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Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1

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Summary. Gastric cancer is one of the most frequent malignancies and its treatment is far from satisfactory. The challenge to oncologists is the characterization of novel chemical entities with greater effectiveness. Prodigiosin is a red pigment produced by various bacteria including *Serratia marcescens*. Here we characterize the apoptotic action of prodigiosin in human gastric carcinoma cell line (HGT-1). Cells were assayed by the MTT assay, fragmentation pattern of DNA, Hoechst 33342 staining and study of actin microfilament architecture. Treatment of these cells with prodigiosin showed a constant decrease in viability by apoptosis. Morphological analysis of prodigiosin-treated cells demonstrated that prodigiosin induces cell shrinkage, chromatin condensation, reorganization of actin microfilament architecture, and detachment of cells from the cell culture substrate. Altogether these results suggest that prodigiosin induces apoptosis in HGT-1 human gastric cancer cells and raises the possibility of its use as a new chemotherapeutic drug.

Key words: Apoptosis, Cancer cell lines, Chemotherapy, Prodigiosin

Introduction

Esophageal and gastric cancers pose a number of challenges for oncologists, gastroenterologists and surgeons. Surgical resection is the most effective treatment in gastric cancer. Nevertheless, in the United States and Europe, curative resections are possible in only 50% to 60% of newly diagnosed gastric cancer patients (Kelsen, 1996). The current chemotherapy treatment for these patients is 5-fluorouracil (5-FU), which alone or in combination with other drugs like cisplatin (Lokich, 1998; Ross et al., 1998), induces apoptosis in tumor cells (Inada et al., 1997).

Apoptosis is characterized by internucleosomal DNA degradation, chromatin condensation and distinct histological features (Kerr et al., 1994). Unlike necrotic death, apoptosis is an active process involving the programmed activation of signaling cascades that are necessary to induce cell death. Apoptosis plays an important role in development and tissue homeostasis, and provides defense against viral infection and oncogenesis (Raff, 1992; Vaux et al., 1994). The importance of apoptosis as an anti-cancer agent has been demonstrated in many tumor cell types in response to a broad range of drugs (Hannun, 1997; Cameron and Feuer, 2000). The identification of novel targets and development of new, more specific chemotherapeutic agents are two of the most important goals of research on cancer therapy. 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). Verotoxin 1, the active component of the bacteriocin preparation from *Escherichia coli*, induces apoptosis in human cancer cell lines (Arab et al., 1998) and eliminates human astrocytoma xenografts (Arab et al., 1999).

A family of natural red pigments called prodigiosins are synthesized from different bacteria. Cycloprodigiosin hydrochloride (cPrG.HCl) and undecylprodigiosin (UP) are members of this family for which immunosuppressive and apoptotic activities have been described (Kawauchi et al., 1997; Songia et al., 1997). Very recently, screening for anticancer agents *in vitro* in our

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laboratory led to the discovery that prodigiosin produced by *Serratia marcescens* 2170 triggered apoptosis in different cancer cell lines, behaving as a rapid, potent and selective drug (Montaner et al., 2000; Montaner and Pérez-Tomás, 2001). The fact that the antiproliferative effect of prodigiosin is p53-independent (Montaner et al., 2000) makes prodigiosin an interesting new antineoplasic candidate to study in cell culture cancer models. We decided to study the effect of prodigiosin in a human gastric cancer cell culture model for the following reasons: a) gastric cancer is the second cause of annual mortality worldwide, with over 600,000 deaths in 1990 (Pisani et al., 1999); b) gastric cancer cells have a low sensitivity to chemotherapy agents (Toge, 1999); and c) at present, chemotherapeutic choices are limited to 5-FU, and the benefits, in terms of tumor regression or improvement of symptoms, are limited.

The purpose of this study was to analyze the effect of prodigiosin in the human gastric carcinoma cell line (HGT-1). We quantified the loss of viability by MTT assay; we also stained DNA with Hoechst 33342 for morphological identification of apoptotic cells and we studied the ladder pattern of DNA after agarose gel electrophoresis, which is a biochemical feature of apoptotic cells. Finally, we examined whether prodigiosin affected the actin cytoskeleton during apoptosis, and whether such an effect was reversible.

Materials and methods

Chemical and reagents

Meat peptone was purchased from Difco (Detroit, Michigan, USA). Glycerol was bought from Merck (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were purchased from Sigma Chemicals Co (St Louis, MO, USA). Deionized water further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Cell lines and culture conditions

HGT-1 (clone 6) human gastric carcinoma cell line was a generous gift from C.L. Laboisse (Laboisse et al., 1982). Cells were cultured in DMEM, purchased from Biological Industries (Beit Haemek, Israel) and supplemented with 10% of heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine (all from GIBCO BRL, Paisley, UK), at 37 °C, 5% CO_2 in air.

Bacteria strain and culture conditions

S. marcescens 2170 environmental isolate is a wild-type strain that produces the characteristic pigment prodigiosin. *S. marcescens* 2170 was inoculated into 25 ml of peptone glycerol (PG) medium, containing 1% meat peptone and 10% glycerol in distilled water, and cultivated for 8 h at 30 ºC with vigorous shaking, and 1 ml was then transferred to 250 ml of PG medium and cultivated for 48 h at 30 °C with vigorous shaking. Bacteria were then harvested by centrifugation at 6,800g for 15 minutes at 4 °C. Prodigiosin was extracted by shaking the pellet with acidic methanol (methanol:1N HCl (24:1)) and centrifuged at 2,000g for 5 minutes at room temperature. The supernatant was evaporated under vacuum and the pigment was re-dissolved in DMSO, divided into aliquots and stored at -20 ºC.

Isolation and purification of prodigiosin

Prodigiosin was measured using the difference in absorption of the concentrated supernatant at 534 and 655 nm. A difference in absorbency of 1.0 between the two wavelengths is equivalent to 19.3 μg of prodigiosin per ml (Goldschmidt and Williams, 1968).

Furthermore, prodigiosin was purified as described previously (Montaner et al., 2000). Briefly, after evaporation under vacuum of the acidic methanol solvent, atmospheric pressure liquid chromatography of the extract was performed on silica gel with chloroform and methanol as solvents. The eluted fractions were pooled and the chloroform/methanol extract was vacuum evaporated, re-dissolved in H_2O and lyophilized. The isolated pigment was re-dissolved in methanol and analyzed by electrospray ionization mass spectrometry (ESI-MS) using a VG-Quattro triple mass spectrometer (Micromass. VG-Biotech, UK). The isolated pigment was re-purified by subsequent semi-preparative HPLC on a Shimadzu instrument (Kyoto, Japan). A Nucleosil C_{18} reversed-phase column (250x4 mm, 10 μm) was used with a 0% to 100% linear gradient in 30 minutes (A: 10 mM ammonium acetate, pH 7.0, B: 100 % acetonitrile). The elution was monitored by using both diode-array UV detector (SPD-M10AVP Shimadzu) and by ESI-MS. After repeated injections the pooled fractions containing the major peak were vacuum evaporated, re-dissolved in H_2O, lyophilized and characterized by ESI-MS and ^{1}H-NMR. ESI, m/z 324.4 $(M+H)^+$, ($C_{20}H_{25}N_3O$ requires 323.4381 (MW average)). ^{1}H-NMR (CD_3OD, 500 MHz, ppm); 10.71 (m, NH), 8.54 (m, NH), 7.08 (s, 1H), 6.95 (s, 1H), 6.88 (m, 1H), 6.83 (m, 1H), 6.30 (m, 1H), 6.25 (s, 1H), 3.96 (s, 3H), 2.43 (t, 2H), 1.58 (s, 3H), 1.2-1.4 (m, 6H), 0.91 (t, 3H).

Cell viability assay

Cell viability was determined by the MTT assay (Mosmann, 1983). Briefly, $20x10^3$ cells were incubated in 96-well microtiter cell culture plates, in the absence (control cells) or in the presence of 0.5 to 4.0 μM of prodigiosin in a final volume of 100 ml. After 4 h. incubation, 10 mM of MTT (diluted in PBS) was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 μl of isopropanol:1N 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±Standard Deviation of triplicate cultures.

Analysis of DNA fragmentation

Analysis of DNA fragmentation by agarose gel electrophoresis was performed as described previously (Montaner et al., 2000). Briefly, $1x10^6$ cells per 2 ml were exposed to 2μM of prodigiosin and incubated for 12 h. Cells were 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 minutes at 4 °C, cell lysates were centrifuged at 14,000g for 15 min and the supernatants were 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 °C. The DNA preparations were phenol/chloroform-extracted twice and DNA was precipitated with 140 mM NaCl and two volumes of ethanol at -20 °C overnight. DNA precipitates were recovered by centrifugation at 14,000g for 10 minutes at 4 °C, 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 °C. 3.2 μl of 6X loading buffer was added to each tube and the DNA preparations were electrophoresed in 1% agarose gels containing ethidium bromide. Gels were placed on a UV light box to visualize the DNA ladder pattern.

Hoechst staining

Cell morphology was evaluated by fluorescence microscopy following Hoechst 33342 DNA staining. HGT-1 cells ($4x10^5$ per ml) were incubated in the absence (control cells) or in the presence of 3 μM of prodigiosin and incubated for 2 and 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 minutes at 37 °C in the dark. After incubation, cells were washed in PBS and the sections were examined with a Leitz Diaplan microscope and photographed with a Wild MPS 45 Photoautomat system. Apoptotic cells were identified by nuclear condensation, formation of membrane blebs and apoptotic bodies.

Analysis of actin microfilament organization

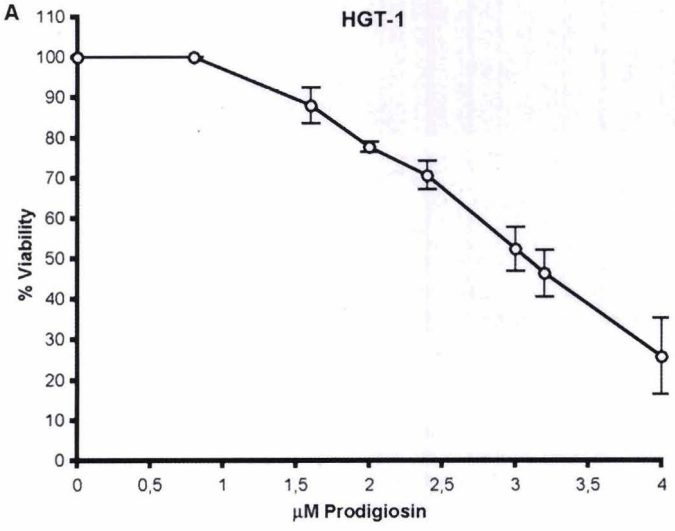
HGT-1 cells were cultured to 70% confluence over sterile coverslips introduced in plates. Cells were treated with 3 μM of prodigiosin and incubated for 1, 2 and 3 h. respectively.

Coverslips with treated and non-treated cells were washed in PBS and fixed for 1 h in Bouin solution at room temperature. After several washes in PBS, cells were incubated for 5 min in 0.1% Triton X-100. The coverslips were incubated with normal sheep serum for 1 h at room temperature and then for 2 h. with mouse monoclonal anti-actin (at a dilution of 1:400 in PBS-NaN_3-BSA from purchased liquid antisera; clone C4, ICN Biomedicals, Inc. cat. # 69100). Coverslips were rinsed in PBS and incubated for 1 h in sheep IgG anti-mouse Ig conjugated with FITC (at a dilution of 1:100 in PBS) as secondary antibody (Boehringer Mannheim. Germany). Cells were examined with microscope and filter.

Results

Isolation and purification of prodigiosin from S. marcescens 2170

Prodigiosin was purified from *S. marcescens* 2170 by methanol/HCl extraction followed by silica gel chromatography and semipreparative reverse-phase



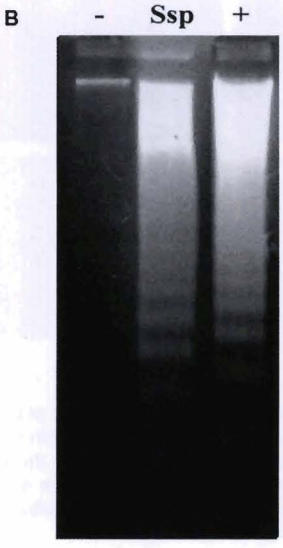


Fig. 1. A. Cell viability in prodigiosin-treated cells. The data were obtained from the treatment of HGT-1 cell line with 0.5 to 4.0 μM of prodigiosin for 4 h. Cell viability was determined by the MTT assay and is expressed as a percentage with respect to control cells. The results are from the assay of three independent experiments. **B.** Induction of DNA fragmentation by prodigiosin. HGT-1 cells are untreated (-) or incubated (+) for 12 h. with 2 μM of prodigiosin. HGT-1 cells are treated with 1mM of staurosporine (Ssp) as a positive control. The fragmented DNA was extracted and analyzed by agarose gel electrophoresis.

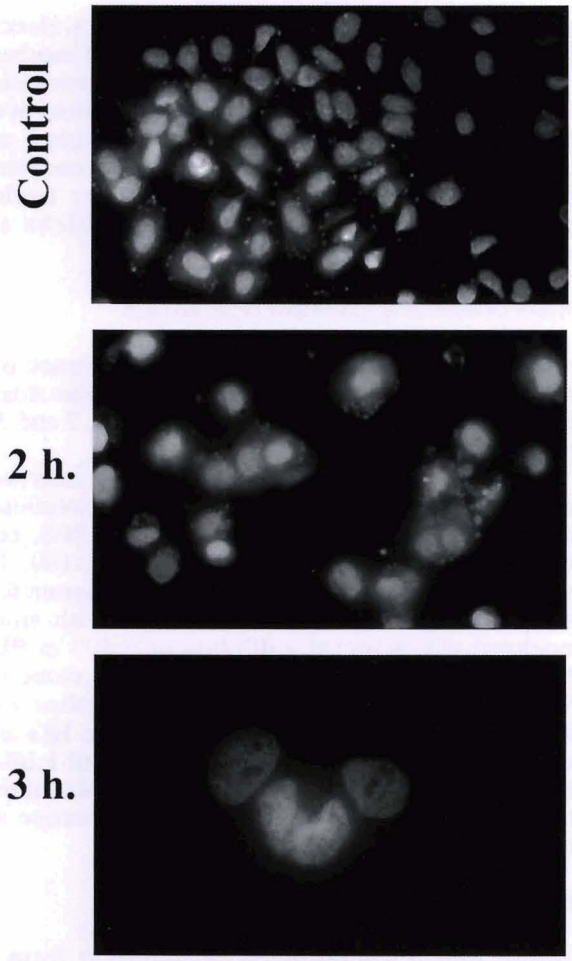


Fig. 2. Fluorescence microscopic analysis of HGT-1 nuclei with Hoechst 33342 staining. Nuclear morphology of cells untreated (control) or treated with 3 μM of prodigiosin for 2 and 3 h. Apoptotic nuclei are condensed and smaller. x 200, x 200 and x 500, respectively.

HPLC. ESI-MS gave a molecular mass of 323.4 Da, consistent with the expected value for prodigiosin ($C_{20}H_{25}N_3O$). The structure of prodigiosin was further confirmed by high-field ^{1}H-NMR spectroscopy. We used the pigment extracted with acidic methanol from *S. marcescens* 2170, re-dissolved in DMSO, as a source of apoptotic activity in the human gastric carcinoma cell line.

Prodigiosin decreased the viability of gastric cancer cells

HGT-1 cells were incubated for 4 h. with doses of prodigiosin ranging from 0.5 to 4.0 μM, and cell viability was then determined by the MTT assay. A significant dose-dependent decrease in the number of viable cells was observed, with an IC_{50} of 3.1 μM (Fig. 1A). The viability study of this cell line treated with prodigiosin at different times (4 to 24 h) did not show differences (data not shown).

Prodigiosin induced apoptosis in gastric cancer cells

In order to determine whether this cytotoxic effect was due to apoptosis, we analyzed whether prodigiosin induced DNA fragmentation. Agarose gel electrophoresis of DNA showed the characteristic ladder pattern of apoptosis in HGT-1 cells incubated for 12 h in the presence of 2 μM of prodigiosin (Fig. 1B).

We corroborated these results at microscopic level using Hoechst 33342 staining. In contrast to untreated cells, the apoptotic nuclei of HGT-1 gave stronger blue fluorescence and were condensed, and occasionally we saw holes in the nuclei of dead cells (Fig. 2). These results demonstrate that prodigiosin induces changes in nuclear morphology characteristic of apoptotic cell death.

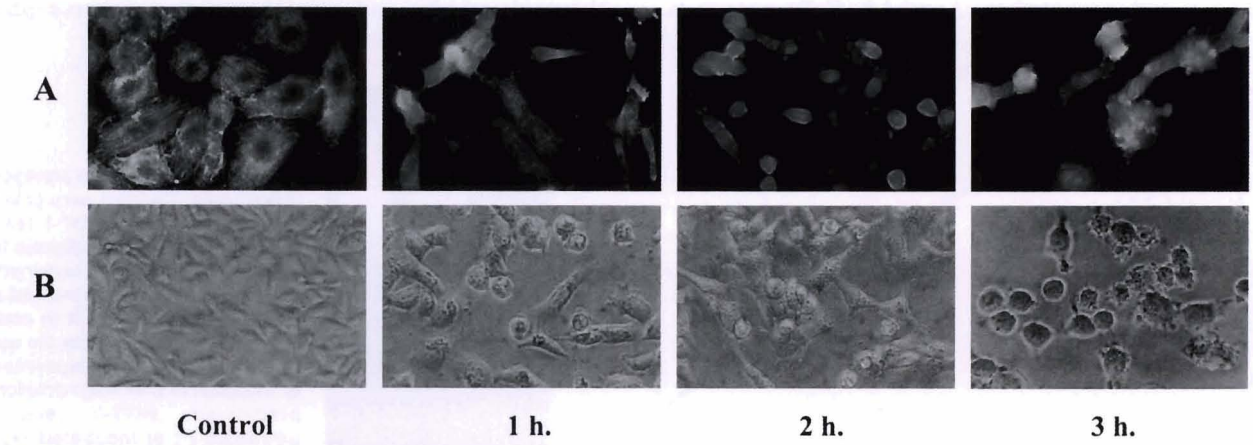


Fig. 3. Morphology of cells untreated (control) or treated with 3 μM of prodigiosin for 1, 2 and 3 h. **A.** Actin immunostaining of untreated cells (control) is observed as an intense and diffuse pattern evenly distributed within the cell (x 200). In treated cells the actin immunostaining is confined around the nucleus, and 3 h after treatment vesicles are observed within the cytoplasm showing intense actin immunostaining. x 160, x 160 and x 160, respectively. **B.** Confluent cells of untreated cells (control) show a morphological change after prodigiosin treatment; the cytoplasm has almost disappeared and the cells are smaller and rounded. After 3 h in culture, the cells have completely detached from plates and the plasma membrane is ruffled. x 60, x 130, x 130 and x 130 respectively).

Effect of prodigiosin on the organization of actin microfilaments

HGT-1 cells grew as monolayers of closely apposed polygonal cells with epithelial morphology. Most cells were mononuclear, but bi- and multinucleated cells were also observed.

Actin immunostaining showed a typical fiber network. Actin microfilaments were seen in a parallel pattern depending on the plane of focus and more concentrated around the nucleus (Fig. 3). Sometimes, punctuate structures of non-filamentous actin were seen in the extensions of lamellipodia. Immunostaining appeared more intense at the point of contact between cells.

Treatment with 2 μM of prodigiosin caused progressive morphological changes. Within the first hour, cells which had previously been almost completely confluent, had retracted and the plasma membrane started to ruffle (Fig. 3). By the second hour of treatment confluent cells had also begun to shrink, the cytoplasm almost disappeared and became rounded. In about 3 h, cells dettached from plates and typical apoptotic bodies were seen at the boundaries of detached cells (Fig. 3).

Vesicles observed within the cytoplasm of treated cells were densely stained with anti-actin antibodies. Apoptotic bodies of rounded detached cells were stained (Fig. 3).

We also saw many rounded cells with ray-like immunostaining, indicating the formation of numerous filopodia or perhaps microvilli (data not shown). Retraction of the cytoplasm was accompanied by condensation of the immunostaining around the nucleus of these cells, which may have come from non-confluent regions of plates. When the cells were exposed to prodigiosin for 2 h, washed in PBS and treated with the culture medium without prodigiosin, they began to grow and attach to the substratum (data not shown). These results demonstrate that the effect of prodigiosin on HGT-1 cells was reversible.

These results indicate that cells treated with prodigiosin detached from the substratum. This induced the reorganization of actin microfilament architecture, and cells died by apoptosis.

Discussion

We have recently reported the apoptotic effect of prodigiosin synthesized by *S. marcescens* 2170 on several human cancer cell lines like Jurkat, NSO, HL-60, Ramos (all of hematopoietic origin), SW-620, and DLD-1 (human colon origin), but not in non-malignant cells like NIH-3T3, Swiss-3T3 and MDCK (Montaner et al., 2001). Here we extend our study of prodigiosin-induced apoptosis to the human gastric carcinoma cell line (HGT-1) and we examine the possibility that actin microfilaments could be a target of this molecule.

The prevention of neoplasia by agents of bacterial origin that inhibit cancer cell proliferation and with no appreciable toxicity for normal cells is an attractive prospect. In this connection, a family of natural red pigments called prodigiosins, which are synthesized from bacteria, have been described. Cycloprodigiosin hydrochloride (cPrG.HCl) and Prodigiosin 25-C (UP) are reported to have immunosuppressive and apoptotic effects (Kawauchi et al., 1997; Songia et al., 1997). For a third member, prodigiosin, Han et al. (1998) described T-cell-specific immunosuppression but no data were reported on the involvement of prodigiosin in apoptotic cell death in human gastric carcinoma. Here we demonstrate that apoptotic cell death is induced in the human gastric carcinoma cell line HGT-1. Analysis of actin cytoskeleton in HGT-1 with FITC-conjugated actin revealed that actin filaments of cells that were not treated with prodigiosin were organized into a dense, dynamic meshwork of actin fibers, whereas the actin fibers of prodigiosin-treated cells were either disorganized, disassembled, or disrupted. These observations suggest that prodigiosin promotes the breakdown of actin microfilament, and this is the first demonstration that prodigiosin causes the reorganization of actin cytoskeleton. Arguably, it may be difficult to assess whether cleavage of a particular protein substrate is part of the endogenous cell death program, or an unrelated consequence of the lethal stimulus. However, Levee et al. (1996) suggest that reorganization of the microfilament network is necessary for the formation of apoptotic bodies, and depolymerization of F-actin may also be necessary for apoptosis.

Mgbonyebi et al. (1999) suggest that cdk inhibitors like roscovitine may be involved in cytoskeletal regulation, by reducing the polymerization of actin microfilaments. We have demonstrated that prodigiosin causes the reorganization of actin cytoskeleton and may promote the breakdown of actin microfilaments. These findings combined with our previous observations of inhibition of cdk2 activity in prodigiosin-treated cells (unpublished data) indicate that prodigiosin follows the same mechanism as roscovitine.

During the past few years, new compounds like cytochalasins, lantrunculins, jasplakinolide, swinholide A and inhibitors or farnesyltransferase (Cooper, 1987; Bubb et al., 1995; Senderowicz et al., 1995; Ayscough et al., 1997; Gibbs and Oliff, 1997), all of natural origin, have been found to modulate actin polymerization and dynamics, often by unique mechanisms (Jordan and Wilson, 1998). These molecules, together with prodigiosin, are likely to become valuable tools for the analysis of actin dynamics and functions in cells. Because they inhibit cell proliferation, they or their derivatives have potential as chemotherapeutic agents in the treatment of cancer.

Interestingly, prodigiosin induces apoptosis in Jurkat and HL-60 cells, both of which are p53-deficient (Montaner et al., 2000). Bcl-2 is an important gene product in the regulation of programmed cell death, acting as a suppressor (Korsmeyer, 1999). However, prodigiosin induced apoptosis in a Jurkat cell line stable

transfected with Bcl-2 with the same efficiency as in Jurkat cells with normal Bcl-2 expression (unpublished data). Oncogenesis is often associated with defects in p53 or with high expression of antiapoptotic genes, such as *Bcl-2* family members. As prodigiosin-induced apoptosis is p53-independent and it is not protected by Bcl-2, this could give it an advantage over other chemotherapeutic drugs (Bunz et al., 1999).

The elucidation of the mechanisms involved in the apoptotic action of prodigiosin and its evaluation as a possible anticancer drug warrants further investigation.

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