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Original Article

Effect of piperlongumine on drug resistance reversal in human retinoblastoma HXO-RB44/VCR and SO-Rb50/CBP cell lines

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Abstract: Piperlongumine (PLGM) was considered as an anti-cancer agent since it was involved in suppressing of many types of cancer. To investigate the functions and mechanisms of PLGM on drug resistance reversal in human retinoblastoma cell lines, drug resistance cell lines HXO-RB44/VCR and SO-Rb50/CBP were established. We found that after treatment with PLGM, drug sensitivity and apoptosis rate of these drug resistance cancer cells were improved, cell cycle was arrested, the expressions of P-gp, MDR1, MRP1, Top-II, GST-π, Survivin, Bcl-2, CDK1, ABCB1 and ABCG1 was decreased, while the activities of caspase-3/8 and intracellular content of Rh-123 was increased. Furthermore, the activities of PI3K/AKT and PKCζ pathways were suppressed following PLGM treatment. Therefore, this study suggests that PLGM could reverse the drug resistance of human retinoblastoma cell lines HXO-RB44/VCR and SO-Rb50/CBP. This drug resistance reversing effect might exert via PI3K/AKT and PKCζ pathways.

Keywords: Piperlongumine-human retinoblastoma-drug resistance

Introduction

Retinoblastoma (Rb) is a rapidly developing cancer that develops from the transformation of the immature cells of a retina, and is the most common malignant tumor of the eye in children [1]. Although Rb is a relatively rare disease, with cumulative lifetime incidence rate of 18000 to 30000 live births worldwide, it is harmful since it could lead to blindness. The priority of Rb treatment is to preserve the life of the patient, then to preserve vision, and then to minimize complications or side effects of treatment. Children with involvement of both eyes at diagnosis usually require chemotherapy and local therapies [2]. In these cases, vincristine and carboplatin are two common chemotherapeutic drugs for treatment. However, in some cases, drug resistance could arise and lead to treatment failure.

Piperlongumine (PLGM) is a natural product constituent of the fruit of the long pepper (*Piper Longum*), a pepper plant found in Southern India and Southeast Asia [3]. Early in 1990s, Duh and colleagues found that PLGM had anticancer potency. However, the profound investigations emerged until 2005, when Bezerra and other researchers disclosed that PLGM has anticancer activities against leukemia, colon carcinoma, melanoma, sarcoma, prostate cancer, head and neck cancer, glioblastoma, and ovarian cancer, both *in vitro* and *in vivo* [4, 5]. In 2011, a letter published on *Nature* showed that PLGM specially targets the stress response to ROS and elevates ROS level in cancer cells, thus selectively killing cancer cells but not normal cells. A further research demonstrated that the ubiquitin-proteasome system in the cancer cells is a target for PLGM in inhibiting cancers [6].

Nevertheless, whether PLGM is able to reverse the chemotherapy resistance has not yet been investigated. In this study, we showed that PLGM could restore the drug sensitivity of drug resistant Rb cancer cells. We also showed that PLGM led to cell cycle arrest and apoptosis. The drug resistance reversing effect of PLGM was related with increasing drug uptake, down-regu-

lating key factors and pathways that involved in drug efflux, cell cycle and apoptosis.

Materials and methods

Cell culture

The human retinoblastoma cell line HXO-RB44 was provided by the cell center of Xiangya Medical College, Central South University, and SO-Rb50 was obtained from the Department of Pathology of the Zhongshan Ophthalmic Center, Sun Yat-sen University. Cells were cultured in RPMI 1640 (Life Technologies) with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL stre

Establishment of drug-resistant cell lines

The drug-resistant cell lines were established via intermittent exposure of the parental cells to gradually increasing concentration of drugs. Use cancer cells that under exponential growth phase and adjusted the cell concentration to 1 × 10^5/mL. For establishment of HXO-RB44/VCR, 75 ng/mL of VCR (QILU Pharmaceutical, Jinan, China) was added to the cell culture for a continuing 2 weeks. Then increase the VCR concentration by 2-fold (150 ng/mL) and culture for another 2 weeks. Displace the drug-contained culture medium with normal culture medium for 2 weeks to facilitate cancer cell recovery before next induction cycle. In the second induction cycle, cells were treated with 150 ng/mL of VCR for 2 weeks and then 300 ng/mL for another 2 weeks. Repeat the induction cycle until the VCR concentration reached 600 ng/mL, and cells could growth normally in such concentration of VCR. For establishment of SO-Rb50/CBP, 7.5 μg/mL of CBP (QILU Pharmaceutical, Jinan, China) was added to the cells for 1 hour and then the drug-contained culture medium was displaced by normal culture medium. Refresh the culture medium in the second day and the following days according to the cell growth state. After cells recovered from the drug shock about 1 month later, increased the CBP concentration by 2-fold (15 μg/mL) and repeated the drug shock-recovery cycle. Such cycle was repeated in the following months with 30, 60, 120, 400, 800, and 1000 μg/mL of CBP respectively. After 10 months induction, cells were able to recover within 1 week from 1 hour shock of 1000 μg/mL CBP. Withdraw drugs treatment for 1 week before following experiments.

MTS assay to determine the drug sensitivity of the cells

Cancer cells were plated in a 96-well plate at a density of 5 × 10^4 cells/well (HXO-RB44/VCR) or 2 × 10^4 cells/well (SO-Rb50/CBP) in RPMI1640 containing 10% FBS. After the cells had been adherent to the wall, 10 or 20 μM PLGM with or without various concentrations of drugs (VCR for HXO-RB44/VCR and CBP for SO-Rb50/CBP respectively) were added to each well accordingly. Six duplicate wells were set up as a group. The culture medium with drugs was replaced at 24-hour intervals to maintain the drug concentration. Forty-eight hours later, 20 μl of 5 mg/ml MTS was added to each well, and the culture was incubated for an additional 4 hours. Then, spectrometric absorbance at a wavelength of 490 nm (A490) was measured. Each experiment was performed three times. The inhibition rate (%) was calculated according to the following formula: (1-experiment absorbance/control group absorbance) × 100%.

Flow cytometry analysis

HXO-RB44/VCR and SO-Rb50/CBP cells were incubated with 10 or 20 μM PLGM for 48 hours. Then cells were suspended in PBS at a density of 1× 10^6 cells/mL. The cells were stained with 10 μM rhodamine-123 (Sigma-Aldrich) for 1 hour and the intracellular concentration of Rh-123 was determined by flow cytometry (BD, FACSCalibur). The expression of p-glycoprotein on the cells surface was determined by using a direct immunoflurescence staining kit purchased from BD Biosciences and performed as described in the product instruction. For cell cycle analysis, the cells were incubated for 10 min in absolute ethanol overnight. Then cells were re-suspended with PBS containing 50 μg/mL RNaseA, 1% Triton X-100 and 40 μg/mL propidium iodide (Sigma-Aldrich) for 30 min at 37°C, and analyzed with flow cytometer at 488 nm wavelength. For apoptosis analysis, Annexin V-FITC/PI dual stain (BD Biosciences) was employed. For caspase-3/8 activation analysis, PE labeled anti-active caspase-3 antibody and anti-active caspase-8 antibody (BD Biosciences) was used respectively.

Western blotting

After treatment with 10 or 20 μM PLGM for 48 hours, the whole cellular proteins were obtained

Table 1. Oligonucleotide sequences used for real-time PCR

Gene	Primer Sequences (5'-3')
MDR1	Forward: AAAAAGATCAACTCGTACCACTC
	Reverse: GCACAAAATACACCAACAA
MRP1	Forward: CTGGGAACATGATTAGGAAGC
	Reverse: GAGGATTTCCCAGAGCCGAC
Topo-II	Forward: CCAGCGGAAACTGAACAGTCAA
	Reverse: TTCCGAGACACGGCACTCAA
GST-π	Forward: TGGTGGACATGGTGAATGAC
	Reverse: ATCTGGTCTCCCACAATGAAG
Bcl-2	Forward: ACGGGGTGAACTGGGGGAGGA
	Reverse: TGTTTGGGGCAGGCATGTTGACTT
Survivin	Forward: GCATGGGTGCCCCGACGT TG
	Reverse: GCTCCGGCCAGAGGCCTCAA
CDK1	Forward: CAG TCT TCA GGA TGT GCT TAT GC
	Reverse: GAGGTTTTAAGTCTCTGTGAAGAA CTC
ABCG2	Forward: CAGGTGGAGGCAAATCTTCGT
	Reverse: ACCCTGTTAATCCGTTCGTTTT
Ku70	Forward: GCTAGAAGACCTGTTGCGGAA
	Reverse: TGTTGAGCTTCAGCTTTAACCTG
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC
	Reverse: GAAGATGGTGATGGGATTTC

Table 2. IC_{50} of HXO-RB44/CVR and SO-Rb50/CBP cells and their parental cells

Cell lines	IC_{50}*
HXO-RB44	45.23 ± 2.5 ng/mL
HXO-RB44/CVR	421.62 ± 5.1 ng/mL
SO-Rb50	5.98 ± 3.6 μg/mL
SO-Rb50/CBP	93.45 ± 4.3 μg/mL

*IC_{50} values were determined by MTS assay. Each value is mean ± SD of the two experiments in which triplicates were assayed.

by RAPI lysis buffer (Millipore) extraction and centrifugation at 12,000 g for 10 min. Total protein concentrations of the supernatants were measured by the BCA kit (Sigma-Aldrich). 20 μg proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes. Target proteins were detected by incubation overnight at 4°C with rabbit anti-MDR1, MRP1, Top-II, GST-π, Bcl-2, survivin, CDK1, β-actin antibody (Santa Cruz). All primary antibodies were diluted according to the manual instruction. Membranes were washed and incubated for 1 hr with peroxidase-labelled anti-rabbit IgG (Santa Cruz, diluted at 1:2000). Finally, membranes were washed three times in TNT and exposed to the Immobilon™ Western chemiluminescent HRP substrate (Millipore) for 1 min, and then exposed autoradiography film for 2~3 min in the dark. The expression and phosphorylation of PI3K, Akt and PKCζ (antibodies also from Santa Cruz) were analyzed by WB as well.

Real-time PCR analysis

After treatment with 10 or 20 μM PLGM for 48 hours, about 3 × 10^6 cells were harvested for RT-PCR analysis. The total mRNA was extracted from the cells by the Dynabeads mRNA Direct Kit (Life Science) according to the manual instruction. Total mRNA was then reverse transcribed for 1 hr at 42°C in incubation buffer containing 250 μM of each deoxynucleotide triphosphate, 5 μM oligo (dT) 20, 25 units of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (Roche Diagnostics). The transcription level of target proteins was detected by semiquantitative real-time PCR using the icycler iQ detection system (Bio-Rad). The PCR condition was as following: decontamination at 50°C for 2 min, denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 20 sec. and at hybridization 60°C for 40 sec. The sequences of primers used in RT-PCR were shown in **Table 1**.

Statistical analysis

Data were expressed as means ± SD., statistical analysis was carried out using One Way ANOVA and $P < 0.05$ indicates statistical significance.

Results

HXO-RB44/CVR and SO-Rb50/CBP cells are highly resistant to chemotherapy drugs

Clones of drug resistant cancer cells were established after several months of continuing exposure to chemotherapy agents. The resistant potency of each cell clone was demonstrated by IC_{50}, as showed in **Table 2**. Both HXO-RB44/CVR and SO-Rb50/CBP were highly resistant to chemotherapy drugs when compared to their parental cells.

PLGM restores drug sensitivity

When administrated alone, 10 or 20 μM PLGM had moderate and dose-dependent effect on the growth of cancer cells, as shown in **Figure**

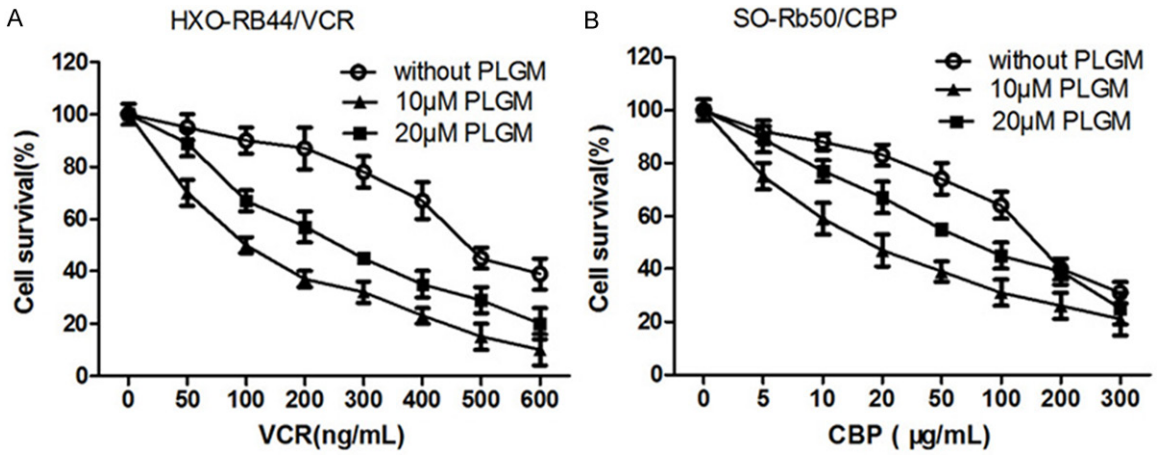


Figure 1. Cell viability of HXO-RB44/CVR and SO-Rb50/CBP cells. Cells treated with or without PLGM for 48 hr, (A) Effect of PLGM on the resistance of HXO-RB44/CVR cells; (B) Effect of PLGM on the resistance of SO-Rb50/CBP cells.

1. Then we examined the sensitivity of each cell clone to its respect drug when co-administrated with 10 or 20 μM PLGM. As shown in **Figure 1** we could see the IC_{50} values of each drug against the resistant cells was lower than that in the control group (without PLGM). These results indicated that PLGM had a synergic anticancer effect with vincristine or carboplatin, and PLGM plays a positive role to reverse the chemotherapy resistance of these cancer cells. The reversing effects of PLGM were also dose-dependent.

Reverse of drug resistance is accompanied with an intracellular accumulation of Rhodamine-123, cell cycle arrest and elevation of apoptosis rate and caspase-3/8 activities

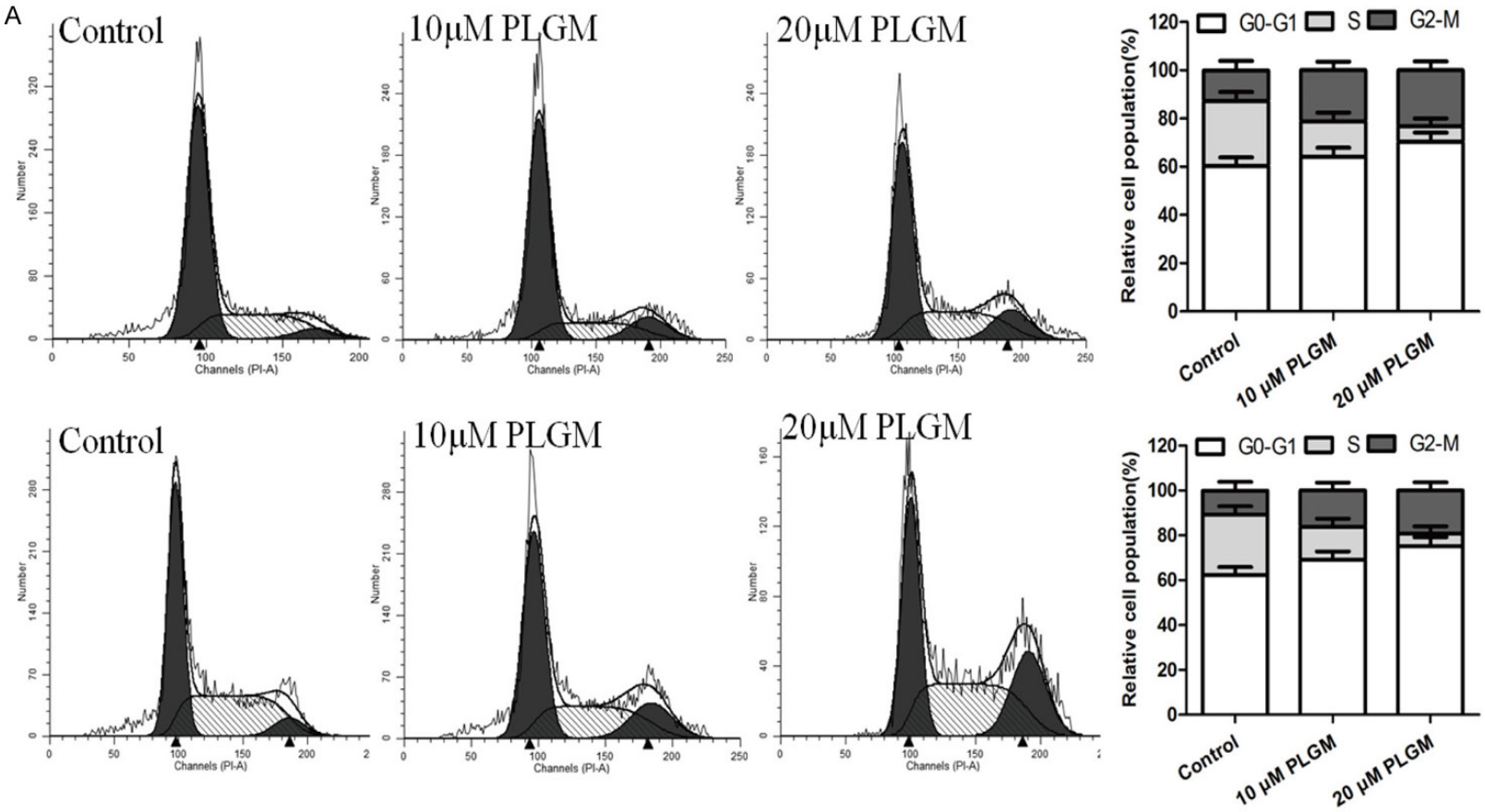
FCM analysis showed that PLGM treated cells had a greater intracellular fluorescent activity after stained with Rh-123, which meant that more Rh-123 was retained inside the cancer cells. Meanwhile, cell cycle analysis showed that after treatment with PLGM, more cancer cells were distributed to G0/G1 and G2/M phases. Moreover, apoptosis rates and caspase-3/8 activities in PLGM treated cells were increased significantly, as shown in **Figure 2**.

Accumulation of intracellular Rh-123 is accompanied by down-regulation of p-gp, MDR1, and MRP1

We investigated whether expression of transporter proteins from ABC family, such as p-gp, MDR1, and MRP1, which have the potency to transport both Rh-123 and chemotherapy agents, were modulated by PLGM. Staining the cells with FTIC-labeled p-gp antibody followed by FCM analysis showed that the mean fluorescence of the cell mass that treated with PLGM shifted to the left significantly when compared to the control cells, which indicated a down-regulation of p-gp. Furthermore, WB and RT-PCR analysis showed that cells from both drug resistant clones expressed more MDR1 and MRP1, whereas, the expression of these proteins was decreased after PLGM treatment (**Figure 3**).

PLGM down-regulates the expression of Top-II, GST-π, survivin, Bcl-2, CDK1, ABCG2 and Ku70

Topo II cut both strands of the DNA helix simultaneously and plays a key role in cell cycle transition from G2 to M phase [7]. GST-π is an intracellular antidotal enzyme that plays an important role in chemotherapy drugs resistance [8]. Bcl-2 and survivin have been implicated in anti-apoptosis, and is also thought to be involved in resistance to conventional cancer treatment [9]. Moreover, CDK1 is a protein which could promote the cell cycle to get through G1/S checkpoint. The ABC subfamily G2 (ABCG2) transporter is critically involved in multidrug resistance of human cancer. These transporters mediate ATP-dependent drug efflux, and are thereby associated with reduction of intracellular drug accumulation. The Ku70 DNA end-joining protein has recently been shown to suppress apoptosis by sequestering BAX from the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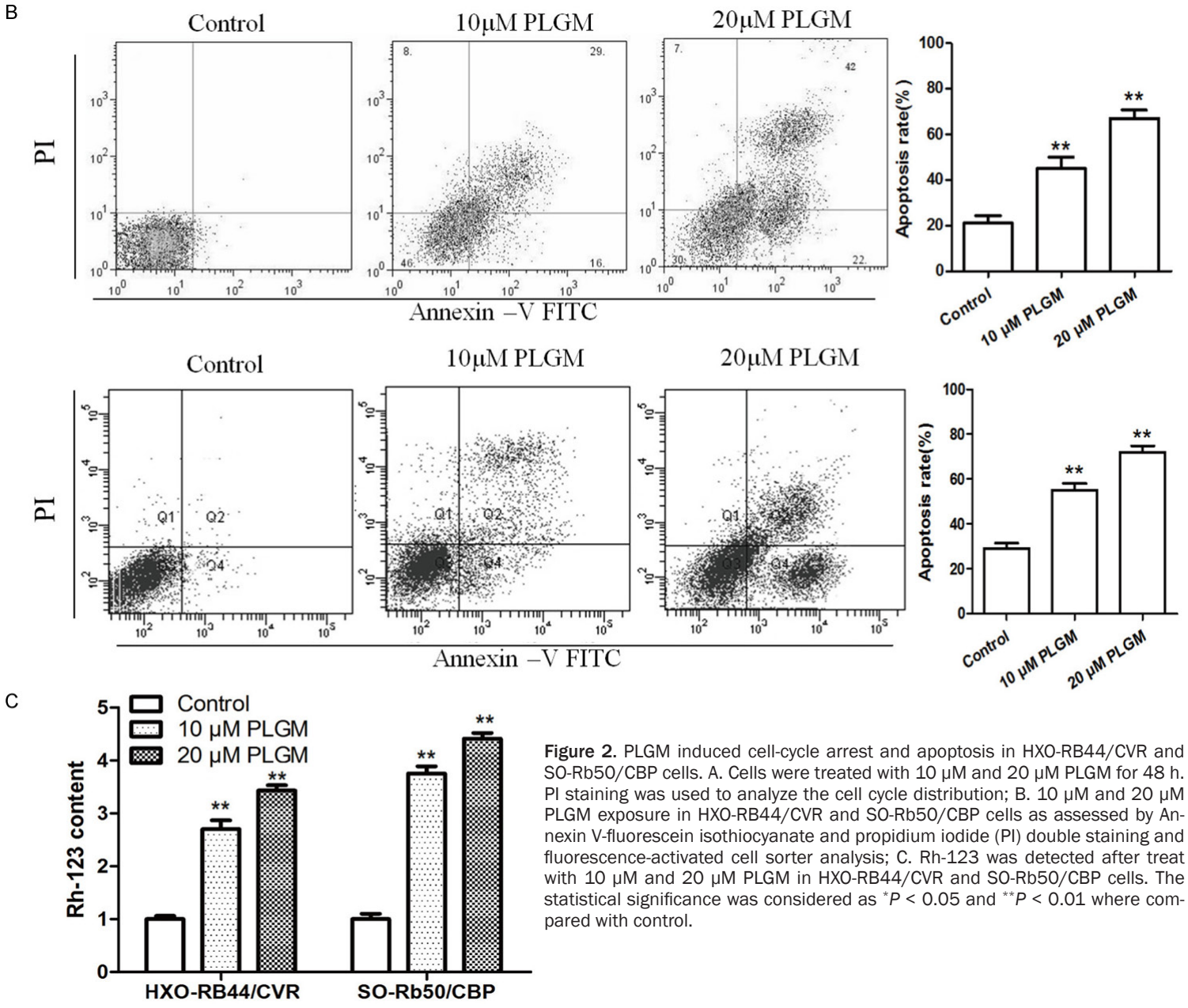

Figure 2. PLGM induced cell-cycle arrest and apoptosis in HXO-RB44/CVR and SO-Rb50/CBP cells. A. Cells were treated with 10 μM and 20 μM PLGM for 48 h. PI staining was used to analyze the cell cycle distribution; B. 10 μM and 20 μM PLGM exposure in HXO-RB44/CVR and SO-Rb50/CBP cells as assessed by Annexin V-fluorescein isothiocyanate and propidium iodide (PI) double staining and fluorescence-activated cell sorter analysis; C. Rh-123 was detected after treat with 10 μM and 20 μM PLGM in HXO-RB44/CVR and SO-Rb50/CBP cells. The statistical significance was considered as $^{*}P < 0.05$ and $^{**}P < 0.01$ where compared with control.

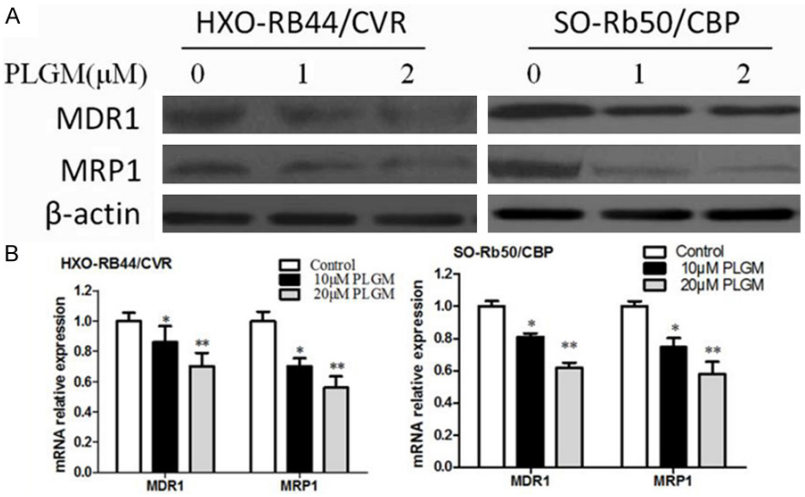

Figure 3. Inhibitory effect of PLGM on expression of MDR1 and MRP1. A. Western blot analysis of protein extracts obtained from HXO-RB44/CVR and SO-Rb50/CBP cells; B. mRNA expressions of MDR1 and MRP1 genes in HXO-RB44/CVR and SO-Rb50/CBP cells. Data presented are means ± SD values, N = 3. Bars indicate SD. Significant differences from control were indicated by $P < 0.05$ (*) and $P < 0.01$ (**).

mitochondria. Ku70 was repoted to binds to and inhibits BAX activation in NSCLC cells in response to gefitinib [10]. As shown in **Figure 4A**, the expressions of Topo-II, GST-π, survivin, Bcl-2 and CDK1 were elevated in the drug resistant cells when compare to their parental cells. However, treatment with PLGM down-regulated the expression of these proteins. Results from RT-PCR were consistent to those from WB, as shown in **Figure 4B** and **4C**.

PI3K/AKT and PKCζ pathways are involved in PLGM mediated drug resistance inversing

The PI3K/AKT pathway is a pathway in cell signaling leading to cell survival by blocking apoptosis. PKCζ pathway leads to increased expression of oncogenes and promotes cancer progression [11]. Here we showed that in drug-resistant cancer cells, the phosphorylation level of PI3K, AKT, and PKCζ was increased significantly, which indicated that they were highly activating. After treated with PLGM, their phosporylation level was decreased significantly, which indicated their activities were attenuated by PLGM (**Figure 5**).

Discussion

In order to investigate the role of PLGM in chemotherapy resistance, we firstly established drug resistant cancer cell lines HXO-RB44/VCR and SO-Rb50/CBP and then determined the anticancer activity of PLGM when being administrated alone or combined with VCR or CBP. In addition to previous studies that indicate PLGM is able to inhibit cancer cell proliferation and viability, our study showed that PLGM had moderate and dosedependent inhibition activities toward drug-resistant cancer cells HXO-RB44/VCR and SO-Rb50/CBP. Moreover, the drug sensitivities of these resistant cancer cells could be restored after treatment with PLGM. Further investigations showed that after PLGM treatment, intracellular accumulation of Rh123 increased, cells cycle was halted at G0/G1 and G2/M phases, and apoptosis rate increased. Therefore, we followed these clues to explore the possible mechanisms of PLGM in mediating resistance reversing.

One major mechanism that involved in drugs resistance is over-expression of special membrane transport proteins which efflux drugs out of cancer cells [12]. The increase of intracellular concentration of Rh-123 after PLGM treatment means that drug efflux by the cancer cells is inhibited by PLGM. By determining the expression of several drug resistant related membrane transport proteins, FCM showed that the expression of p-gp, an important protein of the cell membrane that pumps many foreign substances out of cells, was down-regulated significantly. Then WB and RT-PCR showed that the expression of ABC family transporters MDR1 and MRP1 [13] were down-regulated by PLGM as well. Another drugs resistant mechanism involves increased antidotale activities inside cancer cells [14]. GST-π is a glutathione S-transferase that is able to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification [15]. Here we released that HXO-RB44/VCR and SO-Rb50/CBP over-expressed GST-π, while their expression was lowered after PLGM treatment. Other mechanisms involved

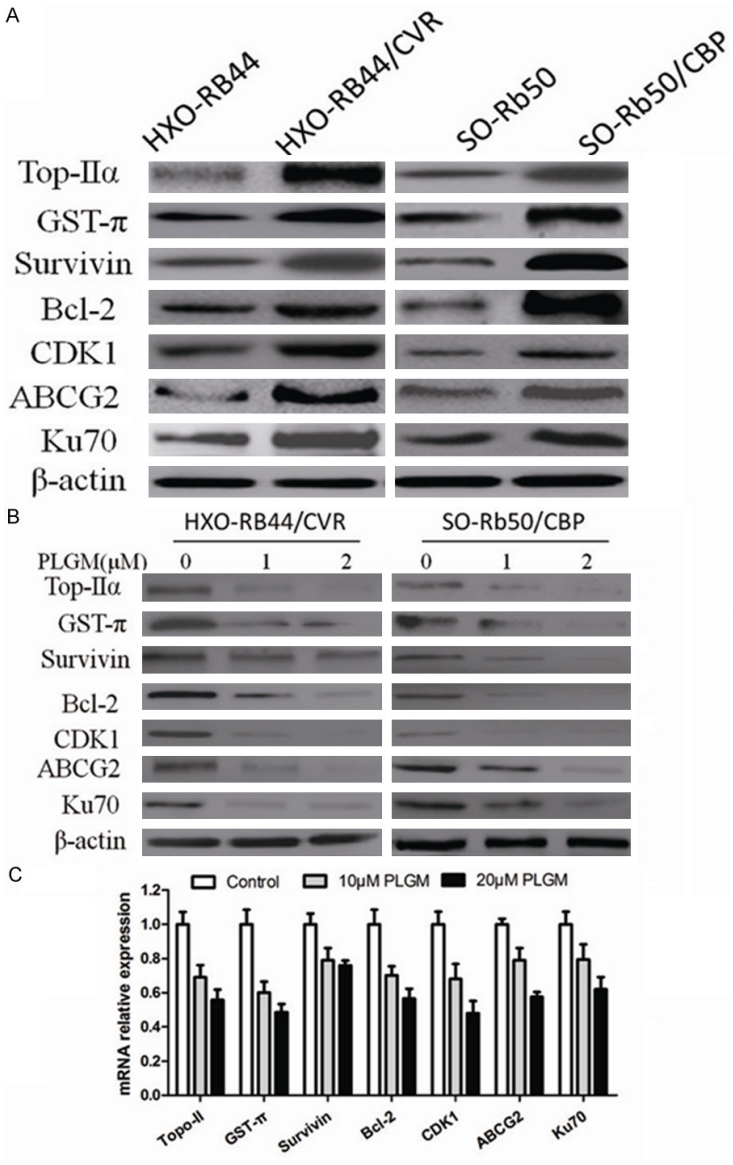

Figure 4. The effect of PLGM on Expression of Drug Resistance Related Gene in HXO-RB44/CVR and SO-Rb50/CBP cells. A. The expression of drug resistance related genes in HXO-RB44/CVR and SO-Rb50/CBP cells compared to their parental cells by western blotting. B. Western blot analysis of protein extracts obtained from HXO-RB44/CVR and SO-Rb50/CBP cells; (mRNA expression of Top-II, GST-π, survivin, Bcl-2, CDK1, ABCG2 and Ku70 genes in HXO-RB44/CVR and SO-Rb50/CBP cells. Data presented are means ± SD values, N = 3. Bars indicate SD. Significant differences from control were indicated by $P < 0.05$ (*) and $P < 0.01$ (**).

in drug resistance include cell cycle promotion and anti-apoptosis effects [16]. Cell cycle analysis showed that PLGM treatment resulted in more cancer cells distributing to G0/G1 and G2/M phases. We found that two key cell cycle modulator proteins, Topo II and CDK1, were increased in HXO-RB44/VCR and SO-Rb50/CBP cells, demonstrating their contributions to drug resistance. However, treatment with PLGM attenuated their expression, which was consistent to the observations that PLGM halted cell cycle of HXO-RB44/VCR and SO-Rb50/CBP at G0/G1 and G2/M phases. FCM analysis also showed that PLGM promoted apoptosis in drug resistant cells. WB and RT-PCR showed that the expressions of Bcl-2 and survivin, which are involved in cancer cell anti-apoptosis, were heightened in HXO-RB44/VCR and SO-Rb50/CBP cells. However, their expressions were down-regulated after PLGM treatment. These results suggest some details about the drug resistance reversing mechanism of PLGM. Firstly, PLGM down-regulates p-gp, MDR1 and MRP1 to alleviate drug efflux and therefore increase intracellular drug concentration in cancer cells. Secondly, PLGM down-regulates GST-π to prevent chemotherapy drug from detoxification. Thirdly, PLGM down-regulates the expression of Topo II, CDK1, survivin and Bcl-2 to halt the cell cycle and promote apoptosis, which will sensitize cancer cells to chemotherapy drugs.

To further investigate whether some critical cell signaling pathways are involved in PLGM mediated drug resistance reversing, we firstly determined the phosphorylation of PI3K and AKT prior and after PLGM treatment. PI3K/AKT pathway is an intracellular signaling pathway important in apoptosis and hence cancer progression and drug resistance. PI3K and AKT are phosphorylated sequentially by many membrane receptors to become active and then trigger the signaling cascade by phosphorylating downstream proteins. Our western blotting assay showed that phosphorylation levels of PI3K and AKT were elevated in HXO-RB44/VCR and SO-Rb50/CBP when compared to their parental cells, but attenuated after PLGM treatment in a dose-dependent manner. PKCζ has

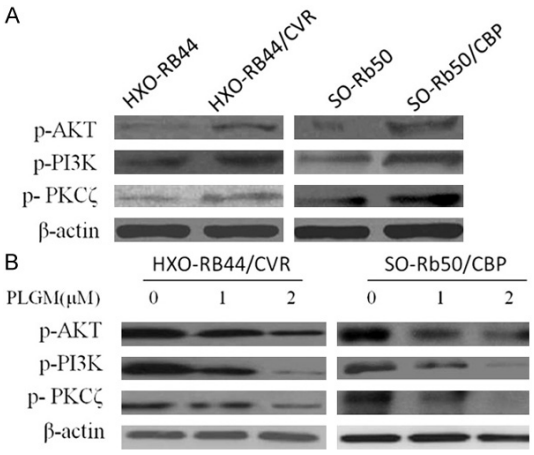

Figure 5. PLGM affect on PI3K/AKT and PKCζ *pathways* signal pathway molecules in HXO-RB44/CVR and SO-Rb50/CBP cells. A. The phosphorylation level of PI3K, AKT and PKCζ in HXO-RB44/CVR and SO-Rb50/CBP cells compared to their parental cells by western blotting; B. Immunoblotting for the proteins PI3K, AKT and PKCζ. Immunoblot analyses were performed on the lysates of HXO-RB44/CVR and SO-Rb50/CBP cells that had been incubated with 10 μM and 20 μM PLGM for 48 h, Equal protein loading was verified by β-actin immunoblotting. Three independent experiments were performed. The statistical significance was considered as $^{*}P < 0.05$ and $^{**}P < 0.01$ where compared with control.

been found in the regulation of the motility of hematopoietic cells and also works coordinately with other PKC subfamily members and other signaling pathways [17]. Those PKCζ mediated pathways lead to increased expression of oncogenes and promoting cancer progressing. PKCζ has an N-terminal regulatory domain, which does not require calcium or diacylglycerol (DAG) to become active, but rather relies on a different second messenger, presumably generated through a PI3K pathway. However, in addition to binding with a second messenger, phosphorylation is essential to the kinase activity of PKC [18]. Thus we measured the phosphorylation level of PKCζ to address its activity. Similar to PI3K/AKT pathways, phosphorylation levels of PKCζ was elevated in HXO-RB44/VCR and SO-Rb50/CBP when compared to their parental cells, but attenuated after PLGM treatment. These results demonstrate that PI3K/AKT and PKC- pathways are involved in both drug resistance of HXO-RB44/VCR and SO-Rb50/CBP and PLGM mediated drug resistance reversing.

In summary, we found that PLGM could reverse chemotherapy resistance of retinoblastoma cancer cells in a dose-dependent manner. Treatment with PLGM leads to increased drug uptake, cell cycle arrest and apoptotic cell death. Our study also suggested that critical proteins, such as g-pg, MDR1, MRP1, Topo II, GST-π, survivin, Bcl2, and CDK1, and cell signaling pathways such as PI3K/AKT and PKCζ were involved in PLGM mediated drug resistance reversing.

Disclosure of conflict of interest

None.

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