



## Gamma-linolenic acid modulates the response of multidrug-resistant K562 leukemic cells to anticancer drugs

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### ABSTRACT

Gamma-linolenic acid (GLA) is known to have selective tumoricidal action. It is also reported that GLA may increase the cytotoxic activity of cancer chemotherapeutic agents in some cancer cells. The present study examined whether GLA pretreatment could modulate the response of multidrug-resistant K562/ADM leukemic cells to anticancer drugs. The cell viability assay results showed that GLA at 10 µg/ml enhanced cell growth inhibition induced by the MDR-type drugs doxorubicin, etoposide and vincristine, but could not enhance or even attenuated cell growth inhibition induced by the non-MDR-type drug cisplatin, mitomycin and fluorouracil in K562/ADM cells. Further flow cytometry results showed that GLA decreased the expression of P-glycoprotein, enhanced the accumulation of doxorubicin and rhodamine 123 in K562/ADM cells and inhibited the efflux of rhodamine 123 from K562/ADM cells, lowered the efflux rate. These results suggest that GLA could modulate the response to anti-cancer agents in P-gp overexpressing multidrug-resistant cells, and the mechanism may be by decreasing the P-gp expression and inhibiting P-gp function.

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### 1. Introduction

Chemotherapy, radiotherapy and surgery are conventional and indispensable in the treatment of many cancers, use of these therapies has greatly improved the outcome for cancer patients. However, along with the success of chemotherapy, the development of drug resistance, during treatment or during retreatment at relapse, remains a major problem (Kong et al., 2006). Of particular concern is the phenomenon of multiple drug resistance (MDR). This form of resistance results from a mechanism that simultaneously confers resistance to many xenobiotics despite an unrelated chemical structure or target of action (Holland et al., 2006). Finding ways to circumvent MDR is now a major challenge in oncology.

The molecular mechanisms of MDR have been shown to be multi-factorial. One mechanism is mediated by drug transporter proteins, such as P-glycoprotein (P-gp), the multidrug-resistance-related protein 1 (MRP1), lung resistance-related protein (LRP) and the breast cancer resistance protein (BCRP). These proteins are found to be over-expressed in MDR cancer cells (Leslie et al., 2005; Majumder et al., 2006; Meschini et al., 2002). Of these, the P-gp mediated multidrug-resistance was the first discovered and probably still is the most widely observed mechanism in clinical

multidrug-resistance (Chu et al., 2008). P-gp is a membrane-resident protein that functions as an energy-dependent pump reducing the intracellular concentration of anticancer drugs. It confers MDR by directly binding and transporting a wide variety of structurally and functionally unrelated anti-cancer agents, such as vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes. Inhibiting the transport function of P-gp and increasing the intracellular accumulation of drugs is an effective way to overcoming MDR (Che et al., 2002). Many compounds called chemosensitizers have shown to reverse the MDR phenotype, such as calcium channel blockers and cyclosporine A. However, the use of effective doses of these agents for systemic chemotherapy has been difficult because of their side-effects. Therefore, less toxic and more potent candidates need to be developed (Balayssac et al., 2005; Gao et al., 2007).

The omega-6 polyunsaturated fatty acid gamma-linolenic acid (GLA; 18:3 n-6), which is found in several plant oils and microorganism oils, has antitumor activity *in vitro*. It is selectively cytotoxic to more than 30 human cancer cell lines at concentrations that do not harm normal cells (Menendez et al., 2002). In recent years GLA has been demonstrated to enhance tamoxifen, faslodex, paclitaxel, vinorelbine and docetaxel (taxotere) cytotoxicity in human breast carcinoma cells (Kenny et al., 2000; Menendez et al., 2004a,b, 2001) and Epirubicin cytotoxicity in superficial bladder carcinoma (Harris et al., 2003). There is also evidence that GLA could increase

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idarubicin uptake in MGH-U1/R resistant bladder cancer cells and could change intracellular drug distribution of mitoxantrone in MCF-7/R resistant human breast cancer cells (Davies et al., 1999), but the mechanisms remain unclear. Our previous study showed that GLA could induce apoptosis in K562/ADM multidrug-resistant leukemic cells (Kong et al., 2006). In the present study, we investigated whether GLA could modulate the response of K562 and K562/ADM leukemic cells to anticancer drugs including MDR-type drugs (doxorubicin, etoposide, vincristine) and non-MDR-type drug (cisplatin, mitomycin, fluorouracil) at concentrations which did not result in cell toxicity, and examined the effects of GLA on the function of P-gp/MDR1 and the expression of P-gp in K562/ADM.

## 2. Materials and methods

### 2.1. Chemicals

RPMI 1640 medium, fetal bovine serum (FBS), trypan blue, penicillin G and streptomycin were obtained from GIBCO BRL. P-glycoprotein (MDR) purified mouse anti-human monoclonal antibody was obtained from BD Biosciences Pharmingen, FITC conjugated goat anti-mouse IgG, rhodamin 123, 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), GLA were obtained from Sigma Chemical Co. USA. GLA was dissolved in 100% ethanol at 100 mg/ml. The subsequent dilutions were made in cell culture media.

### 2.2. Cell culture

The erythromyogenous leukemic cell K562 and its P-glycoprotein-overexpression cell K562/ADM were maintained in RPMI 1640 medium, with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin in humidified air at 37 °C with 5% CO<sub>2</sub>. Cells were maintained in the density range of 0.1–1 × 10<sup>6</sup> cells/ml, and exponentially growing cells were decanted for experimental studies as required. Cell density was determined regularly by using a hemocytometer (Qiuqing, Shanghai). Cell viability was determined by trypan blue exclusion. To maintain drug resistance, doxorubicin was supplemented to K562/ADM cells at regular intervals, but was omitted 2 weeks before any experiment.

### 2.3. Cell viability studies (MTT)

Cells preincubated with 10 µg/ml GLA or not for 24 h were suspended at a final concentration of 1 × 10<sup>5</sup> cells/ml and seeded in 96-microwell flat-bottom plates, and drugs were added at different concentrations. After incubation for 48 h, MTT solution (5 mg/ml in phosphate-buffered saline (PBS)) was added for 4 h incubation at 37 °C. The precipitated formazan was dissolved in 150 µl pre-warmed DMSO. Cell viability was evaluated by measuring the absorbance at 570 nm, using an automated plate reader (Sunrise Co., Ltd.). Cell survival was expressed as percentage of viable cells of drug-treated samples to control samples. All drug concentrations were tested in six replicates and the experiment were repeated three times. IC<sub>50</sub> values were extrapolated from the dose-response curves, as estimated by performing a linear regression:  $Y = \log(fa/fu)$  versus  $X = \log(\text{drug concentration})$ , in which *fa* is the fraction affected by the dose calculated as a ratio (treated cells to untreated cells), and *fu* is the unaffected fraction (i.e., 1-*fa*) according to Chou and Talalay (Chou and Talalay, 1981; Topaly et al., 2001).

### 2.4. Intracellular doxorubicin accumulation

The accumulation of doxorubicin was determined by flow cytometry. Cells were pretreated with 5, 10 µg/ml GLA for 24 h,

and then the cell suspensions were incubated with 5 µg/ml doxorubicin for 1 h at 37 °C. After washed with PBS, the cells were subsequently subjected to flow cytometry with excitation measured at 488 nm and emission measured at 585 nm. The K562 cell was used as a positive control for maximum doxorubicin accumulation.

### 2.5. Rhodamin 123 accumulation and efflux assay

The cells preincubated or not with GLA for 24 h, were exposed to 5 µg/ml rhodamin 123 for 1 h at 37 °C. To detect rhodamin accumulation and efflux, the cells were collected and washed with PBS at 0 min, part of the cells was immediately subjected to flow cytometry to record the green fluorescence produced by rhodamin 123, and the rest was incubated in fresh medium at 37 °C. After 2 h, the cells were subjected to flow cytometry. To determine the rate of efflux, after incubated with rhodamine 123, the cells were washed twice with excess volume of ice-cold PBS, and incubated again with the medium in the presence or absence of GLA. After various incubation times, the cells were washed twice with an excess volume of ice-cold PBS, lysed with 0.1% Triton X-100. The fluorescence intensity was measured with a spectrofluorometer (SAFIRE). The excitation and emission wavelengths were 485 and 532 nm. The efflux rate *K* was calculated by the formula  $F_t = F_0 e^{-kt}$  (Ji et al., 2005).

### 2.6. Measurement of P-glycoprotein expression

P-gp expression was analyzed by immunofluorescence using P-glycoprotein (MDR) purified mouse anti-human monoclonal antibody and determined by flow cytometric analysis. Briefly, cells pretreated with 10 µg/ml GLA for 6 h, 12 h and 24 h were washed and incubated for 30 min at room temperature in PBS containing p-glycoprotein monoclonal antibody (1:64 diluted), washed twice with PBS and incubated with FITC conjugated goat anti-mouse IgG (1:100 diluted). The cells were washed twice with PBS and the level of fluorescent staining was analyzed using Flow Cytometry.

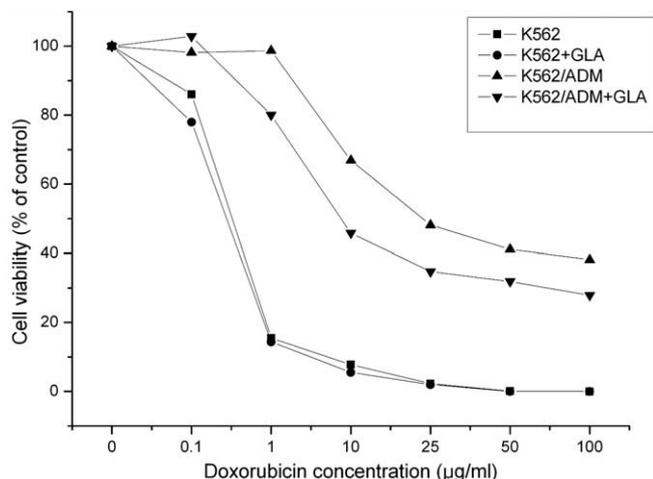
### 2.7. Statistics

Data were expressed as the mean ± S.E. Statistical significance was determined by the one-way analysis of variance (ANOVA), followed by Tukey's pair-wise comparisons at significance level of 0.05.

## 3. Results

### 3.1. Resistance of K562/ADM cells to anti-cancer agents and the effect of GLA on the resistance

K562 and K562/ADM cells were exposed to different concentrations of doxorubicin, etoposide, vincristine, cisplatin, mitomycin and fluorouracil, because these drugs are frequently found in treatment regimes for leukemia. The concentration-cell viability curves of doxorubicin in K562 and K562/ADM treated or not with GLA were shown in Fig. 1 as a represent. IC<sub>50</sub> values were calculated as mentioned in "Materials and methods". The IC<sub>50</sub> values of other anti-cancer agents were also listed in Table 1. As shown in Fig. 1 and Table 1, K562/ADM cells were obviously resistant to MDR-type drugs doxorubicin, etoposide, vincristine. At 10 µg/ml GLA alone had been detected to have no cytotoxic effects on K562 and K562/ADM cells (data not shown). And in this experiment, GLA at this concentration significantly reduced degrees of resistance of K562/ADM cells to doxorubicin, etoposide and vincristine. And compared with K562 cells, K562/ADM had



**Fig. 1.** Growth inhibitory effect of doxorubicin on K562 and K562/ADM pretreated with GLA or without as measured by MTT assay.

also slight resistance to non-MDR-type drugs mitomycin and cisplatin, but GLA could not reverse the resistance or even enhance the resistance. To K562 cells, the IC<sub>50</sub> values of doxorubicin, vincristine, mitomycin were somewhat decreased when the K562 cells were incubated with GLA.

### 3.2. Intracellular doxorubicin accumulation

Accumulation of doxorubicin was measured by flow cytometry. Fig. 2a showed representative histograms of cells pre-incubated with 5, 10 µg/ml GLA for 24 h. And the fluorescence mean values of histograms derived from three independent experiments (Fig. 2b) showed that the fluorescence intensity of doxorubicin in K562 cells was 43% higher than K562/ADM cells and GLA treated K562/ADM can enhance intracellular doxorubicin accumulation in K562/ADM cells, and the effect augmented with the concentration of GLA. The fluorescence intensity of doxorubicin in 10 µg/ml GLA-treated K562/ADM cells increased 22% compared to the untreated K562/ADM cells.

### 3.3. Rhodamine 123 accumulation and efflux

Accumulation of rhodamine 123 was also measured by flow cytometry. Representative histograms of cells pre-incubated with 5, 10 µg/ml GLA for 24 h were shown in Fig. 3a. And the fluorescence mean values of histograms derived from three independent experiments (Fig. 3b) showed that the fluorescence units of rhodamine 123 in K562 and K562/ADM cells were 601.93 units and 293.35 units, respectively. The accumulation of rhodamine 123 by K562/ADM cells was less than half of parental K562 cells, showing significantly difference ( $P < 0.01$ ). In K562/ADM cells, 5 µg/ml and 10 µg/ml GLA increased the intracellular rhodamine 123 accumulation in K562/ADM cells ( $P < 0.05$ ). The fluorescence intensity of rhodamine 123 in 10 µg/ml GLA-treated K562/ADM cells was 373.76 units, which was 27% higher than untreated K562/ADM cells. For the rhodamine 123 efflux assay, we used both flow cytometry and spectrofluorometer. Fig. 4a showed the representative examples after 2 h efflux of rhodamine 123, the fluorescence intensity was significantly descended in K562/ADM after 2 h efflux of rhodamine 123, and compared with the K562/ADM after efflux, the fluorescence intensity was significantly elevated in GLA treated K562/ADM cells. To determine the efflux rate, we detected the fluorescence intensity at various time after rhodamine 123 efflux by spectrofluorometer and calculated the efflux rate ( $k$ ) used the formula  $F_t = F_0 e^{-kt}$ . Fig. 4b showed the fluorescence intensity at various time after rhodamine 123 efflux, and the efflux rate of K562/ADM, K562/ADM + 5 µg/ml GLA, and K562/ADM + 10 µg/ml is 0.024, 0.012, and 0.010, respectively. GLA can lower the efflux rate of rhodamine 123 in K562/ADM.

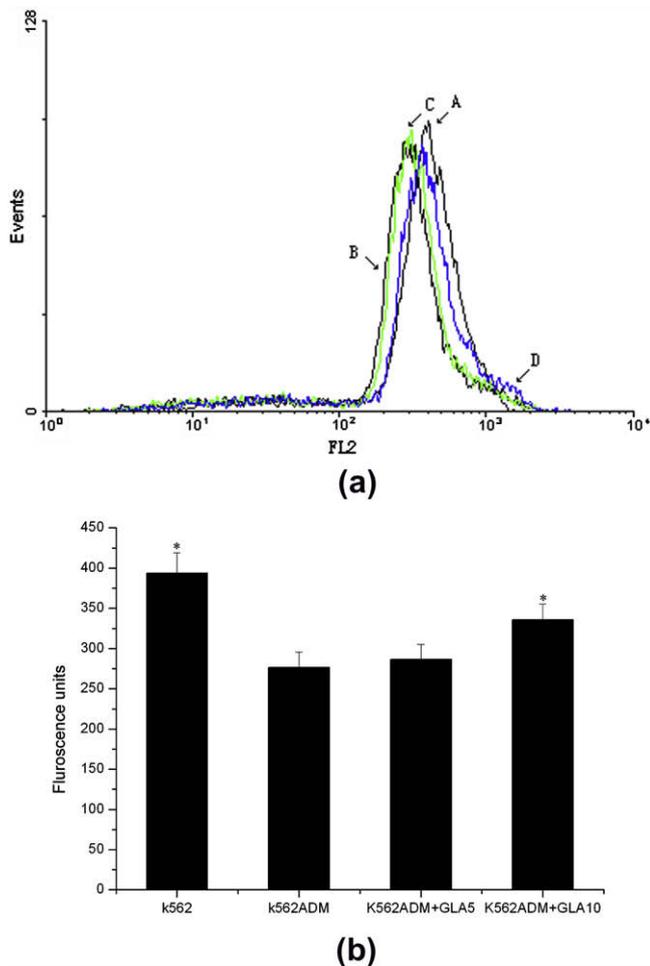
### 3.4. P-glycoprotein expression

To explain the modulating effects of GLA to MDR-type drugs in K562/ADM cells, we analyzed P-glycoprotein expression by flow cytometry. Fig. 5a showed representative histograms of cells pre-incubated with 10 µg/ml GLA for 6 h, 12 h and 24 h. Fluorescence mean values of histograms derived from three independent experiments showed that P-glycoprotein was expressed in K562/ADM cells, while it was not detected in K562 cells. GLA can inhibit

**Table 1**  
Response of GLA-pretreated and untreated K562/WT and K562/ADM cells to cytostatic drugs.

	K562/WT		K562/ADM	
	–GLA	+GLA	–GLA	+GLA
<b>Doxorubicin</b>				
IC <sub>50</sub> (µg/ml)	0.42 ± 0.01	0.37 ± 0.02	21.82 ± 1.26	9.36 ± 0.82
Degree of resistance	1	0.88	51.95	22.29
<b>Etoposide</b>				
IC <sub>50</sub> (µg/ml)	10.16 ± 0.70	9.91 ± 1.09	167.32 ± 5.98	51.33 ± 2.42
Degree of resistance	1	0.98	16.47	5.05
<b>Vincristine</b>				
IC <sub>50</sub> (µg/ml)	0.07 ± 0.01	0.06 ± 0.01	20.69 ± 1.61	4.08 ± 0.06
Degree of resistance	1	0.86	295.57	58.29
<b>Cisplatin</b>				
IC <sub>50</sub> (µg/ml)	0.90 ± 0.01	0.94 ± 0.07	3.06 ± 0.27	4.97 ± 0.33
Degree of resistance	1	1.04	3.4	5.52
<b>Mitomycin</b>				
IC <sub>50</sub> (µg/ml)	2.68 ± 0.37	2.04 ± 0.12	11.16 ± 0.69	11.33 ± 1.13
Degree of resistance	1	0.76	4.16	4.23
<b>Fluorouracil</b>				
IC <sub>50</sub> (µg/ml)	31.98 ± 1.74	32.15 ± 1.98	36.52 ± 2.9	31.34 ± 2.59
Degree of resistance	1	1.01	1.14	0.98

Note: Mean values of three independent experiments with each threefold determination. IC<sub>50</sub>, inhibition concentration 50%. Degree of resistance was determined by dividing the IC<sub>50</sub> values of drugs for K562 and K562/ADM cells with or without GLA by that for K562 cells without GLA.

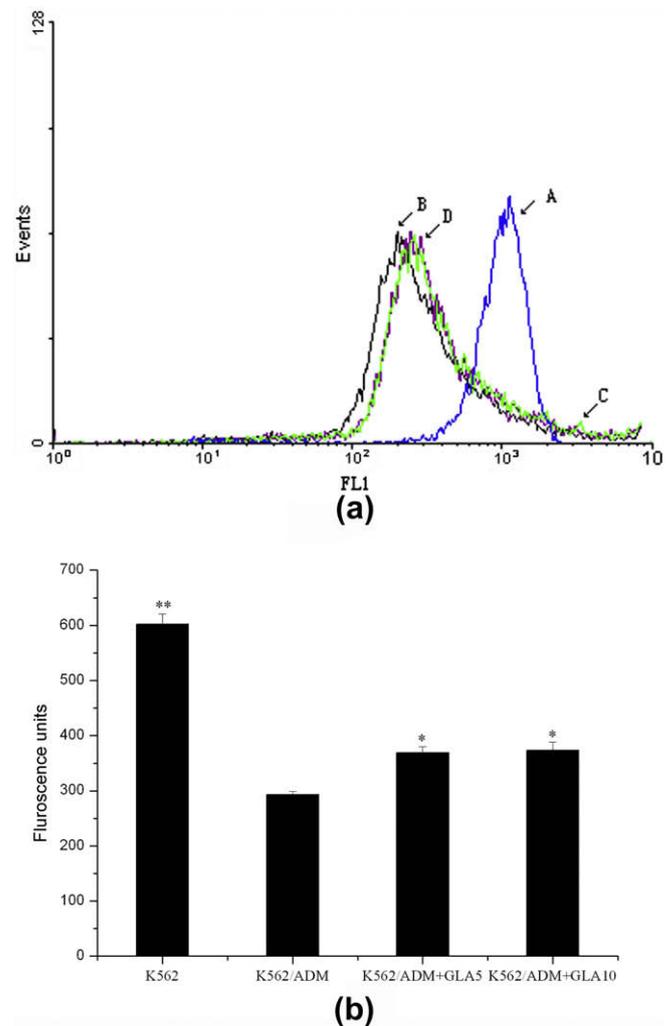


**Fig. 2.** Effect of treatment with GLA on doxorubicin accumulation. Representative examples of three independent experiments are shown. (a) A: K562; B: K562/ADM; C: K562/ADM + 5 µg/ml GLA; D: K562/ADM + 10 µg/ml GLA. The fluorescence units of intracellular doxorubicin were shown in (b). Data were expressed as Means ± S.E. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with K562/ADM.

P-glycoprotein express in a time-dependent manner. The P-gp content in K562/ADM cells treated with 10 µg/ml GLA for 24 h was only 30% of that in untreated K562/ADM cells.

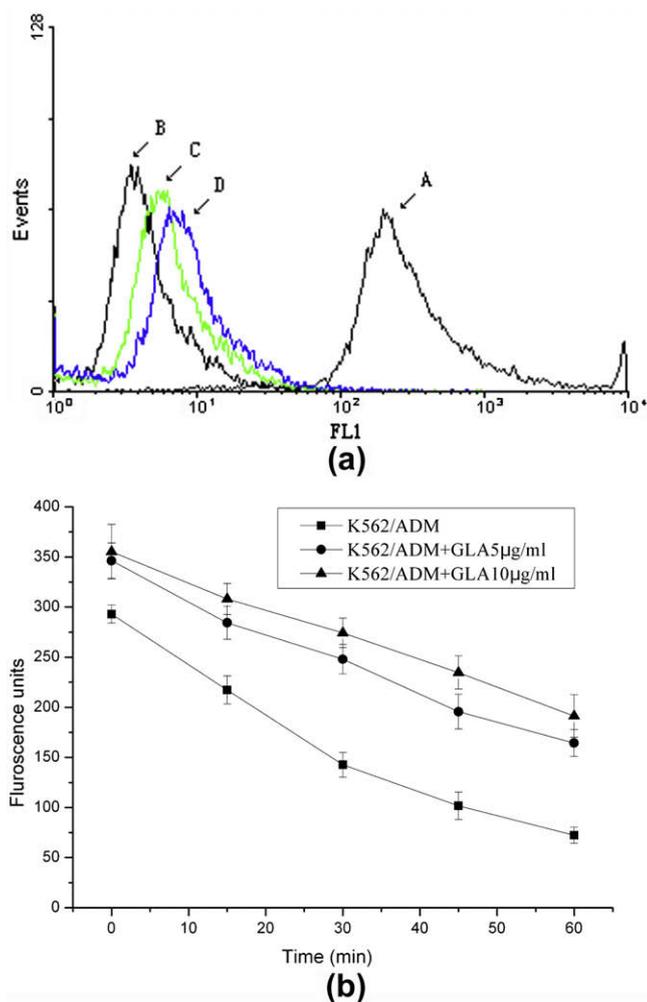
#### 4. Discussion

K562 cell line is a chronic myelogenous leukemia cell with the 9; 22 translocation, resulting in the expression of a fusion oncoprotein Bcr/Abl which exhibits constitutively active kinase activity. K562/ADM is a multidrug-resistant cell line, induced by adriamycin, and the cell line harbors an amplification of the MDR1 gene, shows resistance not only to adriamycin itself, but also to a broad range of structurally and functionally related and unrelated compounds (e.g., doxorubicin, vincristine and etoposide) (Shen et al., 2008). In the present study, K562 and K562/ADM cells were employed to investigate whether GLA could modulate the response of multidrug-resistant cancer cells to anticancer drugs. The results showed for the first time that GLA enhanced cell growth inhibition induced by the MDR-type drugs doxorubicin, etoposide and vincristine, but could not enhance or even attenuated cell growth inhibition induced by the non-MDR-type drug cisplatin, mitomycin and fluorouracil in K562/ADM cells. To K562 cells, the addition of GLA only had a somewhat synergic effect to anti-cancer agents such as doxorubicin, etoposide, vincristine, mitomycin.



**Fig. 3.** Effect of treatment with GLA on rhodamine 123 accumulation. Representative examples of three independent experiments are shown. (a) A: K562; B: K562/ADM; C: K562/ADM + 5 µg/ml GLA; D: K562/ADM + 10 µg/ml GLA. The fluorescence units of intracellular rhodamine 123 were shown in (b). Data were expressed as Means ± S.E. \* $P < 0.05$ , \*\* $P < 0.01$  compared with K562/ADM.

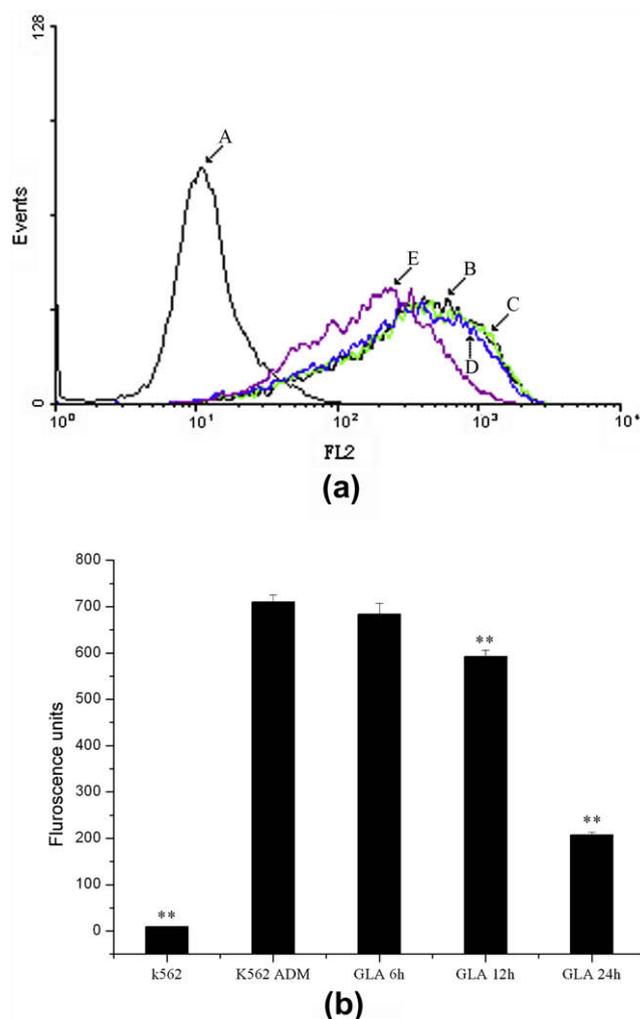
To explain the modulating effects of GLA in K562/ADM cells, we analyzed P-glycoprotein function and expression. Human P-gp is a 170 kDa polypeptide consisting of 1280 amino acids organized in two tandem repeats of 610 amino acids joined by a linker region of ~60 amino acids (Hennessy and Spiers, 2007; Van, 2001). Screening studies for P-gp–drug interactions identified a number of clinically important drugs as P-gp substrates, which are as diverse as anthracyclines (doxorubicin, daunorubicin), alkaloids (reserpine, vincristine, vinblastine), specific peptides (valinomycin, cyclosporine), steroid hormones (aldosterone, hydrocortisone) and local anaesthetics (dibucaine). Even dye molecules (rhodamine 123) and pharmaceutical excipients exhibited P-gp substrate activity (Varma et al., 2003). In this study, doxorubicin and rhodamine 123 were chosen to study P-glycoprotein function since they are good P-gp substrates with an autofluorescence capacity (Ponce and Barrera-Rodriguez, 2005). Our results showed that GLA treated K562/ADM could enhance the accumulation of intracellular doxorubicin and rhodamine 123, inhibit the efflux of rhodamine 123. In contrast, it had no apparent effect on the non-P-gp-expressing parental cell line K562 (data not shown). Furthermore, we investigated the expression of P-gp by flow cytometry, and the results demonstrated the time-dependent inhibition of induced levels of



**Fig. 4.** Effect of treatment with GLA on rhodamine 123 efflux. Representative examples after 2 h efflux of rhodamine 123 are shown. (a) A: K562/ADM (0 h); B: K562/ADM (2 h); C: K562/ADM + 5 µg/ml GLA (2 h); D: K562/ADM + 10 µg/ml GLA (2 h). The fluorescence units of intracellular rhodamine 123 after various time of efflux were shown in (b). Data were expressed as Means  $\pm$  S.E.

P-gp in K562/ADM cells. These indicated the mechanism of the reversal of GLA to K562/ADM is by modulating the P-gp expression and inhibiting transport of the anti-cancer agents.

There is considerable interest in exploiting the effects of GLA in cancer prevention and also as an adjunct to conventional cancer therapy. The ability of GLA, to increase the sensitivity of tumor cells to certain drug therapies has been documented. And some hypotheses have been proposed to explain the action of GLA modulation of chemotherapeutic effectiveness. The first hypothesis is GLA could incorporate into the cell plasma membrane, and this incorporation led to an increase in membrane fluidity and increased drug uptake or caused a shift in the intracellular distribution of certain chemotherapeutic drugs (Davies et al., 1999). This is particularly relevant to many cytotoxic drugs which are thought to enter the cell by passive diffusion (Menendez et al., 2001). The second hypothesis is that GLA could affect the enzymatic antioxidant system, and in return, the antioxidant enzymes influence cancer cell sensitivity to anticancer drugs (Liu and Tan, 2000). In this study, GLA could inhibit P-glycoprotein express and affect P-gp function in K562/ADM cells was found. It is known that unsaturated fatty acids could induce the expression of some early genes, such as *c-jun*, *c-fos*, and *c-myc* in several types of cells such as rat mesangial cells, swiss 3T3 fibroblasts, prostate cancer cells, vascu-



**Fig. 5.** Effect of treatment with GLA on P-glycoprotein expression. Representative examples of three independent experiments are shown. (a) A: K562; B: K562/ADM; C: K562/ADM + GLA (6 h); D: K562/ADM + GLA (12 h); E: K562/ADM + GLA (24 h). The fluorescence units of FITC conjugated antibody were shown in (b). Data were expressed as Means  $\pm$  S.E. \* $P$  < 0.05, \*\* $P$  < 0.01, compared with K562/ADM.

lar smooth muscle cells and hepatocytes and affect the expression of the genes associated with cell adhesion and motility (Liu and Tan, 2000; Chen and Hughes-Fulford, 2000; Chung et al., 2002; Lee and Bae, 2007). They could regulate the activity of NF-kappa B and MAPKs in pancreatic tumor cells, endothelium and macrophages (Babcock et al., 2003; Jump, 2004; Ross et al., 2003; Xue et al., 2006). The cellular protein, p-gp is expressed in many kinds of tissues and cancers, and is always over-expressed in response to chemotherapy to cause efflux of the anticancer drug(s) from cells. In recent years, some of the signaling pathways have been supposed to be attributed to the expression of P-gp including NF-kappa B and MAPK (Barancik et al., 2001; Bentires-Alj et al., 2003; Fujita et al., 2005; Miao and Ding, 2003). Our finding that GLA modulated the P-gp expression may partly due to the transcriptional inhibition. On the other hand, GLA may affect the localization of P-gp in cell plasma membrane by changing cell membrane fluidity, and thus enhance P-gp degradation.

In conclusion, the present study discloses that GLA increased the sensitivity of K562/ADM cells to the MDR-type drugs doxorubicin, etoposide and vincristine, but could not enhance or even attenuated cell growth inhibition induced by the non-MDR-type drug cisplatin, mitomycin and fluorouracil in K562/ADM cells. And the mechanism of the reversal of GLA to K562/ADM may be by

decreasing the P-gp expression and inhibiting P-gp function. Most reversing agents can reverse MDR in cultured cells, but not in patients. One of the reasons for this is that blood concentrations of the reversing agents cannot be elevated to their effective levels, because of their side-effects (Che et al., 2002). GLA is an essential fatty acid in human body, no serious adverse event was observed. GLA may be a potent agent for reversing MDR mediated by P-gp and may be useful to modulate the resistance to doxorubicin, etoposide and vincristine in K562/ADM cells that overexpress P-gp.

### Conflict of interest

None declared.

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