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Original Paper

Prodigiosin Inhibits Proliferation, Migration, and Invasion of Nasopharyngeal Cancer Cells

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Key Words
Prodigiosin • Nasopharyngeal Cancer • Proliferation • Migration • Invasion • Cell Cycle

Abstract
Background/Aims: Nasopharyngeal carcinoma remains a devastating and difficult disease to treat. This study explores the antineoplastic effect of prodigiosin on nasopharyngeal cancer cells. ***Methods:*** Human nasopharyngeal carcinoma CNE2 cells and human normal nasopharyngeal epithelial NP69 cells were obtained and treated with prodigiosin or fluorouracil (5-FU). Colony formation assay was performed to screen for the optimal experimental concentrations of prodigiosin and 5-FU, and MTT assay was used to examine cell proliferative ability. Flow cytometry was used to examine cell cycle distribution, the scratch test was employed to examine cell migration, and Transwell migration assay (Boyden chamber) was used to study cell invasion. ***Results:*** The optimal concentrations of prodigiosin and 5-FU for treatment were 4 mg/L and 0.35 mg/L, respectively. Both prodigiosin and 5-FU inhibited tumor cell proliferation. The percentage of cells in G0/G1 phase was higher and the percentage of cells in S phase was lower in the prodigiosin and 5-FU groups than in the untreated groups. Both prodigiosin and 5-FU inhibited tumor cell migration and tumor cell invasion. ***Conclusions:*** Our results suggest that prodigiosin can inhibit proliferation, migration, and invasion of nasopharyngeal carcinoma cells.

Introduction

Nasopharyngeal carcinoma (NPC) has an unusual ethnic and geographic distribution in Southern China and Southeast Asia, especially among persons of Cantonese origin [1, 2]. The three major etiological factors of NPC include genetic susceptibility, environmental

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factors, and Epstein-Barr virus infection [3, 4]. Most NPC cases are highly malignant, with a high rate of local invasion and early distant metastasis [5]. Due to the anatomical position of NPC and its tendency to present with cervical lymph node metastasis, it is not amenable to surgery. Radiotherapy is therefore the most common treatment for NPC, with chemotherapy playing an important supplementary role. However, conventional chemotherapeutic drugs have inevitable side effects, meaning that the search for safe and effective antitumor drugs is always needed.

The prodigiosin (PG) family comprises red pigments with a methoxypyrrole ring and includes metacycloprodigiosin, undecylprodigiosin, nonylprodigiosin, and cycloprodigiosin. These are secondary metabolites from *Actinomycetes, Serratia marcescens*, and other bacteria. PG was first isolated from *Serratia marcescens* in 1959 [6]. Early studies of PG focused on its biological activities such as immunosuppression and antibacterial or antifungal effects [7, 8]. In recent years, PG has attracted researchers' attention as potential antineoplastic agents in cancer therapy. A study from the American National Cancer Institute showed that PG had a potent anticancer effect on 57 different types of cancer cells, including liver cancer cells, with no toxicity to normal cells [9].

In this study, we investigated the effect of PG, and compared it with that of the conventional chemotherapeutic drug fluorouracil (5-FU), in inhibiting the proliferation, migration, and invasion of nasopharyngeal carcinoma cells.

Materials and Methods

Cell lines and cell culture

Human immortalized nasopharyngeal epithelial (NP69) and human nasopharyngeal carcinoma cell (CNE2) lines were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in an incubator (37°C, 5% CO_2, and saturated humidity) with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The culture medium was replaced every 2 days. When the cells reached 90% confluence, they were passaged at a ratio of 1:2 and then divided into four groups: 1) Normal: untreated NP69 cells; 2) Blank: untreated CNE2 cells; 3) PG: CNE2 cells treated with PG; and 4) 5-FU: CNE2 cells treated with 5-FU.

Colony formation assay

Agar plates were prepared by adding 0.6% agar in RPMI 1640 medium into 6-well plates. The plates were kept at room temperature for 10 min. After the plates solidified, a 0.5-mL CNE2 cell suspension (concentration: 2×10^3 cells/mL) in RPMI 1640 medium supplemented with 10% FBS was added. After cell attachment, PG at various concentrations (0.5, 1, 2, 4, 6, and 8 mg/L) or 5-FU at various concentrations (0.2, 0.25, 0.3, 0.35, 0.4, and 0.5 mg/L) was added into the agar plates. Cell colony formation was observed after 14 d in culture at 37°C. The number of colonies was counted by randomly selecting 5 horizons in each group. Colony forming efficiency was defined as the ratio of the number of colonies formed in culture to the number of cells inoculated.

Cytotoxicity assay

Cells in the logarithmic growth phase were collected and transferred into a 96-well plate after the cell concentration was adjusted to 5×10^6 cells/mL. After the cells adhered to the plate, the PG group was treated with 4mg/L PG and the 5-FU group was treated with 0.35mg/L 5-FU. After 24, 48, 72, and 96 h of culture, 10 μL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added to each well. Subsequently, the cells were cultured for another 4 h in the incubator. After the culture solution was discarded, 150 μL dimethyl sulfoxide was added and the plates were placed in a shaker for 10 min in the dark. Optical density at a wavelength of 490 nm was measured.

Flow cytometry

To establish the proportion of cells in each phase of the cell cycle, cells were trypsinized for 3 min, centrifuged, and washed with phosphate-buffered saline (PBS). The cell pellet was suspended and fixed

on ice for 15 min with 1 mL of cold 70% ethanol. The cells were subsequently centrifuged and the cell pellet was suspended in 1 mL of propidium iodide (PI) solution (0.05 mg/mL PI, 0.02 mg/mL RNase, 0.3% NP40 1 mg/mL sodium citrate) for 1 h at 4°C. Flow cytometry analysis was performed using a FACSort flow cytometer.

Cell migration assay

Matrigel diluent was placed in 24-well plates and the plates were left to air-dry at room temperature for 30 min. Cells were transferred into the prepared plate and cultured in an incubator (37°C, 5% CO_2). When cells fully covered the bottom of the plate, a line was drawn lightly in each well with a sterilized tip, ensuring that the width of each line was the same. A mark was left on the cap of the 24-well plates to ascertain the same visual field in the photograph, and then initial photographs were taken and recorded as 0 h. After incubation at 37°C for 24 h, medium was suctioned and discarded. The plate was washed 3 times with PBS to remove cell debris caused by scratching. Serum-free medium was added and photographs were taken recorded as 24 h. Photographs were taken under an Olympus Inverted Microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with 6 visual fields at a fixed location. The migration distance was calculated with the ImageTool software (Bechtel Nevada Inc., Los Alamos, NM). Migration distance = (width at 0 h – width at 24 h)/2.

Transwell migration assay

Each Transwell chamber (Corning Glass Works, Corning, NY) contained matrigel (3.9 mg/mL, 60–80 μL), and was incubated at 37°C. When the matrigel solidified, the chambers were taken out and placed in a 24-well plate. Medium was pre-warmed in the incubator and then added separately into the upper and lower chambers (0.5 mL per chamber). The chambers were placed in the incubator for a 2-h hydration, and then the medium was suctioned and discarded from the upper and lower chambers. The cell suspension (5×10^4 cells/mL) was prepared after digestion. A total of 0.5 mL complete medium was extracted and placed in a 24-well plate, and then the hydrated chamber was transferred into the 24-well plate, avoiding bubble formation. In total, 0.5 mL cell suspension was extracted and put into the chamber, followed by incubation at 37°C for 24 h. The liquid in the upper and lower chambers was suctioned and discarded. Cotton swabs were used to clean off the cells on the surface of the upper chamber of the Transwell membrane. After three PBS washes, the transferred cells were fixed with ice-cold methanol for 30 min. Cells were then stained with 0.1% crystal violet for 10 min. After this, the cells were washed with running water until no extra crystal violet remained and were air-dried. Finally, data were recorded through direct observation and the cells were photographed under a microscope. Photographs were captured using an Olympus Inverted Microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with 6 visual fields at a fixed location. The number of cells transferred onto the Transwell lower chamber was counted.

Statistical analysis

All data were analyzed with GraphPad Prism version 6 statistical software. Data were expressed as mean ± standard deviation. The t test was used for comparisons between two groups. One-way analysis of variance was applied for comparisons between multiple groups. Statistical significance was assumed for $P < 0.05$.

Results

The antineoplastic effect of PG is concentration-dependent

Colony formation assay revealed that both PG and 5-FU inhibit cell proliferation under all concentrations tested ($P < 0.05$)—the higher the concentration, the better the inhibition. However, when the PG concentration reached 4 mg/L, the inhibitory effect remained unchanged. There was no significant difference between the inhibitory effects of 4, 6, and 8 mg/L PG ($P > 0.05$). Therefore, the PG concentration 4 mg/L was selected as the optimal treatment concentration. Similarly, the inhibitory effect of 5-FU at 0.35, 0.40, or 0.50 mg/L had no significant difference ($P > 0.05$). Therefore, the 5-FU concentration of 0.35 mg/L was used in this study (Fig. 1).

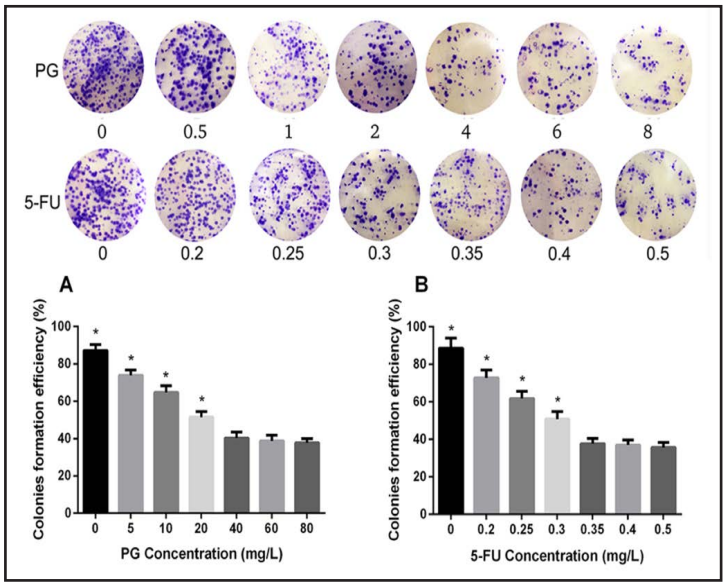

Fig. 1. Colony formation assay. Top: Images of CNE2 cell colonies after treatment with prodigiosin (PG) or fluorouracil (5-FU) at various concentrations. A: Statistics of colony formation after treatment with PG at different concentrations. *P<0.05 compared with PG at 8 mg/L; B: Analysis of colony formation after treatment with 5-FU at different concentrations. *P<0.05 compared with 5-FU at 0.5 mg/L.

PG inhibits proliferation of CNE2 cells

After 24, 48, 72, and 96 h of treatment with PG and 5-FU, the proliferation of CNE2 cells was inhibited in all cases when compared with the Blank group ($P < 0.05$), and the inhibitory effect was time-dependent. The inhibitory effect of 5-FU was stronger than that of PG, but the difference was not significant ($P > 0.05$). Cells proliferated significantly faster in the Blank group than in the other three groups, while the cells in the Normal group proliferated significantly slower than the other three groups ($P < 0.05$; Fig. 2).

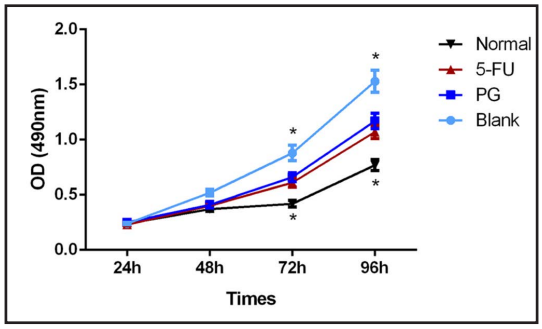

Fig. 2. MTT assay. *P<0.05 compared with the 5-FU group.

PG inhibits CNE2 cell proliferation by interrupting the cell cycle

Flow cytometric analysis showed that the percentage of cells in G0/G1 cell cycle phase in the PG and 5-FU groups were significantly increased compared with the Blank group ($P < 0.05$). In contrast, the percentage of cells in S phase in the PG and 5-FU groups decreased ($P < 0.05$). In the Normal group, the percentage of cells at G0/G1 was higher than in the Blank group but lower than in the other two groups, while that in the S phase was slightly lower than in the Blank group, but showed no significant difference compared with the other two groups ($P > 0.05$). The percentage of cells in G2/M in all groups showed no significant difference (Fig. 3).

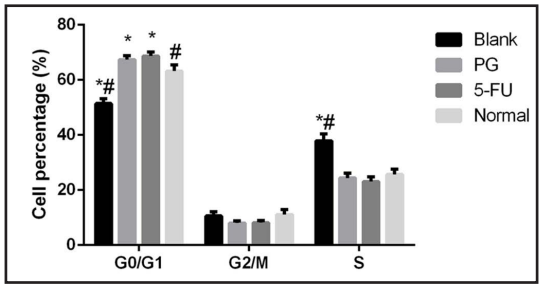

Fig. 3. Flow Cytometry. *P<0.05 compared with the Normal group. #P<0.05 compared with the 5-FU group.

PG inhibits CNE2 cell migration

Scratch adhesion assay showed that CNE2 cell migration was inhibited by PG and 5-FU treatments. Migration distances in the PG and 5-FU groups were significantly lower than

in the Blank group ($P < 0.05$). The migration distance of the PG group was slightly higher than that of the 5-FU group, but the difference was not significant. The Normal group had the lowest rate of cell migration ($P < 0.05$; Fig. 4).

PG inhibits CNE2 cell invasion

Transwell migration assay showed that CNE2 cell invasion was inhibited by PG and 5-FU. The number of cells that penetrated through the Matrigel down to the lower chamber was significantly lower in the PG and 5-FU groups than in the Blank group ($P < 0.05$). The cell number of the PG group was slightly higher than that of the 5-FU group, but the difference was not significant ($P > 0.05$). The Normal group had the lowest extent of cell invasion ($P < 0.05$; Fig. 5).

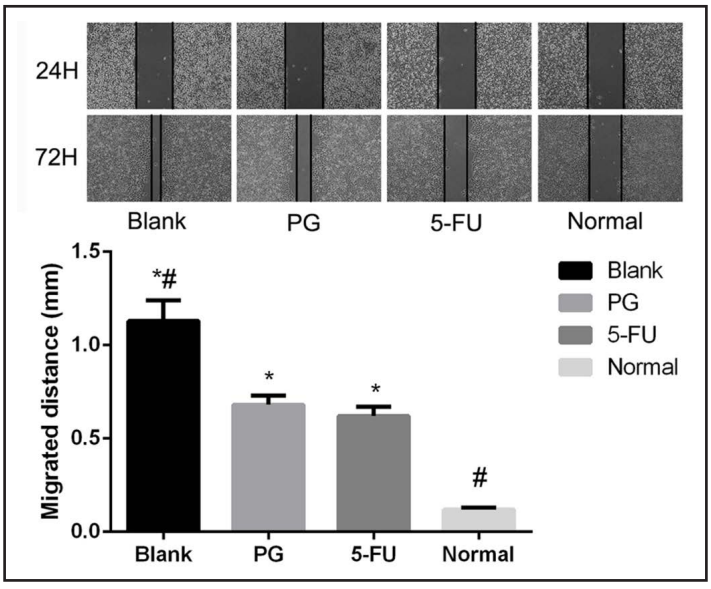

Fig. 4. Cell migration scratch assay. *P<0.05 compared with the Normal group. #P<0.05 compared with the 5-FU group.

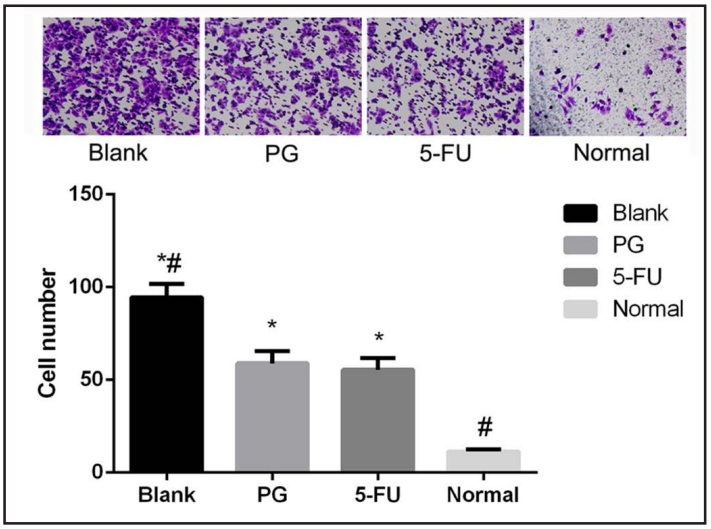

Fig. 5. Transwell migration assay. *P<0.05 compared with the Normal group. #P<0.05 compared with the 5-FU group.

Discussion

Malignant tumor invasion and migration is the most critical stage of tumor progression. Studies show that 90% of malignant nasopharyngeal tumors treated by conventional therapy will recur and metastasize within 1 year. More than 80% of patients with nasopharyngeal tumors die of tumor invasion and migration. The key to controlling tumor development is to inhibit tumor cell proliferation and metastasis. Although traditional antineoplastic drugs achieve certain treatment effects, their specificity is poor and side effects are inevitable. Therefore, new natural drugs with significant efficacy are eagerly being sought. Extraction from secondary metabolites of microorganisms is a widely used method for finding anticancer and antibacterial drugs, and their clinical application has particular advantages.

With 5-FU as a positive control drug, this study showed that the antitumor effect of PG against human nasopharyngeal carcinoma CNE2 cells was similar to that of a conventional chemotherapeutic drug. PG also inhibited tumor cell migration and invasion.

Our study is consistent with earlier studies that showed the anticancer effect of PG. Montaner et al. demonstrated that PG could induce apoptosis in human colon adenocarcinoma

cells DLD-1 and SW-620 and in human gastric cancer cells HGT-1; the effects were dose-dependent [10]. Studies on apoptosis induced by PG were focused on cell survival-related signal transduction. Montaner et al. hypothesized that PG induced cell apoptosis through activation of p38-MAPK phosphorylation. Songia et al. showed that PG blocked proliferation of human lymphocytes by inhibiting the phosphorylation of retinoblastoma and inhibiting cyclin-dependent kinase (Cdk)-2 and Cdk-4 [11].

Yamamoto et al. demonstrated that the antitumor effect of PG arose from PG activating the transmembrane transporter of H^+ and Cl^-, which resulted in dissociation of V-ATPase, leading to cytosol acidification and cell apoptosis [12]. Castillo-Ávila et al. studied the effects of PG on pH value and cytosol lysosomal function in colon cancer cells [13]. The results showed that PG stimulated and decreased pH in lysosomes. Colon cancer cell (HT29) proliferation was arrested in G1, which supported Yamamoto's idea of acidification of cytosol-induced apoptosis. However, this view is controversial. The question remains as to how important a role cytosol acidification plays in apoptosis.

Melvin et al. argued that the antitumor effect of PG could be associated with its nuclease activity [14]. Because PG is electron-rich, it can be oxidized in the presence of Cu^{2+} to form a π-radical cation and Cu^{1+}, which then induce cleavage of DNA double strands, resulting in cytotoxic activity. This effect was studied using agarose gel electrophoresis with microfluorescence technique. Subramanian et al. isolated PG structural analogues from *Micrococcus* sp., and nuclease characteristics and antitumor activities were evaluated [15]. The results showed that the compounds could effectively bind to DNA and promote DNA cleavage and lipid peroxidation mediated by copper ions, and that they inhibit cell proliferation in mouse lymphoma cell line EL4 and human chronic myeloma cell line K562.

Zhang et al. studied the inhibitory effects of PG on the pancreatic cancer cell line H8898 [16]. MTT assays showed that the IC_{50} of PG to H8898 was 75 μmol. Flow cytometry showed that PG inhibited tumor cell mitosis and promoted DNA cleavage. Further studies have shown that PG could enter into cells to stimulate production of cytotoxic reactive oxygen species against tumor cells and promote apoptosis.

Cytotoxic ability varies in PG molecules due to different structures. DNA cleavage induced by PG is closely related to the A ring structure in the molecule. Complete bipyrrole ring chromophore structure is the key for PG to exhibit copper-mediated nuclease activity. When the A ring or B ring in the parent structure is substituted by aromatics, most or all the activity of PG-induced DNA cleavage is lost [14, 17]. Different substitutions of PG C-6 methoxy could also affect potency. Molecular modification based on these results led to the development of new PG derivatives with higher activity indices than that of parent molecule [18].

In summary, we studied the antineoplastic effects of PG on proliferation, migration, and invasion of NPC cells. The potential antitumor mechanism was discussed. Our study confirmed that PG could inhibit proliferation, migration, and invasion of nasopharyngeal carcinoma cells. Our results provide a rationale for the potential use of PG in the treatment of nasopharyngeal cancer, where PG is less toxic to non-tumor cells than conventional chemotherapeutic drugs.

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Disclosure Statement

The authors have declared that no competing interests exist.

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