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INHIBITIVE EFFECT OF PRODIGIOSIN ON THE PROLIFERATION OF HUMAN MALIGNANT PANCREATIC CANCER CELLS

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Abstract. Pancreatic cancer is not only common, but also extremely difficult to treat, for which it has been called "the challenge of the twenty-first century". In this study, we find that prodigiosin could effectively inhibit the proliferation of human pancreatic cancer cells H8898 in a dose-and-time-dependent manner, with an IC_{50} of 75μmol according to the results of MTT and cell proliferation assays. This inhibitive effect may relate to two factors: mitotic arrest and cell death. Results of clone formation and Flow cytometry analysis (FCAS) suggested that prodigiosin has the capability of restraining mitosis by regulating the cell cycle. Prodigiosin also could induce apoptosis of pancreatic cancer cells at low concentration and results in the fragmentation pattern of DNA. Prodigiosin may effectively enter cells and promote the level of intracellular reactive oxygen species (ROSin) in a dose-dependent manner. The generation of ROS may play an important role in the cytotoxic effect. All these results demonstrate that prodigiosin can obviously inhibit the proliferation of pancreatic cancer cells H8898 by arresting the cell cycle and inducing apoptosis. Increased ROS lead this cytotoxic effect.

Introduction

Cancer of the pancreas has become more common in most Western countries over the past three decades. It is the fourth leading cause of cancer-related mortality in the United States, accounting for 30,000 deaths annually.[1, 2] Pancreatic cancer is a deadly disease with a 5-year survival of only 3–5% and the median survival after diagnosis less than 6 months.[3] Surgical resection cures only a very small minority of patients. With limited therapeutic options available at this time, it is critical to find new drugs to fight this devastating disease.[4]

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A family of natural red pigments called prodigiosins are synthesized by various bacteria such as *Serratia marcescens*.[5] Cycloprodigiosin, hydrochloride (cPrG • HCl), uncedylprodigiosin (UP), metacycloProdigiosin, desmethoxyprodigiosin and prodigiosin are congeners. Prodigiosin, with a methoxypyrrole ring, has several biological activities such as immunomodulator, antibacterial, antimycotic, antimalarial and so on.[6-10] Recently, many studies [11-16] imply that prodigiosin has a massive potential in cancer chemotherapy, which draws great public attention. The studies about its anticancer effect mainly focused on inducing apoptosis. It has been reported that prodigiosin could induce apoptosis in haematopoietic, colorectal and gastric cancer cells.[11, 13, 4]

Reactive oxygen species (ROS) are generated during respiration in mitochondria as well as by distinct enzyme systems. ROS, as important modulators, have been implicated in the regulation of diverse cellular functions including intracellular signaling, transcriptional activation, proliferation, and apoptosis.[17]The change of intracellular ROS levels has a marked influence on the cell. So, it has been regarded as an important target site for drugs' development.

Based on this, in the present study we have selected prodigiosin, produced by bioengineering technology, as a candidate and characterized its inhibitory effect on the proliferation of human malignant pancreatic cancer cells H8898. Furthermore, we examined whether the underlying mechanism of this effect relates to intracellular ROS levels. This study establishes a foundation for future research of a potential drug to fight pancreatic cancer.

Methods and materials

Cell lines and culture conditions

Human malignant pancreatic cancer cells H8898 were obtained from the Second Military Medical University. They were cultured in RPMI Medium 1640 (GIBCO BRL, Grand Island, NY, USA) containing 10% dialyzed heat-inactivated bovine serum (BS) (GIBCO BRL) at 37℃ in a humidified atmosphere of 95% air and 5% CO_2.

Purification of Prodigiosin

Prodigiosin purification was performed as described previously.[11] Briefly, prodigiosin was extracted from suspensions with acidic methanol. After centrifugation, the supernatant solvent was evaporated under vacuum. Atmospheric pressure liquid chromatography of the extract was performed on silica gel. The eluted pigmented fractions were pooled and the chloroform/ methanol extract was vacuum evaporated. The isolated pigment was redissolved in methanol and repurified by subsequent semipreparative HPLC with a C_{18} reversed-phase column. Finally, purified prodigiosin was dissolved in H_2O and lyophilized. (Purity > 90%)

Cell Viability Assay

Cell viability was determined by the MTT assay.[18] Briefly, 2×10^5 cell • mL^{-1} of H8898 cells were incubated in 96-well microtiter cell culture plates, in the absence or in the presence of increasing amounts of prodigiosin, in a final volume of 100μl. After 12 h, or indicated incubation times, 10μmol of MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100μl of DMSO and the absorbance measured at 570nm on a microplate reader (Bio-rad, California, USA). The experiment was triplicated. Percent of cell viability= average OD of test team/average OD of control team×100%. IC_{50} means the concentration of the drug under which cells have a survival rate up to 50 percent.

Cell Proliferation Assay

Cells (10^4) were incubated in 24-well microtiter cell culture plates with or without 50μmol of prodigiosin for 5 days. The cells were harvested after trypsinization, and the number of cells was counted using a hemocytometer after stained with trypan blue dye. This experiment was triplicated

Clone Formation Assay

Cells (10^5) were incubated in 24-well microtiter cell culture plates and treated with different concentration of prodigiosin for 12h. After trypsinization, single-cell suspensions in a final density of 10^3 cells • mL^{-1} were plated on10mm culture dishes. After further culturing for 5 days, clones of > 8 cells were counted. This experiment was triplicated.

Effect of Prodigiosin on the Morphology of H8898

1×10^5 cells were placed in 24-well microtiter cell culture plates with or without 12.5μmol of prodigiosin. After 12 h incubation, they were rinsed with PBS, then observed and photographed with an inversion microscope. This experiment was triplicated.

Flow Cytometry Analysis

1×10^6 cells were placed in culture dishes (6cm) and treated with or without different concentrations of prodigiosin for 24h. Then, cells were trypsinized to form single-cell suspensions. After being rinsed twice with PBS, cells were fixed with PBS containing 90 percent methanol for 1 hour at 4℃. Subsequently, they were centrifugated (1100g, 5 min) and washed twice with cold PBS. Flow cytometry analysis technique was applied after cells were stained with 50μg • mL^{-1} propidium iodide (PI) for 30 min. Apoptosis index (AI) = the number of apoptosis cells/the number of total cells×100%. This experiment was triplicated.

DNA Fragmentation Analysis

Analysis of DNA fragmentation by agarose gel electrophoresis was performed as described previously.[19] Briefly, 1×10^6 cell • mL^{-1} of H8898 cells were exposured to 50μmol prodigiosin and incubated for 24h. Cells were washed with PBS and resuspended in ice-cold lysis buffer (10 mmol Tris-HCl pH 7.4, 1 mmol EDTA, 0.2% Triton X-100). After incubating for 15 min at 4℃, cell lysates were centrifuged at 14,000g for 15 min. The supernatant was treated with 0.2 mg • mL^{-1} of proteinase K in a buffer containing (mmol) NaCl 150, Tris-HCl 10 pH 8.0, EDTA 40 and 1% SDS for 6 h at 37℃. The DNA preparations were extracted with phenol/chloroform twice, to remove proteins. DNA was precipitated with 140 mmol NaCl and two volumes of ethanol at -20℃ overnight. DNA precipitates were recovered by centrifugation at 14,0006g for 15 min at 4℃, washed twice in cool 75% ethanol and air dried. DNA pellets were completely resupended in 20 μl of TE (10 mmol Tris-HCl pH 8.0, 1 mmol EDTA) and electrophoresed in 1.2% agarose gels. Gels were placed on a UV light box to visualize the DNA ladder pattern. This experiment was triplicated.

Mensuration of Intracellular Prodigiosin Concentrations

H8898 cells were treated with prodigiosin similar to the Flow cytometry analysis. High-performance liquid chromatography (HPLC) was applied as previously described.[11] After rinsing twice with PBS, cells were resuspended in 1mL methanol and crushed by ultrasonication. Subsequently after centrifugation, the supernatant was used for HPLC assay with a mobile phase (10% ammonium acetate and 90% acetonitrile).

In situ Detection of Cellular Oxidative Stress

Treatment of H8898 cells with prodigiosin was carried out in the same way as for the viability assay. After incubation, cells were rinsed twice with PBS and cultured in phenol red-free RPMI1640 medium containing 10 μM 6-carboxy-2', 7'-dichlorodihydrofluorescein (CDCFH). [20] After 50 min, the fluorescence intensity was measured with a fluorescence plate reader CytoFluor2350 (Millipore, Bedford, MA, USA) with excitation and emission wavelengths of 485 nm and 530 nm respectively. This experiment was triplicated.

Statistical Analysis

The scientific statistic software GraphPad Instat version 2.04 was used to evaluate the significance of differences between groups with statistical significance considered as** $p < 0.01$ or ***$p < 0.001$.

Results

Effect of Prodigiosin on Cell Viability and Proliferation

Effect of prodigiosin on the viability of human pancreatic cancer cells H8898 was studied by MTT assay. The viability of H8898 cells treated with increasing doses of prodigiosin was decreased in a dose-dependent manner (Fig.1A). Prodigiosin at 25and 50 μmol significantly decreased H8898 cell viability by15.6% ($p < 0.01$) and 34% ($p < 0.001$). IC_{50} of prodigiosin for H8898 cells was 75μmol.

H8898 cells were treated with 50μmol prodigiosin at different times (4 - 24h). Every 4 hours, we measured the influence of treatment time by MTT assay. Fig. 1B showed a time course for the effect of prodigiosin on the viability of H8898 cells. The viability of H8898cells was decreased by prodigiosin in a time-dependent manner from 4h. A sharp decrease, about 28 percent, appeared between 8 to 12 hours. The effect of prodigiosin is statistically significant at 12h ($p < 0.001$).

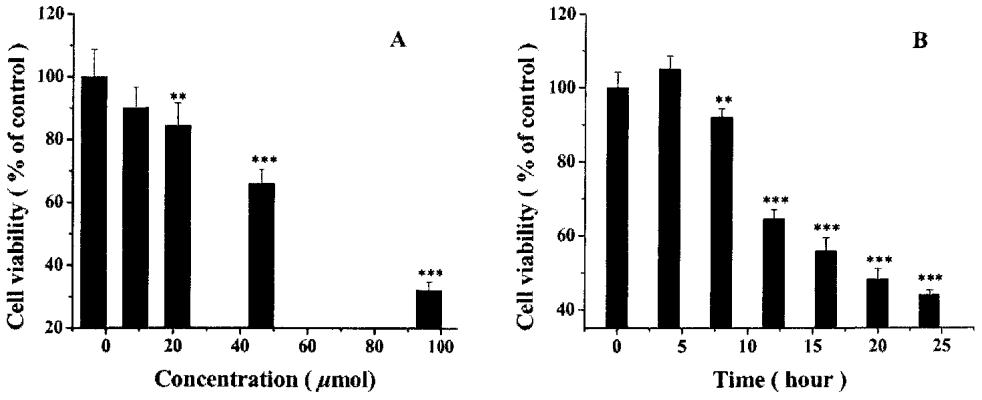


Figure 1. Effect of prodigiosin on cell viability. H8898 cells were treated with different concentrations of prodigiosin for 12h(A), or with 50μmol prodigiosin at different times (B). All data were presented as the mean±SD of three independent experiments in duplicate. Statistical significance of differences between untreated and treated cells with prodigiosin at different conditions was assayed ** $p < 0.01$ or ***$p < 0.001$.

Fig.2 showed the change of the viable cell number when treated with 50μmol prodigiosin for 5 days. Clearly, prodigiosin markedly inhibited the proliferation of H8898 cells. The inhibitory effect first appeared at 2 days and was pronounced at 3 days.

On the base of above results, prodigiosin effectively inhibits the proliferation of human pancreatic cancer cells H8898.

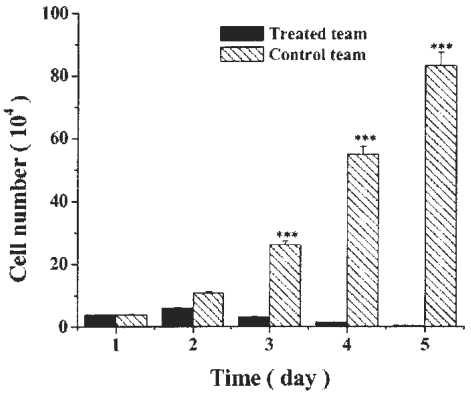
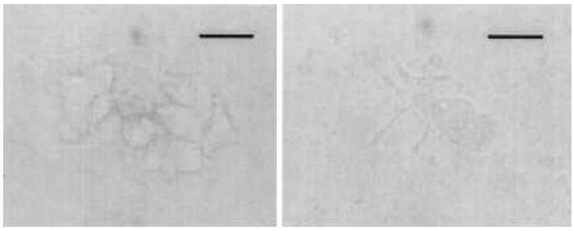


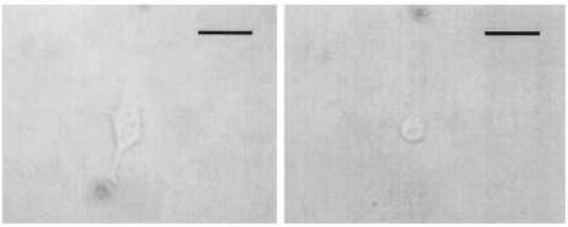
Figure 2. Effect of prodigiosin on cell growth. H8898 cells were treated with or without 50μmol of prodigiosin for 5 days. The number of viable cells were counted daily using a hemocytometer after staining with trypan blue dye. All data were presented as the mean±SD of three independent experiments in duplicate. Statistical significance of differences between untreated and treated cells with prodigiosin at different conditions was assayed ** $p < 0.01$ or ***$p < 0.001$.

Effect of Prodigiosin on Cell Mitosis and Cell Cycle

Following exposure to 0, 12.5, 25, 50μmol prodigiosin, significant inhibition of clone formation was determined and shown in Figure 3. Most of the cells in control group formed clones, while cell mitosis was markedly inhibited with prodigiosin. Cells pretreated with 12.5μmol prodigiosin were only replicated once, while cells pretreated with a higher dose of prodigiosin still remained as a single cell for 5 days. The inhibition of clone formation was not due to toxicity, as determined by the trypan blue exclusion assay.



0 μmol 12.5μmol



25μmol 50μmol

Figure 3. Effect of prodigiosin on clone formation. H8898 cells were pretreated with different concentrations of prodigiosin for 12h. After continuous culture for 5 days, cells were stained with trypan blue and photographed. Scale marker denotes 30μm.

It was presumed that prodigiosin maybe inhibit cell mitosis by arresting the cell cycle. And the results of FCAS assays supported this supposition. Fig.4 indicated that after H8898 cells were exposed to prodigiosin for 24h, the number of cells in the S stage was obviously decreased. This phenomenon was accompanied with an increasing amount in the G0-G1 stage (data not show). This result implied that prodigiosin could retard G1/S cell cycle transition, which could result in the inhibition of cell proliferation.

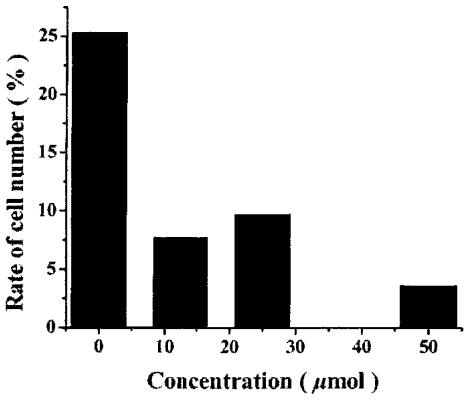


Figure 4. Effect of prodigiosin on the cell cycle. H8898 cells incubated with 0, 12.5, 25, 50μmol prodigiosin for 24h. Flow cytometry analysis technique was applied after cells were fixed and stained. Percent of cell number in S stage were represented.

Prodigiosin Induced Apoptosis in H8898 Cells

Prodigiosin induced apoptosis in H8898 cells, even at low concentration. Figure 5A showed an obvious difference in cell morphology between the test group and controls. The morphology of cells that were treated with 12.5μmol prodigiosin for 12h appeared to have some of the characteristics of apoptosis, such as compaction and cytoplasm leakage into membrane bound-vesicles. But control cells had none of these characteristics.

In order to further determine whether prodigiosin could induce apoptosis, we measured apoptosis in H8898 cells with FCAS analysis. Cells were incubated with 0, 12.5, 25 and 50μmol of prodigiosin for 24 h. Fig.5B showed that the apoptosis index increased directly with prodigiosin concentration.

We confirmed this result by DNA fragmentation assay. Agarose gel electrophoresis of DNA showed the characteristic ladder pattern of apoptosis in H8898 cells incubated 24 hours in the presence of 50μmol of prodigiosin. (Fig.5C)

All these results confirmed that prodigiosin indeed induces apoptosis in H8898 cells. The inductive effect first appeared at 12.5μmol and became very marked at 50μmol.

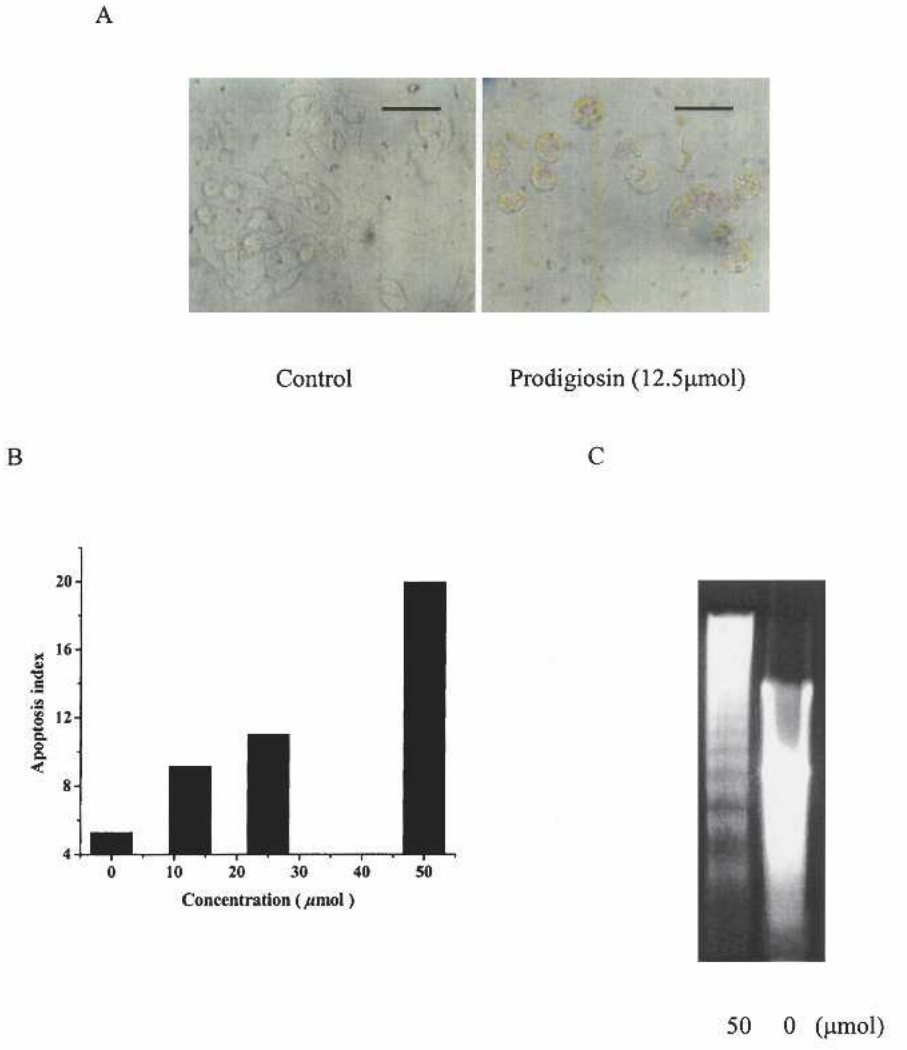


Figure 5. Prodigiosin induced apoptosis in H8898 cells. A: Effect of prodigiosin on the morphology of cells. Scale marker denotes 30μm. B: Incubated H8898 cells with 0, 12.5, 25, 50μmol prodigiosin for 24h. Percent of apoptosis cells in total cells were represented. C: H8898 cells were exposed to 50μmol prodigiosin for 24h. The fragmented DNA was extracted and analyzed by agarose gel electrophoresis.

Intracellular Prodigiosin Concentration

Intracellular contents of prodigiosin were quantified by HPLC at466nm. Fig.6 showed that the peak time of prodigiosin was stable and intracellular prodigiosin concentration was increased in a dose-dependant manner. The result indicated that prodigiosin effectively permeated cells.

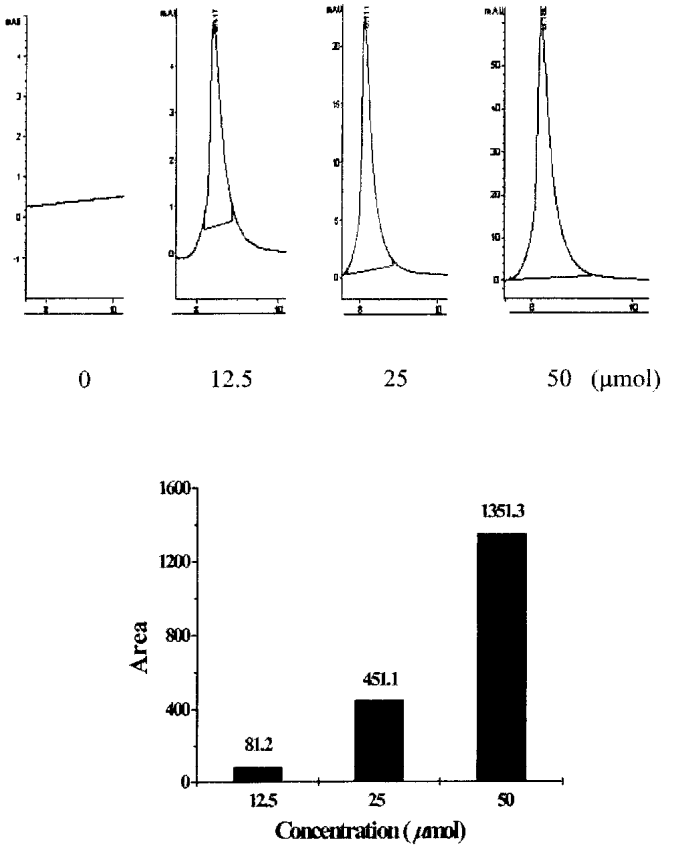


Figure 6. Intracellular prodigiosin concentration. H8898 cells were exposed to different concentration of prodigiosin for 24h. Intracellular contents of prodigiosin were quantified by HPLC at 466nm with mobile phase (10% ammonium acetate and 90% acetonitrile).

Prodigiosin Promoted Intracellular Oxidative Stress

According to the study (Fig.7), the level of intracellular ROS in H8898 cells was higher after 12h incubation with prodigiosin than without. It obviously suggested that the generation of intracellular ROS was promoted by prodigiosin. Prodigiosin increased the intracellular ROS level in a dose-dependent manner.

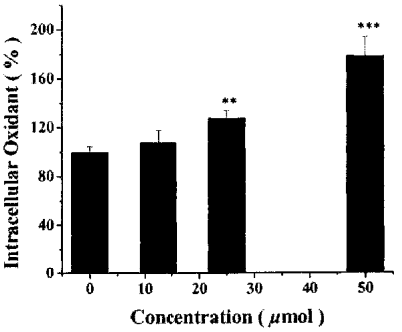


Figure 7. Prodigiosin promoted intracellular oxidative stress. H8898 cells were treated with or without different concentration of prodigiosin for 12h, given CDCFH-DA, and incubated for 50 min, followed by measurement of absorbance at 485 nm. All data were presented as the mean±SD of three independent experiments in duplicate. Statistical significance of differences between untreated and treated cells with prodigiosin under different conditions was assayed ** $p < 0.01$ or ***$p < 0.001$.

Discussion

Pancreatic cancer is hard to cure. Compared with many hematopoietic cancers, achieving a response to therapy in solid cancers is much more intractable, and pancreatic cancers are among the least responsive.[2] In fact, the annual mortality rate of pancreatic cancer almost approximates the annual accident rate. On a case to case basis, cancer of the pancreas has the shortest median survival time for all cancer types.[1, 3] Currently cis-platinum or gemcitabine are the standard chemotherapy for pancreatic cancer.[4] The European Study Group for Pancreatic Cancer (ESPAC)-1 carried out a trial in nearly 600 patients and the results demonstrated that the current best chemotherapy is using bolus 5-fluorouracil with folinic acid. The median survival of patients was extended to nearly 15.5 months.[21] This result is still far from satisfactory. A major challenge now is to find novel chemical entities with less toxicity and greater effectiveness than those in current use.

The present study revealed that prodigiosin released from *S. marcescens* 2170 in culture medium inhibited proliferation of human malignant pancreatic cancer cells H8898, as proved by the MTT and cell proliferation assays.

This effect was due to two factors: one is the inhibition of cell mitosis by arresting the cell cycle. Results of clone formation and FCAS suggested that prodigiosin retards G1/S cell cycle transition, consequently, it inhibits cell proliferation. This conclusion is consistent with a recent report that prodigisin inhibits 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.[15]

The second factor is that it can cause apoptosis by promoting intracellular ROS level. We confirmed that prodigiosin could induce apoptosis at low concentration by DNA fragmentation, FCAS results, and distinctive morphological changes. Although the mechanism underlying the apoptotic effect of prodigiosin is not clear, several hypotheses have been reported. These include: (1) prodigiosin as an intracellular pH modulator promotes H^+/Cl^- symport, induces acidification of the cytosol and thus apoptosis.[22, 23] (2) prodigiosin, as a cell cycle inhibitor, suppresses cyclin-dependent kinase-2 and –4.[7,16] (3) prodigiosin as a DNA cleavage agents intercalates the minor-groove, with preference for AT sites of DNA, and facilitates oxidative copper-promoted double-stranded DNA cleavage.[24-27] (4) prodigiosin as a mitogen-activated protein kinase regulators mediates apoptosis.[28]

The reduction-oxidation (redox) state of the cell is primarily a consequence of the balance between the generation and elimination of ROS.[17] Dramatic elevation of intracellular ROS level can induce cell apoptosis directly, by oxidation of cellular components, and also indirectly, by the sustained activation of signaling pathways and expression of genes that induce apoptosis.[17, 29]

In this study, we confirmed that prodigiosin could effectively promote the generation of ROS. Correlated with the results of HPLC, we draw the conclusion that prodigiosin can effectively permeate into cells and promote intracellular ROS levels. Thus we suggest that the promotion of ROS levels mediates apoptosis.

Numerous reports have demonstrated that promotion of ROS mediates apoptosis induced by many anticancer drugs.[30, 31] Although the exactly mechanism of increasing ROS level inducing apoptosis is

still not clear, it has been stated that ROS can regulate some important signaling pathways such as MAPKs.[29] The p38-MAPK pathway is an oxidant sensitive pathway. Several groups have shown that p38-MAPK can be activated by ROS generated intracellularly.[29, 32] It has been suggested that prodigiosin can induce phosphorylation of p38-MAPK.[33] Taken together, these data suggest that Prodigiosin promotes intracellular ROS levels, which further induce phosphorylation of p38-MAPK resulting in apoptosis.

In conclusion, the present study demonstrates that prodigiosin effectively inhibits the proliferation of human malignant pancreatic cancer cells and induces apoptosis. The underlying mechanism appears to be arrest of the cell cycle and promotion of intracellular ROS. As a new candidate for pancreatic cancer chemotherapy, prodigiosin should be investigated further with emphasis on its molecular mechanism of action *in vivo*

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