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Reversal of *mdr*1-mediated multidrug resistance in human leukemia cells by a new spin-labeled derivative of podophyllotoxin

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GP7 (4-[4″-(2″, 2″, 6″, 6″-tetramethyl-l″-piperidinyloxy) amino]-4′-demethyl epipodophyllotoxin) is a promising anticancer drug of the podophyllotoxin class. However, little is known about its anti-multidrug resistance effects. In the present study, we investigated the effects of GP7 on P-glycoprotein (P-gp) overexpression multidrug-resistant human leukemia K562/ADM cells with the comparison of VP-16 and K562 cells. GP7 inhibited the proliferation of K562/ADM cells in a concentration- or time-dependent manner, and the inhibitory effect of GP7 on K562/ADM cells was 1.50-fold higher than that of VP-16. GP7 caused G_2/M phase accumulation but VP-16 caused S phase accumulation in K562/ADM and K562 cells. GP7 could induce apoptosis of both K562/ADM and K562 cell lines, but there was no significant difference between GP7- and VP-16-induced apoptotic ratios. GP7 could also induce typical apoptotic morphological changes and internucleosomal DNA fragmentation of K562/ADM and K562 cells, but DNA fragmentation induced by GP7 in K562/ADM cells was weaker than that in K562 cells. When treated with GP7 or VP-16 for 48 h, 128–256 μM GP7 induced more DNA fragmentation than VP-16 did, but 32–64 μM GP7 induced less DNA fragmentation than VP-16 did. GP7 could down-regulate the expression of P-gp in K562/ADM cells but VP-16 could not. Our findings suggest that GP7 may reverse multidrug resistance in human leukemia K562/ADM cells via down-regulation of P-gp expression.

1. Introduction

Multidrug resistance (MDR) is a major obstacle to the effective treatment of cancer, and its mechanisms are multifactorial. The best characterized mechanism responsible for MDR involves the overexpression of P-glycoprotein (P-gp or P-170) encoded by the multidrug resistance 1 gene (*mdr*1). P-gp is an ATP-dependent drug pump, which confers drug resistance by the active efflux of chemotherapeutic agents from the cells against a concentration gradient.

VP-16 (etoposide) is an epipodophyllotoxin derivative that inhibits topoisomerase II. It is a widely used anticancer agent for solid tumors and hematological malignancies. However, its effectiveness is sometimes limited by the development of drug resistance (Nooter and Stoter 1996).

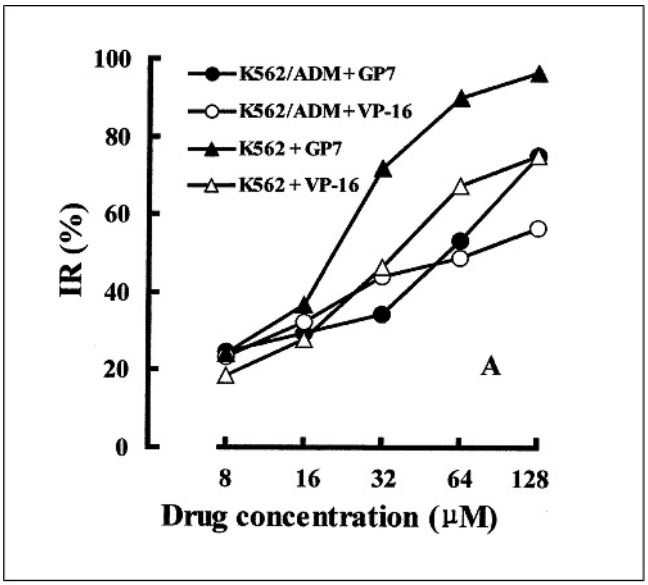
GP7 (4-[4″-(2″, 2″, 6″, 6″-tetramethyl-l″-piperidinyloxy) amino]-4′-demethyl epipodophyllotoxin) is a new spin-labeled derivative of podophyllotoxin semi-synthesized by our university. GP7 has many similarities to clinically used VP-16 but has lower toxicity and higher total chemical yield (based on podophyllotoxin) when compared with VP-16 and thus is a promising anticancer drug of the podophyllotoxin class (Qi et al. 2004, 2006, 2007). Despite its potency, the anti-MDR effects of GP7 still remain to be known.

In the present study, we investigated the effects of GP7 on P-gp overexpression ($P\text{-gp}^+$) multidrug-resistant human myelogenous leukemia K562/ADM cells with the comparison of VP-16 and K562 cells. GP7 inhibited the proliferation of K562/ADM and K562 cells in a concentration- or time-dependent manner, and its inhibitory effect on K562/ADM cells was 1.50-fold higher than that of VP-16. GP7 caused G_2/M phase accumulation but VP-16 caused S phase accumulation in K562/ADM and K562 cells. GP7 induced apoptosis of K562/ADM cells, and 128–256 μM GP7 induced more apoptotic DNA fragmentation than VP-16 did. GP7 also down-regulated the expression of P-gp. Our findings suggest that GP7 may reverse multidrug resistance in human leukemia K562/ADM cells via down-regulation of P-gp expression.

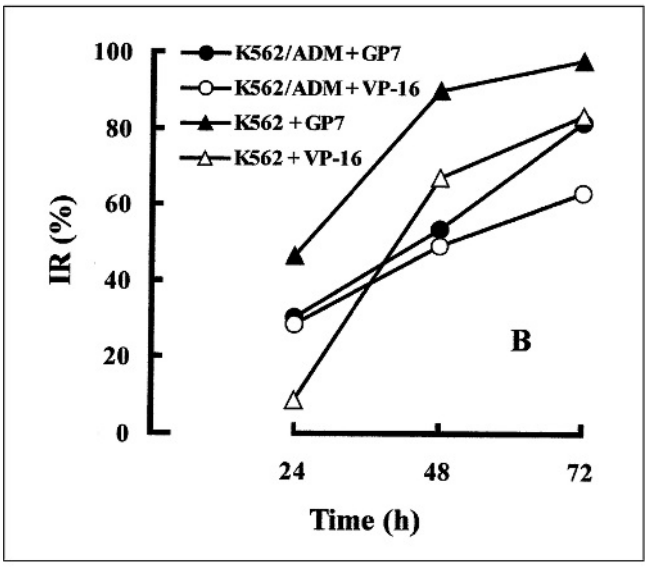
2. Investigations and results

2.1. GP7 inhibits the proliferation of K562/ADM and K562 cells

When treated with 8–128 μM GP7 or VP-16 for 48 h or 64 μM GP7 or VP-16 for 24–72 h, the proliferation of K562/ADM and K562 cells was inhibited in a concentration- or time-dependent manner (Fig. 1). The value of IC_{50} of GP7 or VP-16 in K562/ADM cells was 45.9 ± 1.8 μM or 68.7 ± 4.6 μM respectively while in K562 cells it was 18.4 ± 3.1 μM or 37.5 ± 4.9 μM respectively. The values of IC_{50} indicated that the inhibitory effects of GP7 on the proliferation of K562/ADM or K562 cells were 1.50-fold or 2.03-fold



(A)



(B)

Fig. 1: GP7 inhibits the proliferation of K562/ADM and K562 cells in a concentration- and time-dependent manner. Cell proliferation was detected by MTT assay and IR (inhibitory ratio) was calculated as described in *Experimental*. Results are mean values from 4 separate experiments performed in quadruplicate. (A) Concentration-response curve. K562/ADM or K562 cells were treated with indicated concentrations of GP7 or VP-16 for 48 h. Linear regression analysis displays that the regression equations are as follows: $\hat{Y}_{K562+GP7} = 0.644X - 0.336$; $\hat{Y}_{K562+VP-16} = 0.509X - 0.296$; $\hat{Y}_{K562/ADM+GP7} = 0.415X - 0.191$; $\hat{Y}_{K562/ADM+VP-16} = 0.275X - 0.005$. Bivariate correlation analysis displays the correlation coefficients (r) are as follows: $r_{K562/ADM+GP7} = 0.947$, $P < 0.05$; $r_{K562+GP7} = 0.978$, $P < 0.01$; $r_{K562/ADM+VP-16} = 0.992$, $P < 0.01$; $r_{K562+VP-16} = 0.989$, $P < 0.01$. (B) Time-response curve. K562/ADM or K562 cells were treated with 64 μM GP7 or VP-16 for the indicated time, Linear regression analysis displays that the regression equations are as follows: $\hat{Y}_{K562+GP7} = 1.400X + 8.116$; $\hat{Y}_{K562+VP-16} = 1.289X - 6.539$; $\hat{Y}_{K562/ADM+GP7} = 1.116X + 1.186$; $\hat{Y}_{K562/ADM+VP-16} = 0.875X + 3.727$. Bivariate correlation analysis displays that the correlation coefficients (r) are as follows: $r_{K562/ADM+GP7} = 0.999$, $P < 0.01$; $r_{K562+GP7} = 0.964$, $P < 0.05$; $r_{K562/ADM+VP-16} = 0.988$, $P < 0.05$; $r_{K562+VP-16} = 0.957$, $P < 0.05$

higher than those of VP-16, and the inhibitory effects of GP7 or VP-16 on the proliferation of K562 cells were 2.49-fold or 1.83-fold higher than those on K562/ADM cells.

2.2. GP7 attacks cell cycle progression and induces apoptosis of K562/ADM and K562 cells

When treated with 64 μM GP7 or VP-16 for 48 h, flow cytometric analysis of the cell cycle progression showed that GP7-treated K562/ADM and K562 cells accumulated at G_2/M phase with a corresponding decrease at G_1 and S phases. However, VP-16-treated K562/ADM and K562 cells accumulated at S phase with a corresponding depletion of G_2/M phase (Fig. 2). A fraction of cells with hypodiploid DNA content (sub-G_1 peak) representing apoptosis could also be detected by flow cytometry in K562/ADM and K562 cells (Fig. 2). GP7 and VP-16 increased apoptotic ratios of both cell lines, and induced more apoptosis in K562 cells than in K562/ADM cells. However, there was no significant difference between GP7- and VP-16-induced apoptotic ratios.

2.3. GP7 induces apoptotic morphological changes of K562/ADM and K562 cells

Morphological changes typical of apoptosis could be observed under light microscope when 64 μM GP7 or VP-16 treated K562/ADM or K562 cells for 24–72 h (Fig. 3). Membrane blebbing was observed first followed by chromatin condensation, nuclear pyknosis and fragmentation. Apoptotic morphological changes appeared earlier and more obvious in K562 cells than in K562/ADM cells.

2.4. GP7 induces internucleosomal DNA fragmentation in K562/ADM and K562 cells

Internucleosomal DNA fragmentation, which can be observed as "DNA ladder" on agarose gel electrophoresis, is an important biochemical hallmark of apoptosis. When treated with 32–256 μM GP7 or VP-16 for 48 h, both GP7 and VP-16 induced internucleosomal DNA fragmentation in K562/ADM and K562 cells, and "DNA ladder" was observed (Fig. 4). The amounts of DNA fragmentation were quantified by densitograph gel documentation system. The results showed that there was a concentration-dependent relationship in GP7- or VP-16-induced DNA fragmentation (Fig. 5). When treated with the same concentration, GP7- or VP-16-induced more DNA fragmentation in K562 cells than in K562/ADM cells, and 128–256 μM GP7 induced more DNA fragmentation than VP-16 did in both cell lines, but 32–64 μM GP7 induced less DNA fragmentation than VP-16 did in both cell lines (Fig. 5).

2.5. GP7 down-regulates P-gp expression in K562/ADM cells

When treated with 64 or 128 μM GP7 or VP-16 for 48 h, western blot showed that GP7 could down-regulate the expression of P-gp in K562/ADM cells but VP-16 could not (Fig. 6).

3. Discussion

Overexpression of P-gp is one of the underlying mechanisms of MDR. Studies have found that P-gp can protect leukemia cells against caspase-dependent but not caspase-independent cell death (Johnstone et al. 1999; Ruth and Roninson 2000). GP7 can induce apoptotic DNA fragmentation of human leukemia cells through caspase-dependent and -independent pathways (Qi et al. 2004, 2006, 2007). This incited us to further investigate the effects of GP7 on P-gp$^+$ multidrug-resistant leukemia cells.

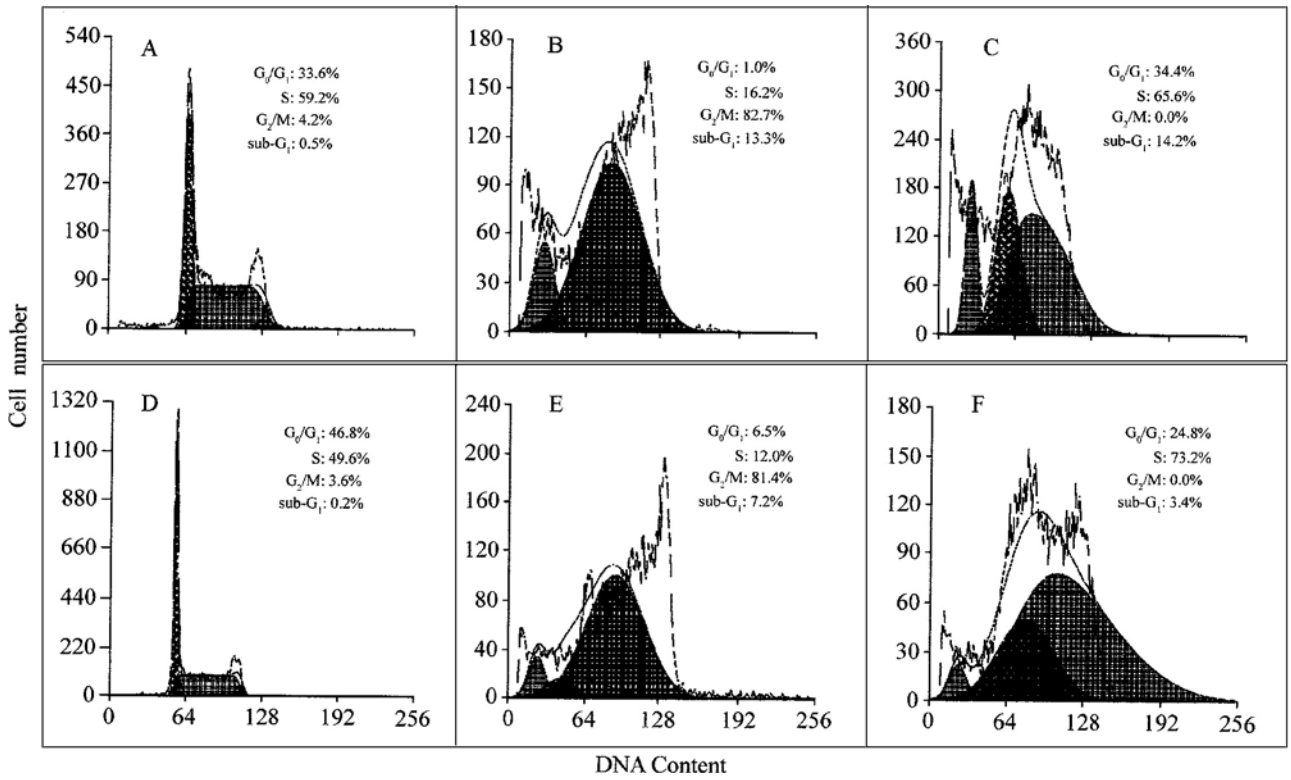


Fig. 2: GP7 attacks cell cycle progression and induces apoptosis of K562/ADM and K562 cells. Cells were treated with 64 μM GP7 or VP-16 for 48 h, and the cell cycle distribution and apoptotic ratio were detected by flow cytometry with PI staining. Results are a representative of three separate experiments. (A) Untreated K562 cells. (B) K562 + GP7. (C) K562 + VP-16. (D) Untreated K562/ADM cells. (E) K562/ADM + GP7. (F) K562/ADM + VP-16

Stable nitroxyl radicals can be utilized to improve anticancer and antioxidant properties of drugs (Zhdanov 1992). The introduction of a nitroxyl moiety can lead to a fast decomposition, higher alkylating, lower carbamoylating activity, better antimelanomic activity, lower general toxicity, and through cell membranes as a transport (Sosnovsky and Li 1985; Zheleva and Gadjeva 2001; Sosnovsky 1992). A previous study has found that a number of nitroxyl spin-labeled derivatives of podophyllotoxin exhibited superior pharmacological properties to the parent compounds (Tian et al. 1997). In the present study, when treated with GP7 or VP-16 for 48 h, the values of IC_{50} show that GP7 is superior to VP-16 in antagonizing the proliferation of both K562/ADM and K562 cells, reflecting the structure superiority of GP7. The inhibitory effects of GP7 on the proliferation of K562 cells were 2.49-fold higher than those on K562/ADM cells, suggesting that overexpression of P-gp in K562/ADM cell extenuates its sensitivity to GP7, and GP7 cannot completely reverse the MDR of K562/ADM cells.

In addition to its ability to efflux toxins, P-gp also inhibits apoptosis induced by a wide array of cell death stimuli relying on activation of intracellular caspases for full function (Johnstone et al. 1999; Ruth and Roninson 2000). In this study, GP7 induced sub-G_1 peak, apoptotic morphological changes and internucleosomal DNA fragmentation in both K562/ADM and K562 cells, confirming that induction of apoptosis is one of the anticancer mechanisms of GP7 (Qi et al. 2004, 2006, 2007). When treated with the same concentration and time, GP7 induced more DNA fragmentation in K562 cells than in K562/ADM cells, suggesting that P-gp overexpression in K562/ADM cell restrains the potency of GP7 to induce its apoptosis, and this is confirmed in part by the increase of DNA fragmentation accompanied by the down-regulation of P-gp when K562/ADM cells were treated with 128 μM GP7 (Figs. 5 and 6). Whether caspases are involved in GP7-induced apoptosis of K562/ADM cells or not needs further investigation.

Both GP7 and VP-16 can induce S phase accumulation and G_2/M phase retardation of Molt 4B cells (Wang et al. 1998).

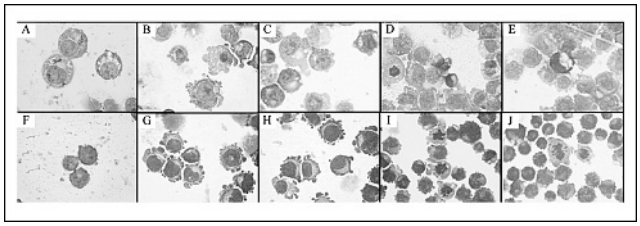


Fig. 3: GP7 induces apoptotic morphological changes of K562/ADM and K562 cells (×1000, original magnification). K562/ADM or K562 cells were treated with 64 μM GP7 or VP-16 for 24–72 h and typical morphology of apoptosis was observed under light microscope after Giemsa staining. (A)–(E) K562/ADM cells. (F)–(J) K562 cells. (A) and (F) Untreated K562/ADM or K562 cells. (B) and (G) Membrane blebbing induced by GP7 at 36 h or 24 h; (C) and (H) Membrane blebbing induced by VP-16 at 36 h or 24 h. (D) and (I) Nuclear pyknosis and chromatin condensation induced by GP7 at 72 h or 48 h. (E) and (J) Nuclear pyknosis and chromatin condensation induced by VP-16 at 72 h or 48 h

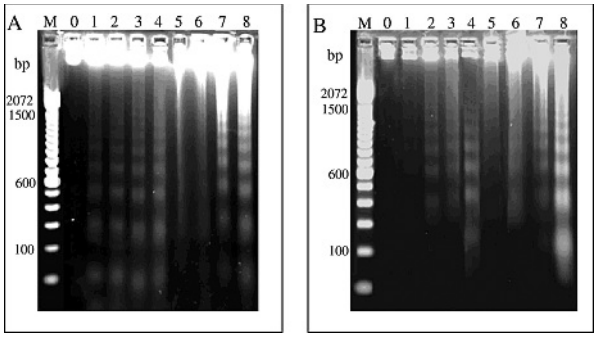


Fig. 4: Agarose gel electrophoresis of DNA in K562 and K562/ADM cells. Cells were untreated (lane 0) or treated with 32, 64, 128 or 256 μM of VP-16 (lanes 1–4, respectively) or GP7 (lanes 5–8, respectively) for 48 h. Results are a representative of three separate experiments. Left lane (M) shows the migration of 100 bp DNA size markers. (A) K562 cells. (B) K562/ADM cells

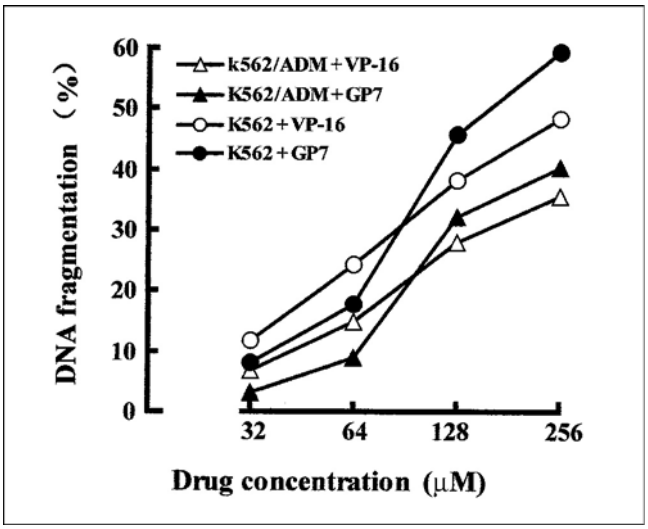


Fig. 5: GP7 induces internucleosomal DNA fragmentation in K562/ADM and K562 cells. Cells were treated with various concentrations of GP7 or VP-16 for 48 h. Cellular DNA was extracted and subjected to agarose gel electrophoresis. The percent of DNA fragmentation was determined from a densitometric scan as described in *Experimental*. Results are a representative of three separate experiments

In the present study, GP7 induced G_2/M phase accumulation but VP-16 induced S phase accumulation in K562/ADM and K562 cells, suggesting that different anticancer mechanisms may exist between GP7 and VP-16 in K562/ADM and K562 cells.

The mechanisms of the resistance to VP-16 include P-gp-mediated MDR, topoisomerase II-mediated MDR (atypical MDR) and multidrug resistance associated protein-mediated MDR (Nooter and Stoter 1996). In this study, VP-16 could not down-regulate P-gp expression in K562/ADM cells. However, GP7 down-regulated P-gp expression in K562/ADM cells, and down-regulation of P-gp expression was accompanied by the increase of IR and DNA fragmentation, implying that the main mechanism of GP7 as MDR modulator is inhibition of P-gp in K562/ADM cells.

4. Experimental

4.1. Materials

GP7 (purity ≥99.9%) was provided by School of Chemistry and Chemical Engineering, Lanzhou University. VP-16 was provided by Jiangsu Hengrui Medical Co., Ltd. (Jiangsu, China. Batch No. H32025583). Stock solution of 10 mM GP7 or VP-16 was made by dissolving reagent in Me_2SO and stored at −20 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), RPMI-1640, fetal calf serum, propidium iodide (PI) and peroxidase-conjugated goat anti-mouse IgG were purchased from Sigma Chemical Co. (St Louis, MO, USA). C219 monoclonal anti-mouse P-gp antibody was purchased from Centocor, Inc. (Malvern, PA, USA). Adriamycin was purchased from Meiji Pharmaceutical Co., Ltd. (Tokyo, Japan). RNase A, proteinase K and 100 bp DNA Marker were purchased from TaKaRa Bio, Inc. (Otsu, Japan).

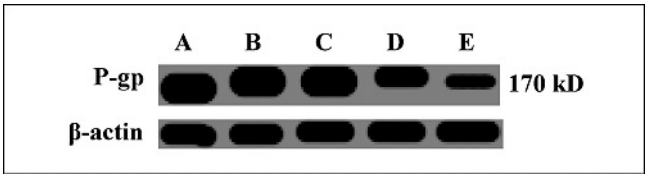


Fig. 6: Western blot analysis of P-glycoprotein expression in K562/ADM cells. K562/ADM Cells were untreated or treated with VP-16 or GP7 for 48 h. β-actin expression was used for the control of equal protein loading. (A) Untreated K562/ADM cells. (B) K562/ADM + 64 μM VP-16. (C) K562/ADM + 128 μM VP-16. (D) K562/ADM + 64 μM GP7. (E) K562/ADM + 128 μM GP7

4.2. Cell culture

Adriamycin-selected and P-gp$^+$ multidrug-resistant human leukemia cell K562/ADM (Tsuruo et al. 1986) and its parental K562 cells were cultured and passaged in our laboratory. All cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum in a humidified atmosphere at 37 °C in 5% CO_2. Adriamycin of 5.2 mM was added regularly in K562/ADM culture medium to maintain the drug resistance. K562/ADM cells were incubated in adriamycin-free medium for over 1 week and used for experiments. During the experiments, all samples, including controls, contained 0.1% Me_2SO, which had no observed effect on any of the assays performed.

4.3. MTT assay for cell proliferation

Approximately 1×10^4 cells per well were incubated in culture medium in 96-well flat bottom microculture plates (Costar, Corning, USA) for various durations, followed by the addition of 10 μL of MTT solution (5 g/L, dissolved in PBS). After 4 h incubation, 100 μL of SDS (10%, w/v, dissolved in 0.01 M HCl) was added and mixed thoroughly to dissolve formazan crystals at 37 °C. After shaking plates for 10 min, the absorbance (A) was measured at 570 nm with a Microplate Reader (EL × 800 Instruments, Bio-TEK, USA). All experiments were performed in quadruplicate and repeated 4 times. The inhibitory ratio (IR) was calculated as follows: IR% = [(mean A value of control groups – mean A value of experimental groups)/mean A value of control groups] × 100%. 50% growth inhibition concentration (IC_{50}) was determined by linear regression method.

4.4. Apoptosis and cell cycle analysis by flow cytometry

After treatment with GP7 or VP-16, cells were washed in PBS by centrifugation, and fixed in ice-cold 70% ethanol at −20 °C for at least 24 h. The cells after fixation were washed in PBS (without Ca^{2+} and Mg^{2+}), and stained with PI solution containing 50 mg/L PI and 50 g/L RNase for 30 min at room temperature in the dark. The sample was read on a Coulter Epics XL flow cytometry (Beckman-Coulter Inc., Fullerton, CA, USA). The percentage of cells in the apoptotic sub-G_1 phase and the distribution of cell cycle were calculated using Multicycle software (Phoenix Flow System, San Diego, CA, USA).

4.5. Analysis of apoptotic morphology by light microscopy

Treated and untreated cells were centrifuged onto slides by cytospin device (Zhengzhou, China) and observed with Giemsa staining by light microscopy.

4.6. DNA extraction and agarose gel electrophoresis

DNA was extracted and electrophoretically separated as described by Prigent et al. (Prigent et al. 1993).

4.7. DNA fragmentation assay

DNA fragmentation was quantified by scanning photographic negatives with a densitograph gel documentation system (ATTO-densitograph, Osaka, Japan) and integrating the area under the curves. Integrated areas were divided at 1500 bp molecular weight marker position into high- and low-molecular weight DNA, and the percentage of DNA fragmentation was determined by dividing the area of low-molecular weight DNA by the total area.

4.8. Western blot analysis of P-gp expression

Plasma membrane fractions from GP7- or VP-16–treated K562/ADM cells were isolated as described by Kawai et al. (Kawai et al. 1990). Membrane-enriched fractions (10 μg proteins) were resolved by electrophoresis in 12% SDS-polyacrylamide gels (SDS-PAGE). The proteins were transferred from the gel to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad) (12 V, 60 min). Membranes were incubated in PBST (PBS with 0.1% Tween-20) containing 5% nonfat dried milk overnight to inhibit nonspecific binding. The membranes were then incubated with monoclonal anti-P-gp antibody C219 for 60 min. After washing in PBST for 30 min, membranes were incubated for another 60 min with peroxidase-conjugated goat anti-mouse IgG. The membranes were then washed and developed with enhanced chemiluminescence (ECL) (Pierce, Rockford, USA). Films were exposed for 1 to 15 min. β-actin expression was used as the internal control.

4.9. Statistical analysis

The data were analyzed using the statistical package SPSS (for Windows). Data obtained from MTT assay were assessed using bivariate correlation and linear regression analysis. Comparisons were made using Student's t-test. $P < 0.05$ was considered statistically significant.

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