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Mitochondria-mediated apoptosis operating irrespective of multidrug resistance in breast cancer cells by the anticancer agent prodigiosin

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Abstract

Prodigiosin (PG) is a red pigment produced by *Serratia marcescens* with pro-apoptotic activity in haematopoietic and gastrointestinal cancer cell lines, but no marked toxicity in non-malignant cells. Breast cancer is the most frequent malignancy among women in the European Union and better therapies are needed, especially for metastatic tumors. Moreover, multidrug resistance is a common phenomenon that appears during chemotherapy, necessitating more aggressive treatment as prognosis worsens. In this work, we extend our experiments on PG-induced apoptosis to breast cancer cells. PG was potently cytotoxic in both estrogen receptor positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines. Cytochrome *c* release, activation of caspases-9, -8 and -7 and cleavage of poly (ADP-ribose) polymerase protein typified the apoptotic event and caspase inhibition revealed that PG acts via the mitochondrial pathway. In a multidrug-resistant subline of MCF-7 cells that over-expresses the breast cancer resistance protein, the cytotoxic activity of PG was slightly reduced. However, flow-cytometry analysis of PG accumulation and efflux in MCF-7 sublines showed that PG is not a substrate for this resistance protein. These results suggest that PG is an interesting and potent new pro-apoptotic agent for the treatment of breast cancer even when multidrug resistance transporter molecules are present.

Keywords: Prodigiosin; Breast cancer; Estrogen receptor positive/negative; Mitochondria-mediated apoptosis; Caspases; Multidrug resistance

1. Introduction

Breast carcinoma represents the third of all cancers diagnosed in women and is the second leading cause of cancer death in Western European and North American women (American Cancer Society). Cytotoxic chemotherapy plays an important role in the management of patients with hormone-insensitive or metastatic breast carcinoma, although most of them ultimately develop recurrences. Therefore, there is a need for novel cytotoxic agents and treatment strategies in patients with advanced breast carcinoma that is refractory to conventional chemotherapy [1].

Apoptosis is a physiologically programmed mechanism of cell death involved in cellular stress response, such as genotoxic agents exposure [2]. One of the major proteins involved in this process is the tumor suppressor protein p53, which mediates either cell cycle arrest or apoptosis [3]. Two major pathways mediating drug-induced apoptosis have been characterized; one requires the activation of cell surface receptors, whilst the other directly targets mitochondria [4]. Both apoptotic signals seem to be integrated at the mitochondrial level and are typically accompanied by the activation of aspartate-specific proteases called caspases [5]. Whilst the former induces caspase-8 activation, the mitochondrial pathway leads to the release of apoptogenic factors such as cytochrome *c* (cyt-*c*), which binds Apaf-1 and procaspase-9, inducing caspase-9 activation in the cytoplasm [6]. Both pathways then activate the effector caspases-3 and -7, which cleave a number of

Abbreviations: ABC, ATP binding cassette; BCRP, breast cancer resistance protein (ABCG2/MXR); ER+/−, estrogen receptor positive/negative; MDR, multidrug resistance; MRP-1, multidrug resistance protein 1 (ABCC1); MTT, methyl-thiazole-tetrazolium; PARP, poly (ADP-ribose) polymerase protein; PG, prodigiosin; P-gp, P-glycoprotein (MDR1/ABCB1); Z-VAD.fmk, Z-Val-Ala-dl-Asp (OMe)-fluoromethylketone

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substrate proteins, including the poly (ADP-ribose) polymerase protein (PARP).

Apoptosis-inducing compounds are candidate antitumor agents. In this view, prodigiosin (PG), a red bacterial pigment with a pyrrolylpyrromethene skeleton, has a number of concentration-dependent effects as an immunosuppressive agent [7,8] and has significant anti-neoplastic activity against a variety of human cancer cells, including haematopoietic and gastrointestinal cells, with no marked toxicity in non-malignant cell lines [9–12]. Furthermore, the apoptotic drug PG triggers the reorganization of actin cytoskeleton promoting the breakdown of actin microfilaments [10], down-regulates the expression of cyclin E-cdk2 and p27, the induction of the cyclin A-cdk2 and cyclin E-cdk2 kinase activity and the phosphorylation of retinoblastoma [13].

Individual tumor cells, after drug treatment exposure, may develop resistance to a broad range of structurally unrelated drugs giving rise to a phenomenon that is known as multidrug resistance (MDR) [14], a significant limiting factor in chemotherapy effectiveness. The ATP binding cassette (ABC), superfamily of membrane transporters, is associated with MDR to anticancer drugs. These ABC proteins act as efflux pumps that cause a decrease in intracellular concentrations of cytotoxic drugs [15]. P-glycoprotein (P-gp/MDR1/ABCB1) [16], multidrug resistance protein 1 (MRP1/ABCC1) [17] and the mitoxantrone resistance protein (MXR/BCRP/ABCG2) [18] are ABC transporters, which participate in the multidrug resistance of tumors.

In the present study, we have examined the effectiveness of PG on ER+ (MCF-7) and ER− (MDA-MB-231) human breast cancer cells and its mechanism of action. In addition, we tested the sensitivity of the BCRP over-expressing MCF-7 MR cells to PG treatment. FACS analysis of uptake and efflux of PG in MCF-7 sublines was used to determine whether PG is a substrate for BCRP or instead acts independently of the presence of such transporter molecules.

2. Materials and methods

2.1. PG purification

2-Methyl-3-pentyl-6-methoxyprodigiosene (PG) was purified from *Serratia marcescens* 2170, as previously described [9]. It was then solubilized and its concentration determined by UV–vis in 95% EtOH–HCl ($\in_{535}$ = 112000/M cm).

2.2. Cell culture conditions

MCF-7 and the mitoxantrone resistant subline MCF-7 MR are human ER+ breast cancer cell lines. MDA-MB-231 is an ER− breast cancer cell line that was purchased from the American type culture collection (ATCC). Cells were cultured in DMEM:F-12 (1:1) (Biological Industries) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 2 mM l-glutamine, 100 u/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamycin and 10 μg/mL insulin at 37°C in a 5% CO_2 atmosphere.

2.3. Cell viability assay

The viability of cultured cells was determined by assaying the reduction of MTT (Sigma Chemical Co.) to formazan [19]. Briefly, 2×10^4 cells were seeded in 96-well microtiter cell culture plates. After 24 h, they were incubated in the absence (control cells) or presence of 0.25–2.75 μM PG in a final volume of 100 μL, for 4, 8, 16 or 24 h. Then, 10 mM MTT (diluted in PBS) was added to each well for an additional 2 h at 37°C. The blue formazan precipitate was dissolved in 100 μL of isopropanol:1N HCl (24:1) and the absorbance was measured at 550 nm in a multiwell plate reader. Cell viability was expressed as a percentage of control. IC_{50} was determined as the concentration of drug that produced a 50% reduction of absorbance at 550 nm.

2.4. Western blot analysis

Cells (5×10^5 cells/mL) were exposed to 0.2, 0.6 and 1 μM PG for 16 h. They were then washed in PBS and a lysis buffer was added (85 mM Tris, pH 6.8, 2% SDS, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 0.1 mM phenylmethanesulfonyl fluoride). Later, 80 μg of protein extracts was separated by SDS–PAGE on a 12 or 15% polyacrylamide gel and transferred to immobilon-P membranes (Millipore). Blots were blocked in 5% dry milk diluted in TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h and then incubated overnight with polyclonal antibodies against cleaved caspase-7 or -9 (Cell Signalling Technology, New England Biolabs, ref. 9491 or 9501S, respectively), anti-PARP or anti-Bax (Santa Cruz Biotechnologies, ref. sc-7150 or sc-526-G) and with the monoclonal antibodies, anti-caspase-8, anti-cytochrome *c* or anti-p21 (Pharmingen, BD biosciences, ref. 559932, 556433, 65951A) or anti-p53 (Neomarkers, ref. MS-186-P1), according to the manufacturer's instructions. Antibody binding was detected with goat anti-rabbit or goat anti-mouse IgG secondary antibodies conjugated to HRP (Biorad) and the ECL detection kit (Amersham).

2.5. Measurement of cytochrome c release

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot (method described above) with some modifications. Cells (5×10^5 cells/mL) were exposed to 1 μM PG over different time periods, from 15 min to 24 h. Later, they were washed with ice-cold PBS and gently lysed for 30 s in 80 μL ice-cold lysis buffer

(250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin and 100 μM phenylmethanesulfonyl fluoride). Lysates were centrifuged at 12,000 × *g* at 4°C for 3 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fractions that contain mitochondria). Supernatants (40 μg) were then electrophoresed on a 15% polyacrylamide gel and analyzed by Western blot using the monoclonal anti-cytochrome *c* antibody (Pharmingen). In experiments with the caspase inhibitor Z-Vad.fmk (Z-Val-Ala-dl-Asp (OMe)-fluoromethylketone) (Bachem), it was added at 100 μM 1 h before PG treatment [20].

2.6. FACS analysis

Approximately 1 × 10^6 MCF-7 or MCF-7 MR cells were loaded with fluorescent drug in a volume of 500 μL for 1 h at 37°C. The final concentration of mitoxantrone was 19 μM, whilst PG was used at a final concentration of 0.48 μM. After loading, cells were spun down, split into two batches and re-suspended in 2 mL of fresh, ice-cold medium without drug. Half of the cells were kept on ice (time zero of efflux) and in the other half, efflux of the drug was allowed at 37°C for 60 min. The efflux was stopped by placing the cells on ice. Experiments were also performed in the presence of 100 nM Ko143, a known BCRP blocker [21], both during the loading and efflux phase. Cells without drug treatment were used as time zero for loading and auto-fluorescence levels. Fluorescence levels were determined with a FACS-Star flow cytometer (Becton–Dickinson) and measured at the appropriate wavelength for PG (excitation 488 nm / emission 575 nm) or mitoxantrone (excitation 635 nm / emission 670 nm). A total of 5000 cells were measured per sample.

2.7. Statistical analysis

Data are shown as mean ± S.E.M. of three independent experiments performed in triplicate. They were analyzed by ANOVA and Student's *t*-test. A *P* value of less than 0.05 was considered significant. IC_{50} values were calculated by non-linear regression analysis of the data.

3. Results

3.1. Cytotoxicity of prodigiosin in breast cancer cell lines

To determine whether PG induced a decrease in the cell viability of human breast cancer cells, the estrogen sensitive MCF-7, its mitoxantrone resistant MCF-7 MR subline and the estrogen independent MDA-MB-231 cell lines were treated with PG in a time- and dose-dependent manner (Fig. 1). The effect of the pigment on viability was measured by metabolism of the tetrazolium salt in a cell titer proliferation assay. PG caused a dose-dependent decrease in viability in every cell line examined. Differences between MCF-7 and MCF-7 MR cells were observed, MCF-7 being more sensitive to PG than MCF-7 MR. The IC_{50} values at 24 h for MCF-7 MR (2.21 μM ± 0.6) were twice as high as for MCF-7 cells (1.10 μM ± 0.04). In contrast, MDA-MB-231 cells exhibited a similar cytotoxic response to PG as MCF-7 cells, showing an IC_{50} value of 1.14 μM ± 0.12 at 24 h.

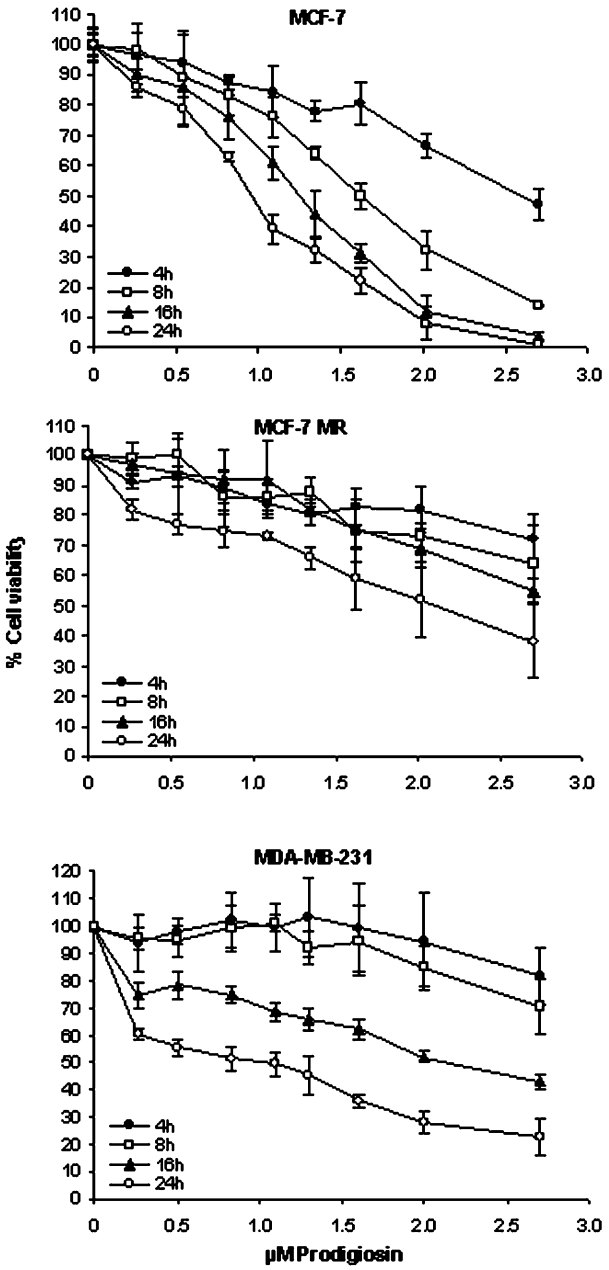

Fig. 1. Cell viability in PG-treated cells. MCF-7, MCF-7 MR and MDA-MB-231 cells (4 × 10^4) were treated with a range of concentrations (0–2.7 μM) of PG over different time periods and their viability was determined by MTT assay. The percentage of viable cells was calculated as a ratio of A_{550} between treated and control cells. Values are shown as mean ± S.E.M. of three independent experiments performed in triplicate.

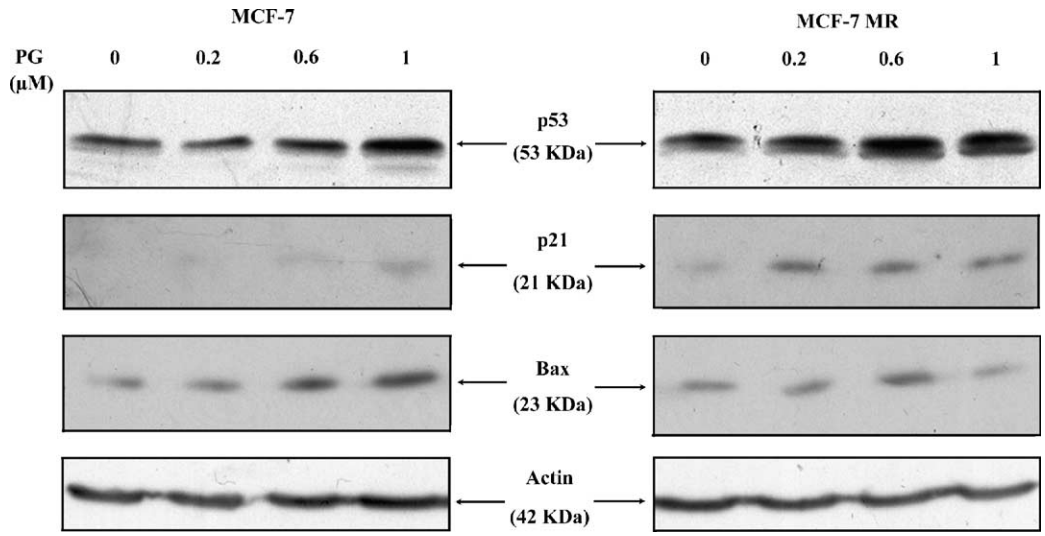

Fig. 2. Effect of PG on p53 and its downstream effectors p21 and Bax. MCF-7 and MCF-7 MR cells treated with PG concentrations ranging from 0.2 to 1 μM for 16 h and incubated with the appropriate antibodies. Results shown are typical examples of data from multiple experiments.

3.2. PG-induced apoptosis in human breast cancer cells

To understand the cell death mechanism induced by PG, MCF-7 and its subline, MCF-7 MR were used to determine whether differences in their sensitivity to PG were related to differences in the molecular process triggered by this cytotoxic agent.

3.2.1. Changes in p53, p21 and Bax protein levels

The p53 has been found to be importantly involved in apoptosis induced by a broad range of agents. We examined, by Western blot analysis, whether PG has any effect on this protein and on p21 and Bax, as it is known that p53 may induce their transcription upon stress signal. As observed in Fig. 2, p53 accumulation was detected in both cell lines upon PG treatment, it being higher in MCF-7 MR. The levels of the downstream effector protein p21 were considerably different between both cell lines starting to increase from as early as 0.2 μM in MCF-7 MR while it was almost undetectable in MCF-7. Conversely, the proapoptotic protein Bax was induced in a clear-way in MCF-7 cells suggesting that different cell effects might have been induced by PG in each cell lines.

3.2.2. Cytochrome c release

During apoptosis, cytochrome *c* is released from mitochondria into the cytosol where it helps in activating caspases. We, therefore, investigated cytochrome *c* release kinetics in response to PG exposure in MCF-7 and MCF-7 MR cells by Western blotting. Fig. 3 shows the time-dependent release of cytochrome *c* into the cytosol upon exposure to PG (1 μM) in both cell lines. Moderately increased levels of cytochrome *c* in the cytosol were detectable in MCF-7 as early as 30 min after PG treatment and a marked increase was observed at 12 h. In cytosolic fractions from MCF-7 MR, cytochrome *c* levels increased as a function of time.

3.2.3. Induction of caspases-9, -8 and -7 activation and PARP cleavage

In both cell lines, PG induced the activation of the main proteases that executes apoptosis such as the initiator caspases-9 and -8 and also the effector caspase-7, as well as cleavage of the caspase substrate called PARP. PG induced processing of caspase-9 and -8, as shown by the appearance of their active form of 37 and 23 kDa, respectively (Fig. 4(A)). In the absence of detectable caspase-3, due to a gene deletion in the MCF-7 and MCF-7 MR

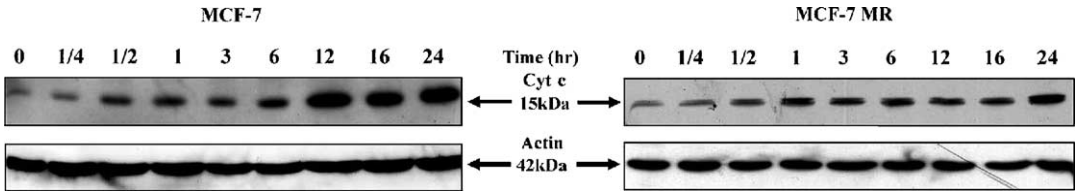

Fig. 3. Time-course of cytochrome c release from mitochondria to the cytosol in response to PG treatment. Cytosolic extracts (40 μg of protein) from MCF-7 and MCF-7 MR cells treated with 1 μM PG for the indicated times were resolved by SDS-PAGE and probed for cytochrome c. Results shown are typical examples of data from multiple experiments.

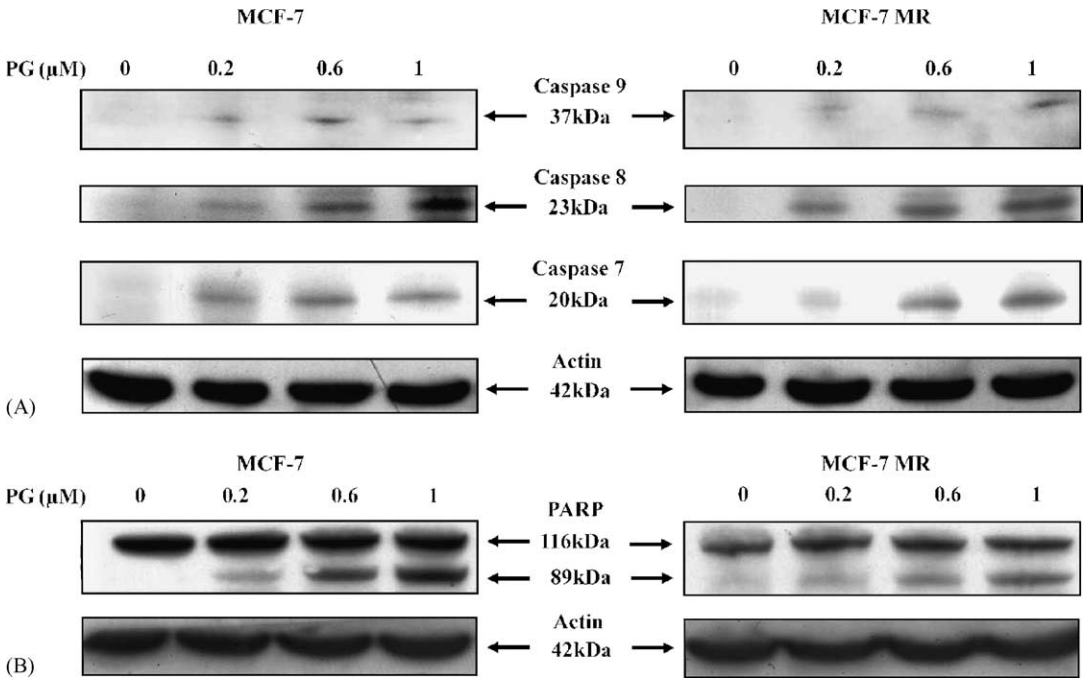

Fig. 4. Western blot analysis of PG-induced apoptosis through caspase activation and PARP cleavage. (A) Cleavage products of procaspase-9, -8 and -7; and (B) PARP cleavage in MCF-7 and MCF-7 MR. Cells were treated with the indicated PG concentrations for 16 h. Results shown are typical examples of data from multiple experiments.

genomes, we analyzed caspase-7 processing as a possible substitute. PG also induced processing of caspase-7, as shown by the appearance of its active form (20 kDa) (Fig. 4(A)). Furthermore, we compared cleavage of PARP, a DNA repair protein, in both PG-treated cell lines as a downstream signalling event indicative of apoptosis. All cells were treated with different doses of PG (0–1 μM) and cell extracts subjected to immunoblot analysis using an anti-PARP antibody that recognizes both the 116 kDa parent PARP and the 89 kDa cleavage product. As shown in Fig. 4(B), dose-dependant PARP cleavage was observed in both cell lines in response to PG treatment.

Finally, morphological changes associated with apoptosis were analyzed using Hoechst 33342 staining. The nuclei of both cell lines gave strong blue fluorescence and were condensed after PG treatment, although apoptotic bodies were not observed due to the atypical apoptosis that these cells undergo (data not shown) [22].

3.2.4. Triggering of mitochondrial apoptotic pathway

To further analyze the apoptotic pathway triggered by PG, we studied whether cytochrome *c* release was dependent or independent of caspase activity. MCF-7 cells were treated with PG in the presence or absence of the caspase inhibitor Z-VAD.fmk. We observed that cytochrome *c* was released from mitochondria even when caspases were not activated. As shown in Fig. 5, although caspase-9 was not active and the caspase substrate PARP was not cleaved in the presence of Z-VAD.fmk, cytochrome *c* was nevertheless released. This indicates that cytochrome *c* release precedes caspase activation, thus suggesting that PG-mediated apoptosis occurs using primarily mitochondria to transduce its death-inducing message.

3.3. No PG transportation by BCRP

FACS analysis of the uptake and efflux of auto-fluorescent PG was performed to determine whether this agent could be a substrate for the MDR transporter BCRP. The cells used in these experiments were the parental MCF-7 cells (very low levels of BCRP) and the MCF-7 MR cells

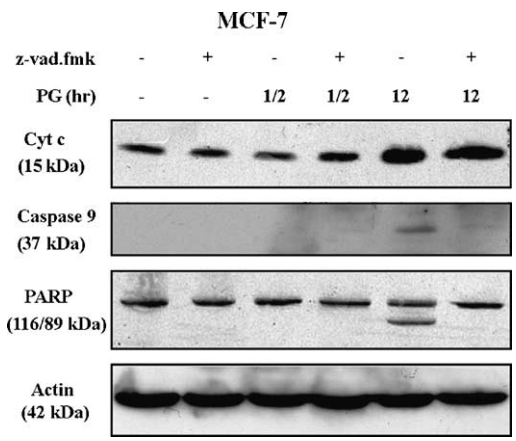

Fig. 5. Effect of PG on cytochrome c release, caspase-9 activation and PARP cleavage. MCF-7 cells were pre-treated with or without 100 μM Z-VAD.fmk 1 h before 1 μM PG treatment. Cytochrome c release and caspase-9 activation were analyzed in the cytosolic fraction, whilst PARP cleavage was observed in the nuclear containing extract. Results shown are typical examples of Western blot data from multiple experiments.

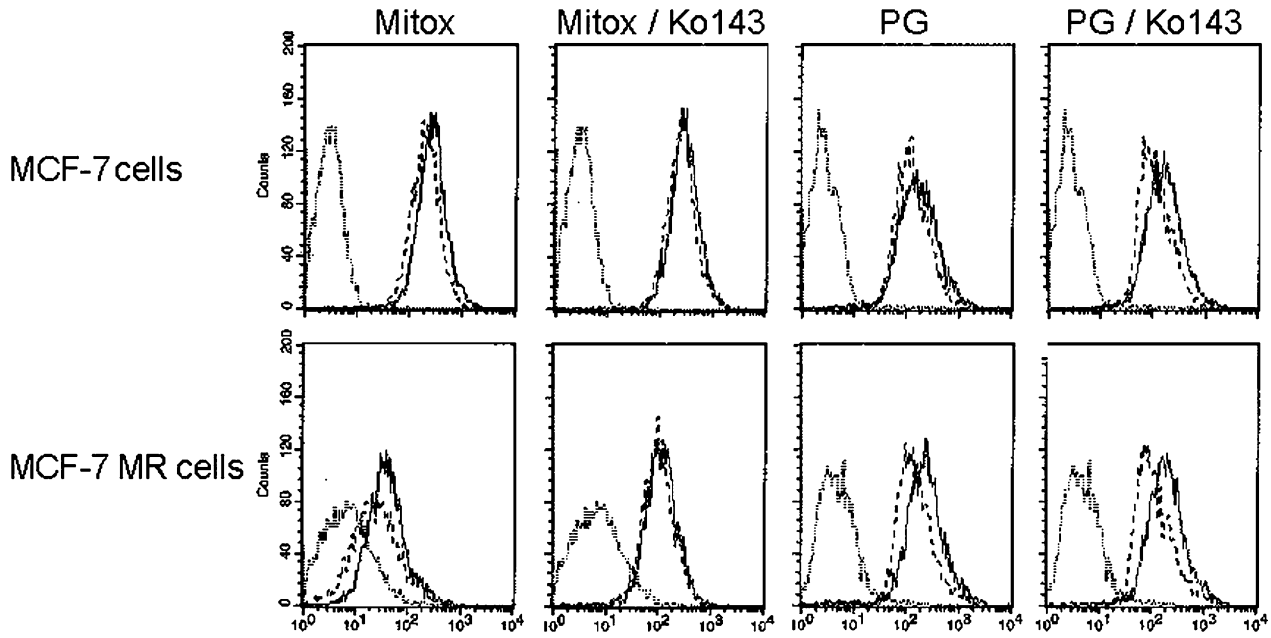

Fig. 6. FACS analysis of uptake and efflux of mitoxantrone and PG in a panel of MCF-7 cells. Upper panel: MCF-7 parent cells; no drug (auto fluorescence; dotted line) accumulation at time zero of efflux (sold line) and fluorescence levels after 60 min of efflux (dashed line) in the presence or absence of Ko143 with the appropriate drug. Lower panel: as above, but for MCF-7 MR (BCRP over-expressing cells).

(high levels of BCRP). As a positive control for BCRP activity, the drug mitoxantrone was used as a substrate. Furthermore, accumulation and efflux experiments were also performed in the presence of Ko143, a specific blocker of BCRP.

As expected, the MCF-7 parent cells accumulated high levels of both mitoxantrone and PG after the loading period and showed almost no active efflux after 60 min in fresh medium. The very low level of mitoxantrone efflux was inhibited when Ko143 was added during the efflux period (Fig. 6, upper panel). MCF-7 MR cells showed high levels of functionally active BCRP, as they accumulated much less of the typical BCRP–substrate mitoxantrone than the parental cells. After 60 min of efflux, the levels decreased even further. The accumulation of the drug could be significantly enhanced, almost to the levels of the parental cells, when the experiment was performed in the presence of Ko143. Under these conditions, no efflux of mitoxantrone was observed after 60 min, again confirming mitoxantrone as a typical substrate for the BCRP transporter. In contrast, PG accumulated to high levels in these MCF-7 MR cells, as high as observed in the parental cells. Some decrease in fluorescence levels after 60 min of efflux was noted, but the accumulation and efflux levels were not affected by the presence of Ko143 during the loading and/or efflux period (Fig. 6, lower panel).

These results indicate that PG is not a substrate for BCRP and it is unlikely that the effectiveness of this antineoplastic agent will be affected by the presence of the MDR transporter in tumor cells.

4. Discussion

Prodigiosins is a family of naturally occurring polypyrrole red pigments produced by a restricted group of microorganisms including some *Streptomyces* and *Serratia* strains. Some members of this family, including PG, have shown immunosuppressive [7,23,24] and apoptotic activities [9–12,25,26]. In the present study, we have extended our experiments on PG-induced apoptosis to breast cancer cells. Our results indicate that PG is an effective inducer of apoptosis in ER+ and ER− human breast cancer cells. The p53 accumulation, cytochrome *c* release, caspase activation, cleavage of PARP and distinctive morphological changes in the nucleus typified the apoptotic process. In addition, experiments with the caspase inhibitor Z-VAD.fmk elucidated the specific molecular pathway via mitochondria, triggered by PG. Finally, FACS analysis of PG accumulation and efflux in MCF-7 sublines showed that PG is not a substrate for the MDR transporter BCRP.

PG caused dose- and time-dependent cytotoxicity (reduction of cell number below the initial plating density) in MCF-7, MCF-7 MR and MDA-MB-231 cells. Cycloprodigiosin hydrochloride (*c*PrG·HCl) also has similar cytotoxic properties in many cancer cell lines, especially in breast cancer cells [25]. However, our cytotoxic assays were performed over shorter periods due to the more potent effect of PG observed in different cell lines [9,10,12]. The different effect could be a consequence of the C-6 methoxy substituent in PG since its substitution by a larger alkoxy substituent progressively reduced the activity of this compound [27].

Other novel anticancer agents for breast cancer have been reported. Among them, the marine compounds neoamphimedine and dehydrothyrsiferol [28,29] have been shown to have anti-neoplastic activities in both MCF-7 (IC_{50} = 1.8 μM at 72 h) and in MDA-MB-231 (IC_{50} = 14.8 μg/ml ± 1.2 at 48 h), respectively. Both of them are less effective than PG as shown by the lower PG IC_{50} values even at shorter times. Furthermore, paclitaxel, a drug currently used in breast cancer treatment, also showed a higher IC_{50} value for MDA-MB-231 cells in vitro (25 ± 1 μM at 24 h) [30]. Taken together, these results are very promising, especially for the estrogen-independent MDA-MB-231 cells as this kind of cancer is associated with a poorer prognosis and shorter survival.

Upon stress signals, p53 accumulation may induce two different sets of genes acting on growth control, undergoing cell cycle arrest due to an increase in p21 levels or on apoptosis and up-regulating the proapoptotic Bcl-2 family member Bax [3]. In MCF-7 cells, we have observed an increase in p53 levels as well as its DNA-binding activity followed by protein Bax expression leading to apoptosis as occurs with other drugs [31,32]. On the other hand, MCF-7 MR response to PG seems to be different at the doses examined since p53 is increasing p21 levels suggesting that these cells might try to undergo cell cycle arrest but finally the apoptotic process is driven. This could explain why the MCF-7 MR IC_{50} is slightly higher than that from its parental cell line. However, we have previous observations indicating that PG is able to induce apoptosis in a p53-independent manner [9,12] The ability of PG to induce apoptosis without the involvement of p53 may prove useful in therapy because p53 mutation is also associated with multidrug resistance in breast cancer [33].

The subcellular fractionation experiments revealed that PG induced mitochondrial cytochrome *c* release to the cytosol, indicating that outer mitochondrial membrane permeabilization is an early event in PG-induced apoptosis. It has been reported that this organelle has a central role in the apoptosis induced by many anticancer drugs, such as vitamin E isoforms in breast cancer cells [34]. Next, the activation of caspases-9, -8 and caspase-7 (as a caspase-3 alternative), as well as PARP cleavage, as a substrate for caspases, was also observed in PG-treated MCF-7 and MCF-7 MR cells. The lack of inhibition of cytochrome *c* release to the cytosol in the presence of the caspase inhibitor Z-VAD.fmk indicated that PG-induced apoptosis occurs via the mitochondrial pathway. Furthermore, in agreement with the previous data [22], we confirmed that MCF-7 cells do not express caspase-3 (data not shown). PG, even in the absence of caspase-3, induced a potent apoptosis in MCF-7 cells. Interestingly, the apoptotic process induced by doxorubicin and etoposide (currently used in breast cancer treatment), as well as cisplatin, an active chemotherapeutic agent used in clinical oncology, were all strongly enhanced by restoring caspase-3 in MCF-7 cells [35,36]. It remains to be seen whether a similar enhancement of PG cytotoxicity could appear in the presence of functional effector caspase-3.

The anthracyclines (doxorubicin, epirubicin) and taxanes (paclitaxel, docetaxel) are considered the most active agents for patients with advanced breast cancer [1]. However, some tumors do not respond and others eventually acquire resistance to several unrelated drugs. Some members of the ABC superfamily of transporter proteins can contribute to multidrug resistance in cancer chemotherapy. P-gp, MRP1 and the half-transporter BCRP are particularly implicated in this respect [37]. Over-expression of MDR1 P-gp confers resistance to vinblastine, vincristine, doxorubicin, daunorubicin, etoposide, teniposide, paclitaxel, docetaxel and many other drugs, whereas BCRP has relatively high affinity for mitoxantrone, topotecan and prazosin [15]. The MDR breast cancer cell line employed in this study was MCF-7 MR, a mitoxantrone-resistant cell line with a non-P-gp, non-MRP1 phenotype and elevated levels of BCRP mRNA [38–40]. These cells displayed a very high degree of resistance to mitoxantrone (1208-folds) [39], which is an inconvenience in cancer treatment, whilst the resistance showed to PG is very low (only 2-folds). In agreement with this low level of resistance to PG in this cell line, FACS analysis of accumulation and efflux of PG showed that this pro-apoptotic agent is not a substrate for the BCRP transporter. Furthermore, similar FACS experiments in the MDR1 P-gp over-expressing MCF-7 Dox40 cell line indicated that PG is also a rather poor substrate for this MDR transporter (data not shown). Moreover, PG has been shown to operate independently of the presence of the MRP-1 protein in a study performed in doxorubicin resistant small lung cancer cells that over-express MRP-1 [41].

In conclusion, the data reported here indicate that PG is a novel pro-apoptotic agent with potential as an anticancer agent, which may be effective irrespective of the presence of MDR transporter molecules.

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