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Effects of the proapoptotic drug prodigiosin on cell cycle-related proteins in Jurkat T cells

R. Pérez-Tomás and B. Montaner

Departament de Biologia Cel.lular i Anatomia Patològica,

Cancer Cell Biology Research Group, Universitat de Barcelona, L'Hospitalet (Barcelona), Spain

Summary. Prodigiosin (PG) is a red pigment produced by *Serratia marcescens* with immunosuppressive and apoptotic activities. In this study, we sought to examine the effect of PG on cell cycle-related proteins. The antiproliferative activity of PG was tested using human Jurkat leukaemia T cells in culture. PG-inhibited cell proliferation was determined using thymidine incorporation assay. PG-arrested cell cycle was analysed using immunoblot analysis with specific antibodies against cell cycle-related proteins and kinase assays of cdk2. Apoptosis was determined by Hoechst staining and analysis of DNA fragmentation. PG inhibited cyclin E, cdk2, p27 and p21, the induction of the cyclin A-cdk2 and cyclin E-cdk2 kinase activity, and the phosphorylation of Rb in leukaemic Jurkat cells. We confirmed that PG induces apoptosis by the characteristic DNA laddering pattern and condensed nuclei or apoptotic bodies identified by fluorescence microscopy. These results indicate that PG and other family members form a new group of molecules with a common mechanism of action and specific molecular targets, raising the possibility of their therapeutic use as antineoplastic drugs.

Key words: Apoptosis, Cell proliferation, Chemotherapy, Immunosuppressor, Prodigiosin

Introduction

In the search for new anticancer drugs several bacterial pathogens have been identified as mediators of apoptosis *in vitro* and during pathogenesis (Zychlinsky and Santonetti, 1997). Bacterial toxins like leukotoxin, α-toxin and haemolysin form pores in the eukaryotic cell membrane and disrupt the cell via osmotic swelling (Hildebrand et al., 1991; Mangan et al., 1991; Jonas et al., 1993). Other toxins like diphtheria toxin and exotoxin A inhibit protein synthesis, causing apoptosis in eukaryotic cells (Morimoto and Bonavida, 1992; Kochi and Collier, 1993).

A family of natural red pigments called prodigiosins are synthesised by bacteria. Prodigiosin 25-C (undecylprodigiosin, UP) and cycloprodigiosin hydrochloride (cPrG•HCl) are synthesised by *Streptomyces spp* and *Pseudoalteromonas denitrificans*, respectively, and they have immunosuppressive and apoptotic qualities (Tsuji et al., 1992; Kawauchi et al., 1997; Songia et al., 1997; Azuma et al., 2000; Lee et al., 2000; Yamamoto et al., 2000a). UP inhibits proliferation of both T and B human lymphocytes but not proliferation of transformed leukaemic cell lines (Songia et al., 1997). Very recently, screening for anticancer agents in vitro in our laboratory led to the discovery that PG produced by *Serratia marcescens* 2170 triggered apoptosis in several cancer cell lines but had no marked toxicity in non-malignant cell lines (Montaner et al., 2000; Díaz-Ruíz et al., 2001; Montaner and Pérez-Tomás, 2001). Songia et al. (1997) and Mortellaro et al. (1999) suggested that cell cycle-related proteins such as retinoblastoma (Rb) and cyclin-dependent kinase-2 and -4 (cdk-2 and cdk-4) are the target molecules of UP and PNU-156804 (a synthetic analogue of UP) to induce growth arrest in G1 phase in human T and B lymphocytes. However, Kawauchi et al. (1997) and Azuma et al. (2000) suggested that apoptosis is the mechanism of action of cPrG•HCl to induce suppression of T cell proliferation. cPrG•HCl inhibits proliferation and induces apoptosis in liver carcinoma cell lines (Yamamoto et al., 1999) and in human breast cancer cell lines (Yamamoto et al., 2000a), and also induces differentiation in the human promyelocytic leukaemia cell line HL60 (Yamamoto et al., 2000b).

In this study, we examine for the first time the relationship between cell cycle-related proteins such as Rb and cdk-2 and the efficient inhibition of Jurkat T cell proliferation induced by PG.

Offprint requests to: Dr. Ricardo Pérez-Tomás, Dept. Biologia Cel.lular i Anatomia Patològica, CCBR Group, Pavelló Central, 5a planta, LR 5.1, C/ Feixa Llarga s/n, E08907 L'Hospitalet (Barcelona), Spain. Fax: 34-93 4024213. e-mail: rperez@bell.ub.es

Materials and methods

Cell lines and culture conditions

Acute human T cell leukaemia cells (Jurkat clone E6-1) were cultured in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel), supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL, Paisley, UK), and 2 mM L-glutamine (Sigma Chemicals Co, St Louis, MO, USA), at 37 ºC, 5% CO_2 in air.

Isolation and purification of PG

PG was isolated from a culture broth of *S. marcescens* 2170 as described previously (Montaner et al., 2000). Stock solutions were prepared in methanol and concentrations were determined by UV-Vis in 95% EtOH-HCl.

Thymidine incorporation assays

The effect of PG on cell proliferation was determined in a concentration-dependent fashion. Briefly, Jurkat cells were plated at $5x10^5$ cells/ml and untreated or treated with 12-64 ng/ml PG. After 2 h incubation, cells were labelled with 4 μCi/ml of [^{3}H]-thymidine for a further hour in the continuous presence of PG. Then, cells were washed twice with PBS and 1 ml of cold 5% TCA (Sigma) was added for 30 min at 4 ºC. Consequently, samples were washed twice with cold PBS and with 100 % ethanol and dried at 37 ºC. Finally, precipitates were treated with 0.7 ml of 300 mM NaOH for 1 h at 4 ºC and 0.5 ml of each sample was neutralised with 0.1 ml 1.5 M HCl and added at 5 ml of scintillation liquid and the radioactivity was counted (Beckman LS5000TA, USA).

Western blot analysis

After cells ($5x10^5$/ml) were treated with 12, 32 and 64 ng/ml of PG for 3 h, they were lysed with 85 mM Tris pH 6.8, 2% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.1 mM PMSF. Protein extracts were quantified using Pierce's BCA Assay Kit. Then, 50 μg were electrophoresed on 12% polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). Membranes 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 rabbit polyclonal (rp) or mouse monoclonal (mm) antiserum according to the manufacturer's instructions: anti-Rb (Santa Cruz Biotechnologies (SCB), CA, USA, ref. C-15 sc-50, rp, diluted 1:200), anti-cdk2 (Upstate Biotechnology (UBI), NY, USA, 06-505, rp, diluted 1:1000), anti-cdk4 (SCB, C-22 sc-260, rp, diluted 1:1000), anti-p21 (SCB, C-19 sc-397, rp, diluted 1:200), anti-p27 (UBI, 06-445, rp, diluted 1:3000), anti-cyclin A (SCB, C-19 sc-596, rp, diluted 1:5000), and anti-cyclin E antibody (SCB, HE12 sc-247, mm, diluted 1:400). Secondary antibodies conjugated to HRP were goat anti-rabbit IgG (BioRad, UK, ref. 170-6515) or goat anti-mouse (BioRad, ref. 170-6516), diluted 1:1000 in 5 % dry-milk-TBS-T, for 1 h at RT. Peroxidase was developed by incubating the membrane with 2.25 mM luminol (Sigma) and 0.45 mM p-iodophenol (Fluka, Buchs, Switzerland) in 50 mM Tris-HCl pH 9.0 plus 0.03 % H_2O_2 for 1 min at RT and enhanced chemiluminescence in an autoradiography film.

Protein expression on Western images were quantified using the image analysis software program Phoretix 1-D advanced. Results are presented as % of control (relative to the densitometry values).

Immunoprecipitation and kinase assays

To measure cdk2 activity, cells untreated or treated with 32 and 64 ng/ml of PG were lysed in IP buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 1 μg/ml aprotinin and 0.1 mM PMSF). Lysates (100 μg) were immunoprecipitated with a normal rabbit serum (NRS), anti-cdk2, anti-cyclin A or anti-cyclin E antibodies (UBI, ref. 06-138 and 06-459, respectively). The immunoprecipitated complexes were washed in kinase buffer (25 mM HEPES-Na pH 7.4, 5 mM $MgCl_2$, 0.5 mM dithiothreitol) and then incubated in kinase buffer containing 1 mM ATP, 3 μg of histone H1, and 10 μCi of [^{32}P]ATP for 30 min at 30 ºC in a final volume of 50 μl. The samples were electrophoresed, and gels were stained with Coomassie Blue, dried, and exposed to X-ray films at -80 ºC.

Statistical analysis

All data points shown are mean value ± s.e.m. of three independent experiments. Statistical significance of differences was assessed by ANOVA (Fisher's PLSD test). Differences between untreated and treated cells with PG are indicated by *P<0.05; **P<0.01; ***P<0.001.

Hoechst staining

Cells were stained with the intercalating DNA dye Hoechst 33342 (Sigma) to reveal their nuclear morphology. Jurkat cells ($5x10^5$/ml) were incubated in the absence (control cells) or in the presence of 64 ng/ml of PG for 3 h. Cells were then washed in PBS and resuspended in PBS plus Hoechst 33342 to a final concentration of 2 μg/ml and incubated for 30 min at 37ºC in the dark. After incubation, cells were washed in PBS and the sections were examined fluorometrically (emission above 420 nm, excitation 330-385 nm) with a Leitz Diaplan microscope and photographed with a Wild MPS 45 Photoautomat system.

Analysis of DNA fragmentation

DNA fragmentation was analysed by agarose gel

electrophoresis, as described previously (Montaner et al., 2000). Briefly, $1x10^6$ cells per ml were untreated (control) or treated (12-64 ng/ml of PG) for 3 h, then washed in PBS and resuspended in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.2% Triton X-100). After incubating for 15 min at 4 ºC, cell lysates were centrifuged at 14,000 X g for 15 min and the supernatant was treated with 0.2 mg/ml of proteinase K in a buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 8.0, 40 mM EDTA and 1% SDS, for 4 h at 37 ºC. The DNA preparations were phenol/chloroform extracted twice to remove proteins. DNA was precipitated with 140 mM NaCl and two volumes of ethanol at -20ºC overnight. DNA precipitates were recovered by centrifugation at 14,000xg for 10 min at 4 ºC, washed twice in cool 70% ethanol and air dried. DNA pellets were resupended in 15 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and treated with DNase-free RNase (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37 ºC. 3.2 ml of loading buffer was added to each tube and the DNA preparations were electrophoresed in 1% agarose gels which contained ethidium bromide. Gels were placed on an UV light box to visualize the DNA ladder pattern.

Results

Since PG had been shown to induce apoptosis in haematopoietic cancer cell lines (Montaner et al., 2000), we determined the effects of PG on Jurkat cell line proliferation in a dose-response study. As shown in Figure 1, PG at the dose 64 ng/ml inhibits the uptake of [^{3}H]-thymidine by about 70%.

We analysed the induction of several cyclins and

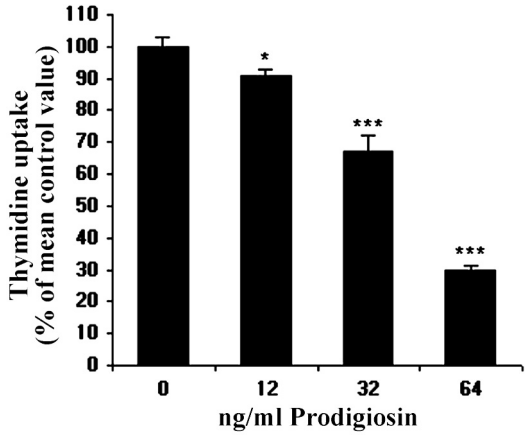

Fig. 1. PG inhibits the proliferation of Jurkat cells in a concentration-dependent fashion. In the thymidine incorporation assay, cells are untreated (control) or treated with 12, 32 or 64 ng/ml PG for 3 h. Data are mean values obtained from three independent experiments. Statistical significance of differences between untreated cells and cells treated with PG at different doses are assayed by ANOVA. Fisher's PLSD are indicated by *P<0.05 and ***P<0.001.

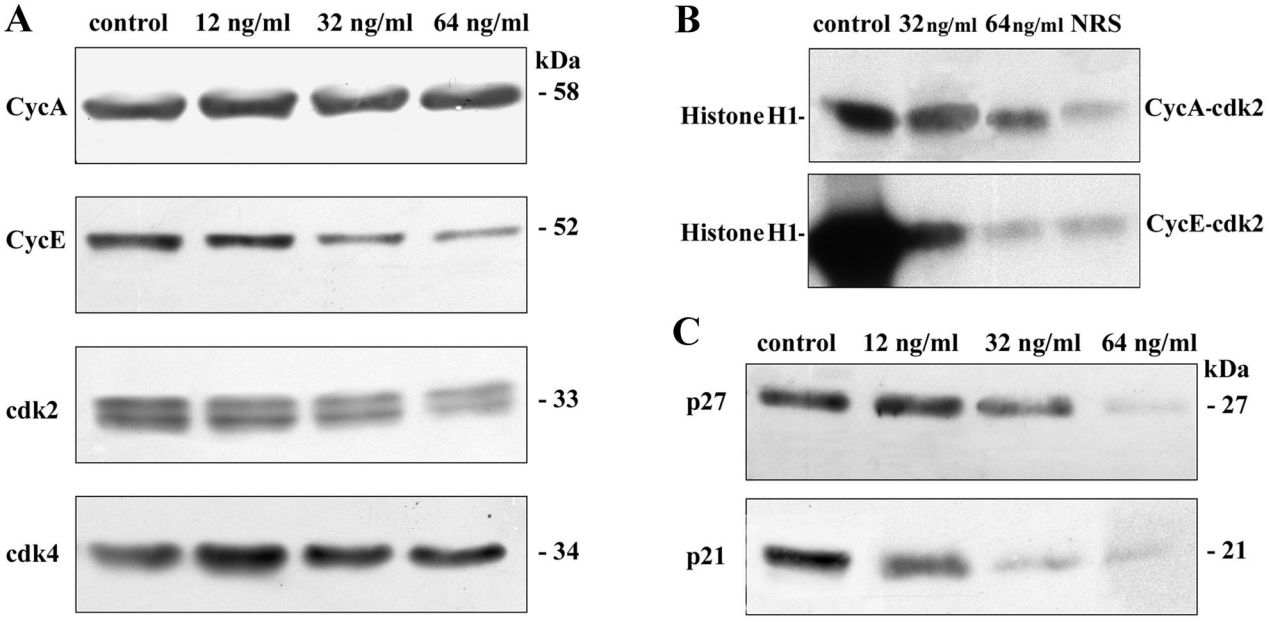

Fig. 2. Effect of PG on cell cycle-related proteins in a dose-response study. **A.** Jurkat cells are untreated (control) or treated with 12, 32 or 64 ng/ml PG for 3 h. Equal amounts of whole cell lysate (50 μg) are subjected to electrophoresis and analysed by Western blotting for the presence of cyclin A, cyclin E, cdk2, and cdk4. **B.** Effect of PG on cdk2 activity. Jurkat cells are untreated (control) or treated with 32 and 64 ng/ml PG for 3 h. Then, extracts of these cells are immunoprecipitated with anti-cyclin A, anti-cyclin E antibodies or a NRS and analysed by Western blot using phospho-cdk2 antibodies. **C.** Jurkat cells are untreated (control) or treated with 12, 32 or 64 ng/ml PG for 3 h, then, 50 μg of whole cell lysate are subjected to electrophoresis and analysed by Western blotting for the presence of p27 and p21.

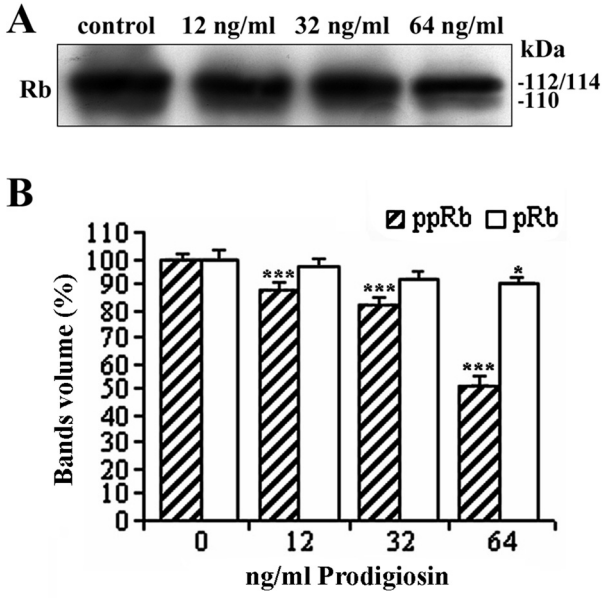

Fig. 3. Effect of PG on retinoblastoma. **A.** Western blot analysis of phosphorylated Rb protein from Jurkat cells untreated (control) or treated with 12, 32 or 64 ng/ml PG for 3 h. 50 μg of whole cell protein extracts are subjected to electrophoresis. In all cases, a representative study is shown; two additional experiments yielded similar results. **B.** Quantification of Rb immunoreactive bands intensity. The densitometric analysis of the hyperphosphorylated (ppRb) and hypophosphorylated (pRb) forms of Rb is performed. Bands densitometry is presented as the % of control. Statistical significance of differences between untreated cells and cells treated with PG at different doses are assayed by ANOVA, Fisher's PLSD are indicated by *P<0.05 and ***P<0.001.

cdks involved in G1 progression and G1-S transition. The analysis was at the protein level by Western blot. Total cell extracts from an equivalent number of cells were loaded in each lane. That equal amounts of protein were loaded per lane was verified by Ponceau red staining of the unused part of the blot (data not shown).

We performed dose-response experiments in order to demonstrate that the inhibition of expression of these molecules paralleled the inhibition of proliferation (Fig. 1). PG inhibited cyclin E protein expression, with an IC_{50} of 40 ng/ml but had no effect on cyclin A (Fig. 2A). The expression of cdk2 and cdk4 was also analysed and only cdk2 was seen significantly inhibited in its expression by PG, with an IC_{50} of 62 ng/ml (Fig. 2A). To verify that PG indeed inhibited the kinase activity of cdk2, a kinase assay was performed on cdk2 immunoprecipitates, with histone H1 as substrate. As shown in Fig. 2B, PG almost totally abolished both the cyclin A-cdk2- and the cyclin E-cdk2-dependent kinase activity, with an IC_{50} of 62 ng/ml and 27 ng/ml, respectively.

Cell cycle progression in T cells is also regulated by cdk inhibitors (ckI). In particular, the ckI p27 and p21 are known to be expressed in T cells. We therefore analysed the effect of PG on p27 and p21 expression. As shown in Fig. 2C, PG completely abolished the expression of p27 protein at the concentration of 64 ng/ml (IC_{50}: 42 μg/ml). Finally, the Jurkat cell (following PMA stimulation) was accompanied by the induction of p21. This induction was almost completely abolished by PG (IC_{50}: 31 ng/ml) (Fig.2C).

Therefore, we went on to investigate the effect of PG on Rb phosphorylation. As shown in Figure 3A, Rb

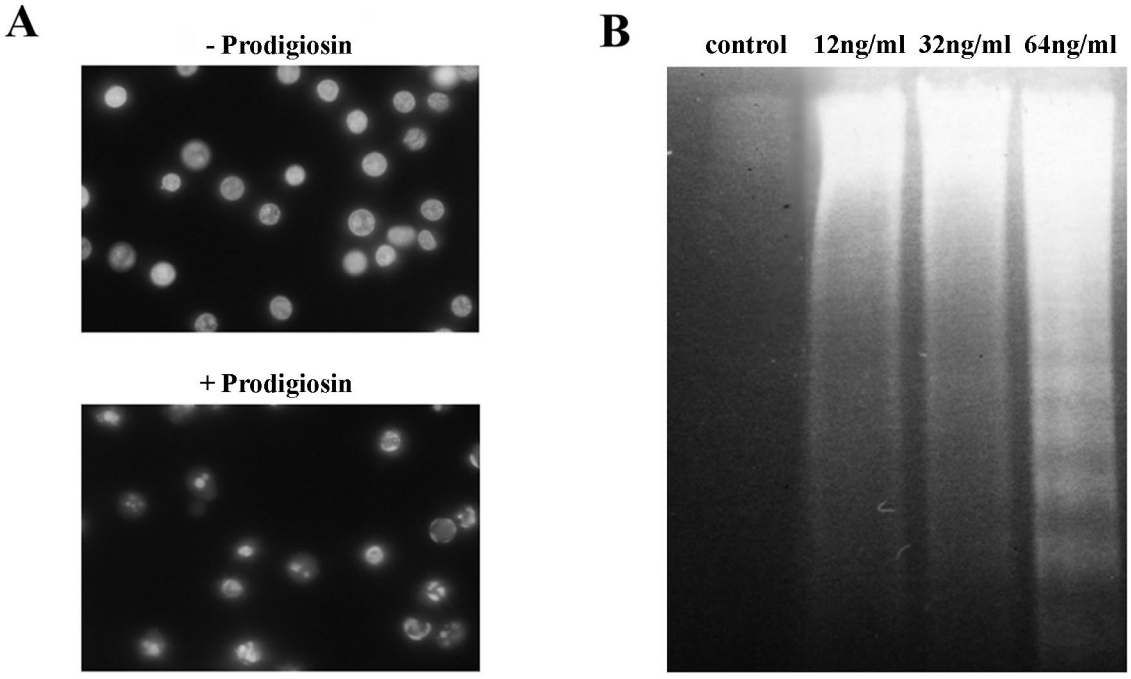

Fig. 4. PG induces apoptosis in Jurkat cells. **A.** Fluorescence microscopic analysis of Jurkat nuclei with Hoechst 33342 staining. Cells are untreated (- Prodigiosin) or treated (+ Prodigiosin) with 64 ng/ml PG for 3 h. PG-treated cells show apoptotic signs with chromatin condensation and nuclei fragmentation. **B.** DNA fragmentation induced by PG is observed in the agarose gel electrophoresis. For this assay, the cells are untreated (control) or treated with 12-64 ng/ml PG for 3 h.

hyperphosphorylation was evident in untreated Jurkat cells and was partially, although not completely, inhibited by PG. A densitometric analysis was done using the "Phoretix-1D gel analysis software" showing underphosphorylation of the hyperphosphorylated Rb protein (Fig 3B).

All the data demonstrated that PG strongly inhibits the expression of cyclin E, cdk2, p27 and p21, the induction of cyclin A-cdk2 and cyclin E-cdk2 kinase activity, and the phosphorylation of Rb.

In order to determine that PG had an apoptotic effect at these conditions, Jurkat cells were treated with graded doses of PG for 3h. Apoptosis was determined by nuclear condensation (Fig. 4A) and typical DNA fragmentation (Fig. 4B), which indicated that the suppression of T cell proliferation was a result of apoptotic cell death.

Discussion

New drugs associated with apoptotic cell death that are expected to be effective against high-proliferation tumours like leukaemia are being screened for new anticancer drug treatment (Cameron and Feuer, 2000). PG, UP and cPrG·HCl, three members of the prodigiosin family, were found to have interesting immunosuppressive properties (Tsuji et al., 1992; Songia et al., 1997; Han et al., 1998; Mortellaro et al., 1999) and apoptotic effects on cancer cell lines (Kawauchi et al., 1997; Azuma et al., 2000; Lee et al., 2000; Montaner et al., 2000; Yamamoto et al., 2000a; Díaz-Ruíz et al., 2001; Montaner and Pérez-Tomás, 2001, 2002a,b) and on hepatocellular carcinoma xenografts (Yamamoto et al., 1999). Along these lines, UP and PNU-156804, a new analogue of UP, inhibit equally well both T and B lymphocyte proliferation, but not transformed leukaemic Jurkat cells (Songia et al., 1997; Mortellaro et al., 1999). However, cPrG•HCl suppresses Jurkat proliferation as a result of apoptotic cell death (Kawauchi et al., 1997). In view of these contradictory results we extended our study in order to elucidate the mechanism of action of PG on the proliferation of the human T cell-derived Jurkat cell line.

PG at 64 ng/ml inhibited the uptake of [3H]-thymidine by about 70 % (Fig. 1), whereas it decreased Jurkat cell viability by only 30% (Montaner et al., 2000). Nevertheless, 50 ng/ml UP did not affect Jurkat cell growth for 2 days. The percentage inhibition of thymidine uptake paralleled the percentage increase in cell death only at 4- to 6-fold higher concentrations, suggesting that UP is toxic to cells at these concentrations (Songia et al., 1997). The differences in the chemical structures of the A-pyrrole rings between PG and UP plays a key role in the cytotoxic potency (Melvin et al., 2000), and it is tempting to speculate that they are responsible for these different effects.

The fact that PG blocked cell proliferation suggested that some general cell cycle-associated proteins required for G_0-G_1-S phase transitions could be a target of PG action. We demonstrated that PG abolished cyclin E protein expression, but had no effect on cyclin A. However, drugs that also prevent mitogen-induced G1-S progression, like doxazosin, inhibit cyclin A expression (Kintscher et al., 2000). PG almost totally abolished both the cyclin A-cdk2- and the cyclin E-cdk2-dependent kinase activity.

Interestingly, the resistance of cancer to chemotherapy may be due in part to the high frequency of mutation in p53 that impairs p53-dependent apoptosis. Many anticancer agents such as doxorubicine, etoposide or 5-fluorouracil induce apoptosis via the p53-dependent pathway (Lowe et al., 1993). Thus, identifying chemotherapeutic agents like PG that act independently of the p53 pathway (Montaner et al., 2000) is of major importance and could lead to such agents having an edge over other chemotherapeutic drugs.

Cell cycle progression in T cells is also regulated by cdk inhibitors (ckI). In particular, the ckI p27 and p21, both of which are p53-inducible genes that work to block the transition of the cell from G_1 to S, are known to be expressed in T cells. PG abolished the expression of p27 protein and in PMA stimulated Jurkat cells, the induction of p21 was also abolished by PG. Thus, the conventional view is that the role of p27 determines the proliferative threshold to mitogenic growth factors. However cells under stress, in states such as growth factor deprivation, have lower or no p27 protein levels and they exit the cell cycle and undergo apoptosis (Hiromura et al., 1999). It has also been shown that $p21^{Cip1,Waf1}$ and p27 are cleaved during growth factor deprivation-induced apoptosis in endothelial cells (Levkau et al., 1998). Our results support a different perspective on the biological function of p27 and suggest that p27, in the presence of PG, coordinates the final outcome of proliferation or death of the cell.

We show that PG, like UP and PNU-156804 (Songia et al., 1997; Mortellaro et al., 1999), induces a p27 protein decrease and inhibits cyclin A-cdk2 and cyclin E-cdk2 kinase activity. Cyclins E and A in association with cdk2 serve as positive regulators for mammalian cell cycle progression through the G_1-S transition and subsequent cell proliferation. However, Hiromura et al. (1999) showed that in serum-starved $p27^{-/-}$ cells cyclin E-cdk2 activity decreased, while cyclin A-cdk2 activity increased. Mgbonyebi et al. (1999) suggest that cyclin-kinase inhibitors like roscovitine induces apoptosis and may be involved in cytoskeletal regulation by reducing the polymerisation of actin microfilaments. These findings are consistent with the results shown here and, together with the fact that PG causes the reorganisation of actin cytoskeleton and may promote the breakdown of actin microfilaments (Díaz-Ruíz et al., 2001), they indicate that PG follows the same mechanism as roscovitine.

Furthermore, PG inhibited Rb phosphorylation This result is in concordance with the findings shown above, since proteins like cyclins, cdks and Rb itself can check cell cycle progression, and hold cells in quiescence or

even lead cells to commit suicide.

Recent studies have shown that PG interact with DNA and uncoil the double-stranded helix (Melvin et al., 2000). Furthermore, we have demonstrated that PG binds directly to DNA by intercalation and that it cleavages the double-strand DNA, inhibiting the topoisomerase I enzyme (manuscript in preparation). These findings may explain the effect of PG in cell cycle-related proteins that we have shown here.

In summary, this study shows that prodigiosin family members form a new group of molecules with a common mechanism of action inducing G1-S transition arrest in association with the down-regulation of the expression of cyclin E, cdk2, p27 and p21, induction of the cyclin A-cdk2 and cyclin E-cdk2 kinase activity, and the phosphorylation of Rb. Further studies of PG activity in primary tumours and the characterisation of the PG internalisation pathway are now underway in our laboratory.

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