High cytotoxic sensitivity of the human small cell lung doxorubicin-resistant carcinoma (GLC4/ADR) cell line to prodigiosin through apoptosis activation

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In the present study, we describe the cytotoxicity of the new drug prodigiosin (PG) in two small cell lung carcinoma (SCLC) cell lines, GLC4 and its derived doxorubicin-resistant GLC4/ADR cell line, which overexpresses multidrug-related protein 1 (MRP-1). We observed through Western blot that PG mediated cytochrome *c* release, caspase cascade activation and PARP cleavage, thereby leading to apoptosis in a dose–response manner. MRP-1 expression increased after PG treatment, although that does not lead to protein accumulation. The MTT assay showed no difference in sensitivity to PG between the two cell lines. Our results support PG as a potential drug for the treatment of lung cancer as it overcomes the multidrug resistance phenotype produced by MRP-1 overexpression. *Anti-Cancer Drugs* 16:393–399 © 2005 Lippincott Williams & Wilkins.

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Introduction

Apoptosis is involved in the action of many (if not all) chemotherapeutic agents. In most cases, apoptosis is accompanied by cytochrome *c* release from the mitochondria into the cytosol. Then caspases can be activated and generate the characteristic apoptotic morphology (chromatin condensation, membrane blebbing, cell shrinkage, DNA cleavage, etc). Resistance to chemotherapy is the main cause of failure in the treatment of human cancer. One major mechanism of resistance is linked to decreased intracellular accumulation of anticancer drugs through enhanced cellular efflux of the antitumor compound [1]. MRP-1 is an efflux pump that belongs to the family of ABC transporters and is frequently overexpressed in clinical samples from patients with small cell lung cancer (SCLC) [2]. Cytotoxic drugs irrespective of their intracellular target cause cell death in sensitive cells by inducing apoptosis [3]. Some members of a family of natural bacterial pigments called prodigiosins (PGs) induce apoptosis in several human cancer cell lines [4–6] and in hepatocellular carcinoma xenografts [7]. The aim of this study is to describe the apoptosis induction by PG treatment in a doxorubicin-resistant SCLC cell line compared to its parental cell line. Here we studied the ability of PG to overcome the multidrug resistance (MDR) phenotype as well as the cytotoxic effect induced in the MRP-1-overexpressing GLC4/ADR cell line, finding interesting parallels with what we have previously described in the doxorubicin-sensitive GLC4 cell line [8].

Methods

Cell lines and culture conditions

The human lung cancer GLC4 cell line and its doxorubicin-resistant subline GLC4/ADR were derived in the laboratory of N. H. Mulder [9]. GLC4/ADR cells were exposed to 1172 nM doxorubicin (Sigma, St Louis, MO) during 48 h once every 15 days to maintain their resistance characteristics. All the experiments using GLC4/ADR were performed after 7 days of non-exposure to doxorubicin treatment. Both cell lines were cultured in RPMI 1640 medium with 10% FCS (Biological Industries, Beit Haemek, Israel) supplemented with 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml (Sigma) at 37°C with 5% CO_2.

Purification of PG

PG was isolated from a culture broth of *Serratia marcescens 2170* as described previously [4]. Stock solutions were prepared in methanol and concentrations were determined by UV/vis in 95% EtOH–HCl ($\epsilon_{535} = 112\,000$ M/cm).

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Cell viability assay

Cell viability was determined by the MTT assay [10]. Briefly, 5×10^4 cells were incubated in 96-well microtiter cell culture plates, in the absence (control cells) or presence of 20–240 nM PG to a final volume of 100 µl. After 4, 8, 16 or 24 h incubation, 10 µl of MTT (diluted in PBS) was added to a final concentration of 10 mM for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 µl of isopropanol:1 N HCl (24:1) and the absorbance at 550 nm was measured on a multiwell plate reader. Cell viability was expressed as a percentage of control. Data are shown as the mean value ± SD of triplicate cultures.

Western blot analysis

Cells (5×10^5 cells/ml) were exposed to 100, 150 or 200 nM PG for 16 h, except when MRP-1 was analyzed (100, 200 or 300 nM PG for 24 h), they were then washed twice with PBS and lysed with ice-cold lysis buffer (85 mM Tris–HCl, pH 6.8, 0.4% SDS, 0.1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin). Protein concentration was measured using the micro BCA Protein Assay Reagent Kit (Pierce, Rockford, MD). Protein extracts were electrophoresed on a polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% dry-milk diluted in TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween-20) and incubated overnight at 4°C. The rabbit polyclonal antibodies used were as follows: cleaved caspase-3 (Asp175) (New England Biolabs, Beverly, MA), caspase-8 (BD PharMingen, San Diego, CA), cleaved caspase-9 (37 kDa) (New England Biolabs) and PARP (New England Biolabs). Monoclonal antibody to MRP-1 (human) MRPm6 (Alexis Biochemicals, Lausen, Switzerland) and purified mouse anti-cytochrome *c* monoclonal antibody (BD PharMingen) were also used.

The peroxidase-conjugated secondary antibodies used were goat anti-rabbit IgG (170-6515; Bio-Rad, Hercules, CA) and goat anti-mouse (170-6516; Bio-Rad). Peroxidase was then developed by incubating the membrane with the enhanced chemiluminescence (ECL) detection kit (Amersham, Little Chalfont, UK). Protein expression of Western blot images was quantified using the image analysis software program Phoretix 1-D advanced. Results are presented relative to the control densitometry values.

Cytochrome *c* detection assay

In time-course cytochrome *c* detection assays, cells were harvested after a 15-min to 12-h exposure to 200 nM PG and prepared as previously described [11], with slight modifications. Briefly, cells were lysed for 30 s in 50 µl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 0.1 mM PMSF). Lysates were centrifuged at 13 000 *g* at 4°C for 2 min to obtain the supernatants (cytosolic extracts) and the pellet (fraction with mitochondria).

Analysis of DNA fragmentation

DNA fragmentation was analyzed by agarose gel electrophoresis, as described previously [6]. Briefly, 5×10^6 cells/ml were treated with 100 or 200 nM PG for 16 h or were left untreated (control). DNA preparations were electrophoresed in a 1% agarose gel containing ethidium bromide. Gels were placed in a UV light box to visualize the DNA ladder pattern.

Gene expression analysis

Cells (5×10^5) were treated with 0 (control), 100, 200 or 300 nM PG during 24 h. Total RNA extraction was performed using Ultraspec RNA (Biotex, TX). cDNA synthesis was obtained using random hexamers and MuLV reverse transcriptase after washing the RNA pellet twice in 75% ethanol, dissolved in DEPC-treated water, following the manufacturer's instructions. The final concentration of cDNA was 1 µg in 50 µl. Each cDNA sample was analyzed for expression of MRP-1 using the fluorescent TaqMan 5′ nuclease assay. Oligonucleotide primers MRP-1 (Hs00219905) and actin (Hs99999903) and probes were initially designed and synthesized as Assay-on-Demand Gene Expression Products (Applied Biosystems, Warrington, UK). The 5′ nuclease assay PCRs were performed using the ABI Prism 7700 sequence detection system for thermal cycling and real-time fluorescence measurements (Applied Biosystems). Each 50-µl reaction consisted of 1 × TaqMan Universal PCR MasterMix (PE Biosystems), 1 × Assay-on-Demand mix containing forward primer, reverse primer and TaqMan quantification probe (Applied Biosystems), and 100 ng cDNA template. Reaction conditions comprised an initial step of 92°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The levels of MRP-1 obtained were normalized by mRNA expression of actin. The relative mRNA expression for MRP-1 was thus presented as relative to the control. Data were analyzed using The Sequence Detector Software (SDS version 1.9; Applied Biosystems).

Statistical comparison of mean values was performed using Student's *t*-test.

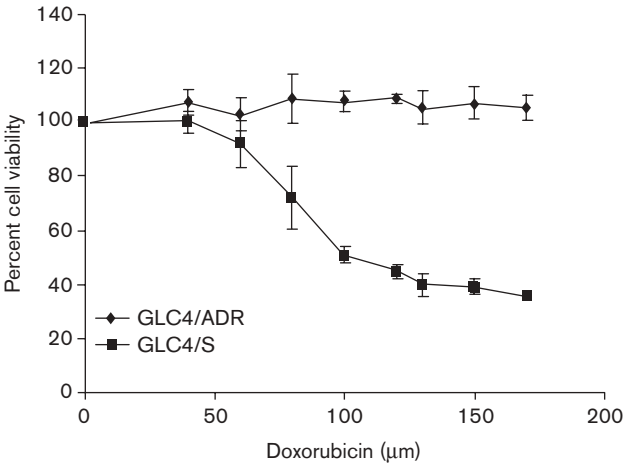
Results

PG decreases the viability of GLC4 and GLC4/ADR cells

First at all, we proved the doxorubicin sensitivity and resistance phenotypes of both cell lines (Fig. 1). Then, the effect of PG on the viability of human SCLC cell lines (GLC4, GLC4/ADR) was studied. Cell lines were incubated for 4, 8, 16 or 24 h with several doses of PG, ranging from 20 to 200 nM, and cell viability was then determined by the MTT assay. A significant dose-dependent decrease in the number of viable cells was

observed in GLC4 and GLC4/ADR cells and no marked differences were detected between them (Fig. 2A and B). Time-course experiments showed a marked decrease in the IC_{50} value as incubation time increased. GLC4 IC_{50} was 129.40 ± 17.10 and 104.59 ± 5.72 nM at 16 and 24 h of PG incubation, respectively. In contrast, GLC4/ADR presented an IC_{50} value of 143.16 ± 24.57 nM at 16 h, which decreased to 111.40 ± 4.27 nM at 24 h. Therefore, we can conclude that there is no significant difference between the viability of GLC4 and GLC4/ADR cells when giving the same PG treatment.

Fig. 1



Doxorubicin resistance. Samples of 2×10^6 cells per condition were incubated for 24 h in a 96-well plate at the indicated doxorubicin concentrations. As expected, resistance to doxorubicin was proved in GLC4/ADR, but not in its parental cell line. Results depicted represent the mean of three independent experiments. Error bars represent SD.

Apoptotic features

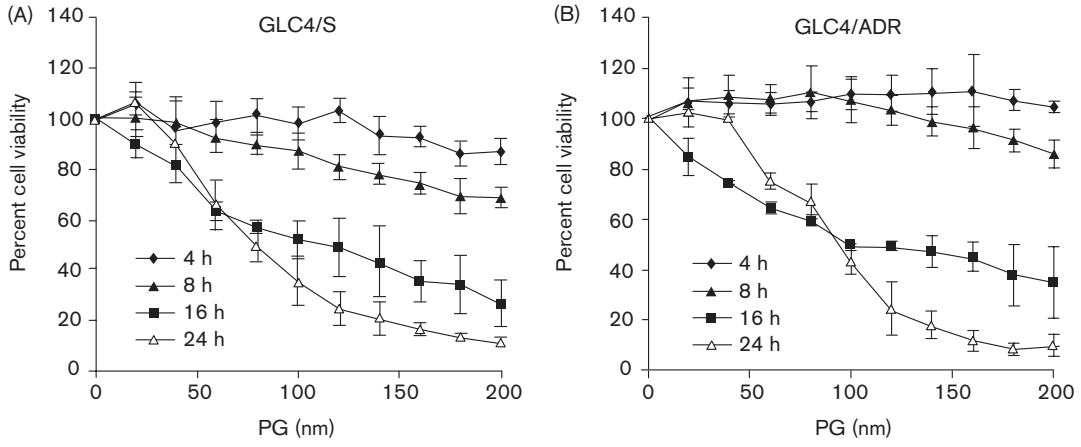
One of the main biochemical features associated with apoptosis is caspase activation. PG induced the processing of caspases, as shown by the appearance of the active cleavage products of caspases-8 (23 kDa) and -3 (17 kDa) (Fig. 3). The appearance of the caspase-9 intermediate cleaved product (37 kDa) and the disappearance of the precursor form were also determined by Western blot in whole-cell extracts (Fig. 3).

PARP cleavage, as a result of caspase-3 activation, was analyzed on protein extracts from cells incubated with 100, 150 and 200 nM of PG by immunoblotting as a specific marker of caspase activity. In PG-treated cells, both the native PARP (116 kDa) and the cleavage product (85 kDa) were observed (Fig. 4A). Agarose gel electrophoresis showed the characteristic DNA ladder pattern induced in the apoptotic process in the two cell lines when incubated for 16 h in the presence of 100 and 200 nM PG (Fig. 4B).

Cytochrome *c* involvement

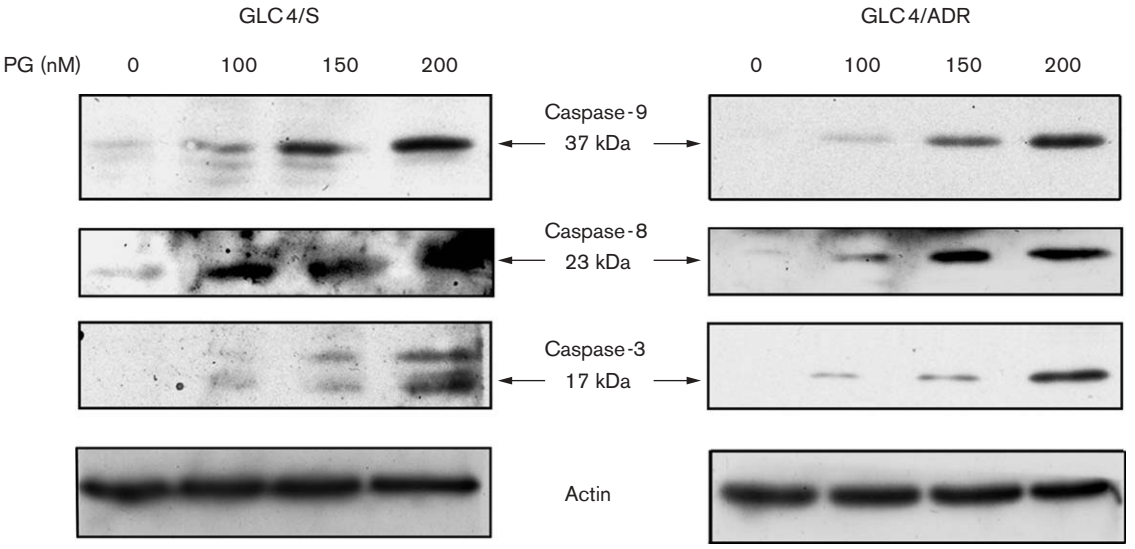
There is evidence that mitochondria play an essential role in many forms of apoptosis by releasing apoptogenic factors as cytochrome *c*. To analyze the involvement of cytochrome *c* release in PG-induced apoptosis, cytosolic and mitochondrial fractions were obtained and analyzed for the presence of cytochrome *c* by Western blot. Cell lines were incubated for 16 h with three doses of PG (100, 150 and 200 nM). PG induced the appearance of cytochrome *c* in the cytosolic fractions in a dose–response manner in both cell lines (Fig. 5A). In time-course experiments (Fig. 5B), we demonstrated that PG induced the appearance of cytochrome *c* in these fractions after 15 min of drug exposure in both cell lines.

Fig. 2



Effect of PG treatment on the viability of GLC4 (A) and GLC4/ADR (B) cell lines by the MTT assay. Cell viability decreases in a dose–response manner, and no significant differences between sensitive and resistant cell lines are observed. The results represent the mean of three independent experiments. Error bars show SD.

Fig. 3



PG induces the activation of caspases. Western Blotting of 50 μg of whole-cell protein extract was used. Cleavage of caspase-9, -8 and -3 was observed after 16 h of PG treatment in a dose–response manner. A representative result from three independent experiments is shown. Control of protein loading by actin is shown in the bottom panel.

Densitometric analysis of the blots corresponding to Figs 3, 4(A) and 5 to quantify the intensity of the bands confirmed no differences between both cell lines (data not shown).

Quantification of MRP-1 mRNA and MRP-1 protein

Our study demonstrates that PG circumvents the MDR phenotype acquired by the GLC4/ADR cell line (mainly caused by MRP-1 overexpression) as PG treatment induces similar cell viability loss and biochemical apoptotic features in both sensitive and resistant cell lines. The PG treatment effect in MRP-1 mRNA expression was measured by quantitative PCR (Q-PCR). The relative levels of MRP-1 mRNA expression in GLC-4/ADR cells (Fig. 6A) increased slightly after PG exposure. However, at the protein level, MRP-1 decreased in a dose–response fashion after the first dose (Fig. 6B and C) and it was hardly detectable at the highest dose of PG ($p < 0.05$). The levels of MRP-1 mRNA and protein were also studied for GLC4, but no effect of PG was observed in this cell line (data not shown).

Discussion

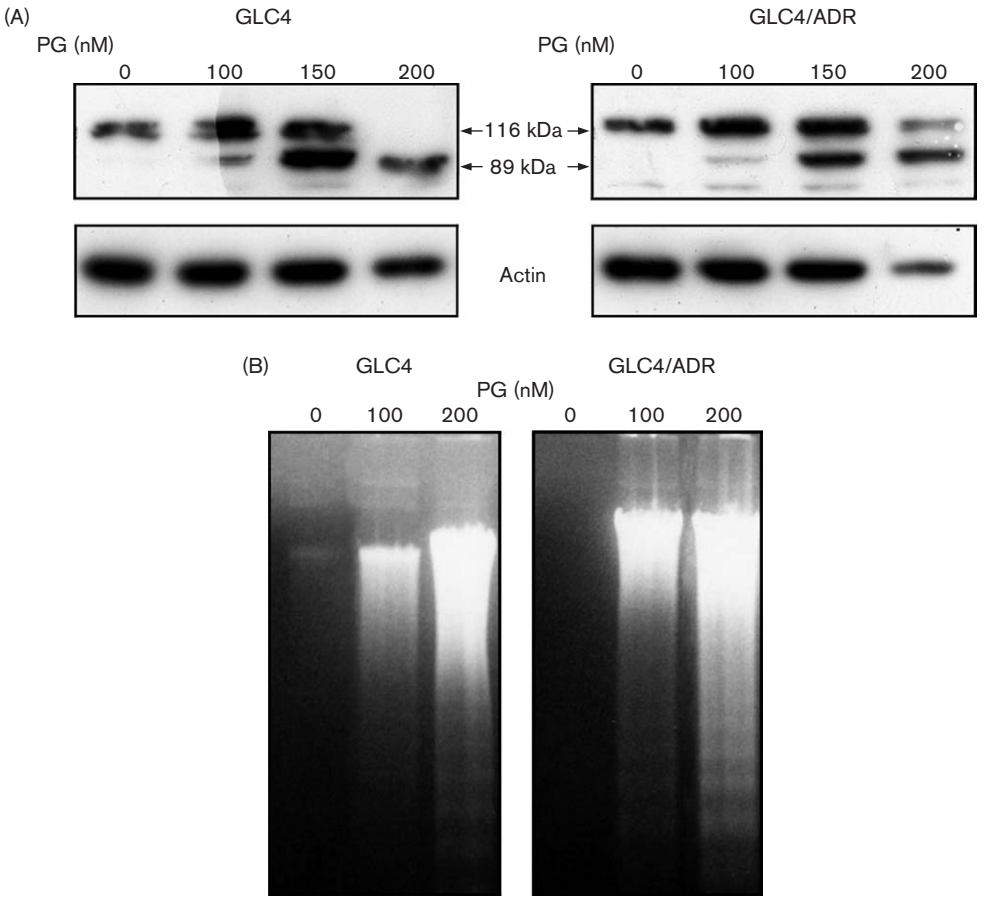
PG exerts its cytotoxic effect in the MDR phenotype GLC4/ADR cell line and its parental GLC4 cell line in a dose-dependent manner. GLC4 and GLC4/ADR have been used as a model to study the effect of several novel or established lung cancer chemotherapy agents [12,13]. The resistant cell line shows cross-resistance not only to doxorubicin, but also to topotecan and paclitaxel [14]. However, PG treatment results in an equivalent decrease in cell viability for both cell lines. It is important to note the low IC_{50} concentration obtained for PG in these cell lines when compared with cisplatin which is one of the most commonly used drugs in SCLC treatment in the US [15], i.e. the IC_{50} values of cisplatin at 72 h are 2000 and 3000 nM in GLC4 and GLC4/ADR, respectively [14].

PG induces apoptosis in hematopoietic, colon and gastric cancer cell lines [4–6]. Cycloprodigiosin hydrochloride, another member of the PG family, also has a pro-apoptotic effect in hepatocarcinoma cells *in vitro* and *in vivo* [7]. Moreover, an apoptotic effect has also been described in human primary cancer cells [16]. However, the mechanism by which PG induces apoptosis remains unclear, although several pathways have recently been hypothesized [17].

Here, we have confirmed the activation of the apoptotic process by analyzing biochemical events such as caspase activation, PARP cleavage and DNA ladder pattern formation as well as the cytochrome *c* release involvement in a doxorubicin-resistant SCLC model. We observed a slight delay in caspase-8 activation in GLC4/ADR, but the final apoptosis execution is performed by caspase-3, which is activated in the same way in both sensitive and resistant cell lines.

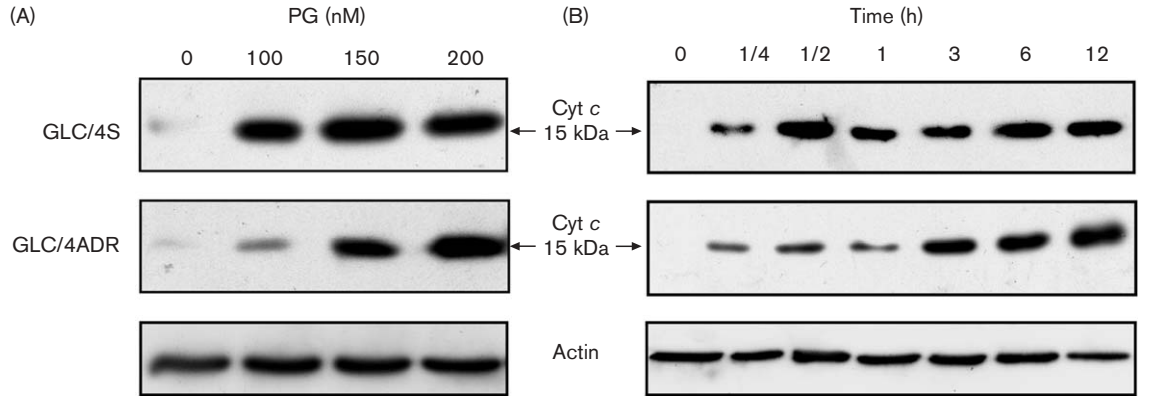
Our study demonstrates that PG circumvents the MDR phenotype acquired by the GLC4/ADR cell line (mainly caused by MRP-1 overexpression) as PG treatment

Fig. 4



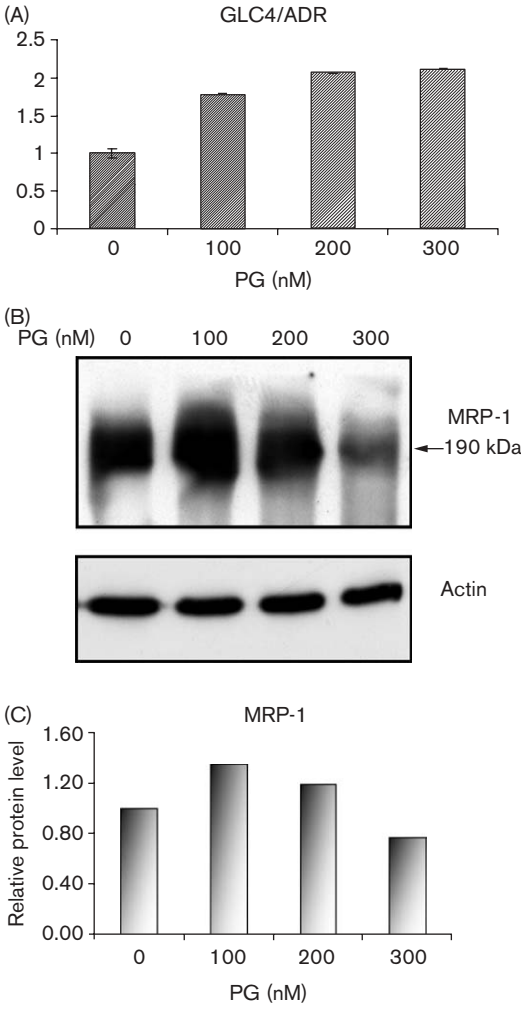
PG apoptotic induction. (A) PARP cleavage in a dose–response assay after PG treatment is observed by Western blot. The bottom shows actin as a loading control. (B) DNA fragmentation induced by PG is detected in agarose gel electrophoresis.

Fig. 5



Western blot analysis of cytochrome *c* release from mitochondria in PG-treated cells. Samples of 30 μg of protein from the cytosolic fraction were used. (A) PG induces the appearance of cytochrome *c* in the cytosolic fraction in a concentration–response manner. (B) Time-course assay shows release of cytochrome *c* after 15 min of 200 nM PG treatment. A representative study of three independent experiments is shown. Bottom shows actin as a loading control.

Fig. 6



PG effect in MRP-1 (A). The results are shown as the relative expression of MRP-1 mRNA after cell treatment with PG normalized by actin mRNA. A slight increase in the resistance protein is observed. Error bars represent SD. (B) MRP-1 protein detected by Western blot. Samples of 30 μg of protein were electrophoresed. MRP-1 slightly decreases after PG treatment. Figures show a representative result from three independent experiments. (C) Western blot quantification is represented by bars. MRP-1 protein increases at low PG concentration (100 nM), but decreases when higher doses are added.

induces similar cell viability loss and biochemical apoptotic features in both sensitive and resistant cell lines. We have previously reported that PG is not a substrate for another ABC transporter family member [BCRP (breast cancer resistance protein)] [18]. Results presented here indicate that PG might not be a substrate for MRP-1. The relative levels of MRP-1 mRNA expression in GLC-4/ADR cells increased slightly after PG exposure although MRP-1 protein decreased in a dose–response fashion after dose 1. This finding indicates that PG could have a novel and useful activity in this aspect, as the increase in MRP-1 has been reported in most SCLC patients, and in leukemia, esophageal carcinoma and non-SCLC [19]. Interestingly, Versantvoort and collaborators hypothesized that the GLC4 cell line, probably like most cell lines *in vitro*, reacts to low chemical selective pressure by increasing the MRP-1 detoxifying protein [20], thereby allowing the cell to pump chemotherapy agents such as doxorubicin, epirubicin, etoposide, vincristine and methotrexate [21]. We have also observed an increase of MRP-1 protein at the lower dose followed by decrease when higher doses of PG were used. Here we report that PG blocks the increase in MRP-1 protein levels, an effect that supports its use in combined therapy as well as describes a new property that could be added to those of PG already described [17].

To our knowledge, this is the first study that shows the cytotoxic activity of a member of the PG family in a doxorubicin-resistant SCLC cell line. Given the high sensitivity of the GLC4 and GLC4/ADR cell lines to PG compared with other commonly used drugs, we conclude that PG is a potential novel chemotherapy agent for lung cancer, particularly for SCLC.

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