that P-gp plays an important role in the process. In this study, we found that after treatment with CA, the suppression of the

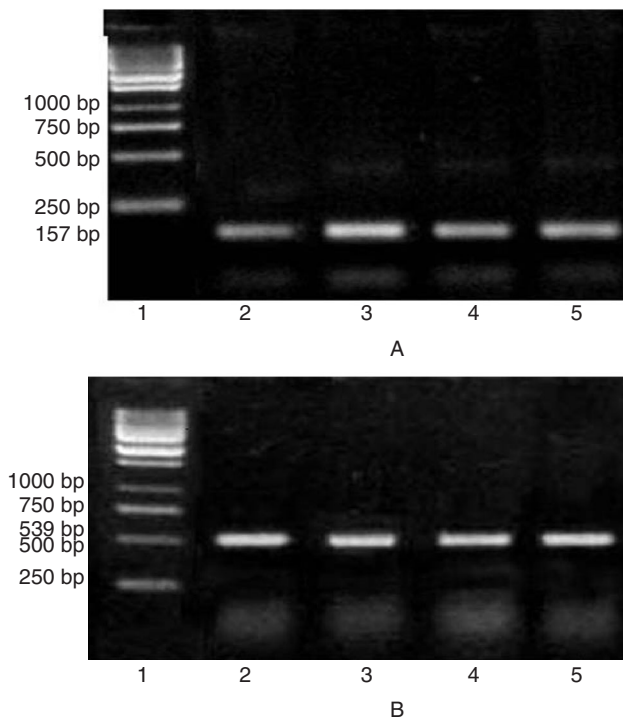


Fig. 5. Agarose gel electrophoresis of semiquantitative RT-PCR. A: *mdr1* gene B: β -actin the marker is shown in lane 1. In K562 cells (lane 2), the expression of *mdr1* was lower, as compared to K562/AO2 cells (lane 3). After treatment with CA (lane 4), VRP (lane 5) in K562/AO2 cells, the expression of *mdr1* was decreased. There were significant differences between untreated K562/AO2 cells and treated K562/AO2 cells ($P < 0.01$).

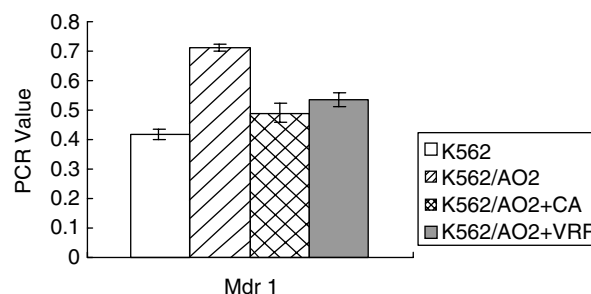


Fig. 6. The mean PCR values of the ratio relative to the β -actin gene. As demonstrated by semiquantitative RT-PCR, the expression of the *mdr1* gene in K562/AO2 cells was higher than that in K562 cells. After treatment with reversal agents, the expressions of *mdr1* in CA-treated K562/AO2 cells was decreased in CA-treated K562/AO2 cells as it did in verapamil-treated cells ($P < 0.05$).

expression of *mdr1* was observed along with the recovery of drug sensitivity in K562/AO2 cells. This is consistent with our finding that the cell surface expression of P-gp decreased in CA-treated K562/AO2 cells. These changes indicated that CA could

reverse MDR by inhibiting the expression of *mdr1* at the mRNA level.

Our study also suggested that after the potent inhibition of P-gp by CA, intracellular drug concentration was increased and subcellular drug distribution changed, which led to the reversal of MDR in K562/AO2 cell line.

In conclusion, this study has demonstrated that CA is a potent agent for reversing P-gp-mediated MDR through the down-regulation of *mdr1* mRNA and P-gp expression. Moreover, CA has the ability to recover the subcellular distribution of adriamycin. We therefore conclude that CA may be a useful therapy in reversal of MDR phenotype of tumor cells. However, we only conducted the experiments in K562/AO2 cells so additional studies are needed to identify reversal effect of CA in multiple cancer cell lines.

Acknowledgments

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