

Treatment with 2-methyl- 3-pentyl-6-methoxyprodiginine isolated from *Serratia marcescens* decreases cell viability and induces apoptosis in acute lymphoblastic leukemia cells

Sommayeh Ghoreishi¹, Mohammad Reza Sam^{2*}

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Abstract

Background & Aims: Acute lymphoblastic leukemia (ALL) is the most common malignancies in the world. Despite advances in treatment of patients with ALL, a subset of patients will have recurrent disease or refractory to chemotherapy and hematopoietic stem cell transplant. Consequently, assessment of the effectiveness of natural compounds with high efficacy and minimal side effects is warranted. In this regard, it has been shown that some of bacterial pigments such as prodigiosin isolated from cell wall of *Serratia marcescens* have dramatic anti-cancer activities. The aim of this study was to evaluate the effects of prodigiosin on the cell viability and cell number, cell proliferation and apoptosis in CCRF-CEM cell line that serves as a model for ALL cells.

Materials & Methods: Malignant cells were treated with 100, 200 and 400 nM prodigiosin for 24, 48 and 72 h and cell proliferation-rates were measured by performing WST-1 assay. Furthermore, malignant cells were treated with the indicated concentrations of prodigiosin for 48 h and cell viabilities and cell numbers along with apoptotic-rates were determined by trypan blue staining method and flow cytometer respectively.

Results: Treatment of cells with increasing concentrations of prodigiosin significantly decreased Proliferation -rates in a dose- and time-dependent manner compared to untreated cells. Specifically, after 72 h treatments with 100, 200 and 400 nM prodigiosin, proliferation-rates were measured to be $77.3 \pm 1.5\%$, $63 \pm 2\%$, and $46.3 \pm 3.2\%$ respectively as compared to untreated cells. Furthermore, following 48 h treatments with indicated concentrations of prodigiosin, the cell numbers and viabilities were decreased in a dose-dependent manner. Specifically, treatment with 400 nM prodigiosin resulted in 44% (4.5×10^5 cells) and 63% for cell number and viability respectively as compared to untreated cells. At the same conditions, apoptotic-rates (Early + Late) were measured to be 33.8% to 72.8% at the indicated prodigiosin concentrations ranging.

Conclusion: Prodigiosin decreased cell number and viability as well as cell proliferation-rates. This compound also increased apoptosis in CCRF-CEM cells. Therefore, this compound with high pro-apoptotic capacity represents an attractive anti-leukemic agent in ALL.

Keywords: Acute lymphoblastic leukemia (ALL), Prodigiosin, *Serratia marcescens*, Cell proliferation, Apoptosis

Address: Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Shahid Beheshti Street, Urmia, Iran. P. O. Box: 165.

Tel: +98-4433440199

Email: m.sam@urmia.ac.ir; s_mohammadreza@yahoo.com

¹Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, Iran

²Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, Iran (Corresponding Author)

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in Iran and the world. This type of leukemia is the most common pediatric cancer that some patients do not have an appropriate response to chemotherapy and hematopoietic stem cell transplant. Since most anti-cancer drugs are chemically synthesized and have many side effects on the patient, the researchers focused on natural ingredients with the aim of replacing them with chemical drugs. Among natural anti-cancer drugs, the red pigment isolated from various microorganisms such as prodigiosin isolated from cell wall of *Serratia marcescens*. It has been shown that this pigment leads to a significant reduction in cell proliferation and the induction of apoptosis in several types of cancer cells. It has been shown that prodigiosin causes death of cancer cells through induction of different conditions. These conditions are acidifying the cytoplasm, cell cycle arrest with increased expression of P21WAF / CIP1, damage to DNA molecules by blocking the function of I and II enzymes topoisomerase, decline in the mitochondrial ATP production. It has been shown that this compound has high efficiency apoptosis induction in cancer cells. Since the favorable conditions of an anti-cancer drug is to induce apoptosis rather than cell necrosis, this property of prodigiosin is important. In this study, CCRF-CEM cell line (T cell type and acute lymphoblastic leukemia) treated with different concentrations of prodigiosin and the number of cells, their survival rates and cell proliferation, as well as induction of apoptosis have been studied in these cells.

Materials and Methods

RPMI-1640 culture medium, fetal bovine serum, penicillin and streptomycin were purchased from Austrian company (PAA). WST-1 reagent, 2-methyl-3-pentyl-6-methoxy prodigiosin and dimethyl sulfoxide (DMSO) were obtained from Sigma Company

Germany. CCRF-CEM cell line was purchased from Pasteur Institute. Trypan Blue was purchased from Merck, 6 and 96-plate plates as well as cell culture flasks were purchased from Jet Biohyphen Company, Korea. Staining Kit of Annexin / PI was purchased from Roche, Germany.

Prodigiosin preparation and cell treatment:

Prodigiosin was dissolved in ethanol for preparation of stock 20 µg / ml. Then stored at -80 ° C. 100 to 400 nM concentrations of prodigiosin were prepared for all tests.

Cell culture:

CCRF-CEM cell line was cultured in RPMI-1640 medium containing 10% fetal bovine serum in T-25 culture flask and kept in CO₂ incubator (5%) and 90% humidity at 37 ° C.

Treatment and Cell Count:

5 x 10⁵ of CCRF-CEM cells were added to a 6-well plate in 2 ml culture medium. Cell culture medium replaced with supplemented mediums with 100, 200 and 400 µM of prodigiosin, respectively, and 2 wells were considered as controls which contained the untreated cells. Then the plate was placed in incubator for 48 hours at 37 ° C with 90% humidity and 5% CO₂. At the end of the treatment, the supernatant of each well was removed and the number of cells were counted using neobar lam by providing a uniform cell suspension.

Determination of cell Viability:

The viability of cancer cells treated with prodigiosin was determined using trypan blue. Live cells were impermeable to trypan blue entrance, while the dead cells absorb the color of trypan blue. To prepare a uniform cell suspension, 50 µl of the suspension was added to 50 µl trypan blue and the number of viable cells were counted after a few

minutes using the slide neobar and the viability rate was calculated by the following formula.

$$\text{Viability} = A/B \times 100$$

A : The number of living cells

B :The total number of cells (live + dead)

Evaluating cell proliferation:

evaluate cell proliferation, 10^4 cells in 200 μ l of culture medium were added to 96-well plate. After 24 hours, the cell culture medium was replaced with fresh medium with the indicated concentrations of prodigiosin and plates were incubated for 24, 48 and 72 hours in an incubator with 5% CO₂ at 37 ° C. At each stage after incubation, 100 microliters of medium were removed from each well and 10 microliters of WST-1 solution was added on 100 μ l culture medium to each well, and plates were placed at 37 ° C for 4 hours. Then, the medium became homogeneous slowly with a sampler. The intensity of the yellow color was recorded by ELISA reader at 492 nm and the growth rate and cell proliferation was calculated by the following formula.

$$\text{Cell proliferation (\%)} = \frac{A_{\text{Sample}} - A_{\text{Blank}}}{A_{\text{Control}} - A_{\text{Blank}}}$$

Flow cytometry analysis:

105×5 cells were plated in 2 ml of culture medium in each well of 6- well plate.. After 24 hours, prodigiosin was added to each part at concentrations of 100, 200 and 400 nM.. Then the plates were incubated with 5% CO₂ for 48 hours at 37 °. After incubation, the cells were collected separately and sediment cell suspensions were obtained by centrifugation. The supernatant was discarded and precipitated cells were washed with sterile phosphate buffered. Then, the supernatant was discarded and 2 μ l of Annexin solution and 100 μ l cell binding buffer was added to the sediment and incubated on ice and in the dark for 15 minutes. Centrifugation was performed and the supernatant was discarded. Next, 1.5 ml of sterile PBS buffer plus set 2 μ l".

of PI color was added to the cells and the results were recorded by flow cytometry in less than 5 minutes. The cells stained with Annexin are at the Earlyapoptosis stage and cells stained with PI necrosis and cells that have attracted both colors have been experiencing Late apoptosis. In this method, the cell without the treatments have been stained with the same manner and the results were recorded by flow cytometry.

Results

The effect of prodigiosin on the number of cells and their viability rates:

CCRF-CEM cells were treated with different concentrations of prodigiosin. The number of cells at concentrations of 100, 200 and 400 nM were 10260000, 370000, 330000, 287000, respectively (Figure 1). Also the cell viability at concentrations of 100, 200 and nM were $77.3\% \pm 1.5$, $72.7\% \pm 1.5$ and $63\% \pm 4$, compared to the control cells. Based on these results, we found that the number of cells and the viability rate depended on the concentration of the drug. with an increase in drug concentration, the cell number and survival rates reduced (Figure 1a: and b).

Prodigiosin effect on cell growth and proliferation Assay:

Cell proliferation of malignant cells was decreased on a dose and time dependent manner.. After 24 hours of treatment with prodigiosin at concentrations of 100, 200 and 400 nM, the cells growth was $92\% \pm 2.5$, $82\% \pm 4$ and $64\% \pm 3$, respectively, compared to untreated control cells. At 48-hour treatment with indicated prodigiosin concentrations, cells growth was $85\% \pm 1$, $72\% \pm 3.8$, $52\% \pm 10.1$ compared to untreated control and at 72-hour treatment the growth rates were $77\% \pm 3.2$, $64\% \pm 2$, $46\% \pm 3.2$ compared to untreated control cells. Based on the results, the maximum

reduction in cell growth and proliferation have been recorded within 72 hours after treatment (Figure 2). It's worth noting that no cytotoxic effects on PBMCs cells was observed in with the indicated prodigiosin concentrations.

The effect of prodigiosin on apoptosis nduction:

In order to analyze the effect of prodigiosin on apoptosis induction, cancer cells were prepared for flow cytometry analysis after 48 hours treatment with prodigiosin at concentrations of and $100, 200$

400 nM. Based on the results, prodigiosin induces apoptosis in CCRF-CEM cells and the necrosis at different concentrations was very low. Apoptosis (early + secondary) in different prodigiosin concentrations ranging was 33.8-72.8%. The highest rate of apoptosis was seen in nM of prodigiosin (Figure 3 and Table 1). Thereby, reduction in cell proliferation in this study was due to the induction of apoptosis in CEM - CCRF cells.

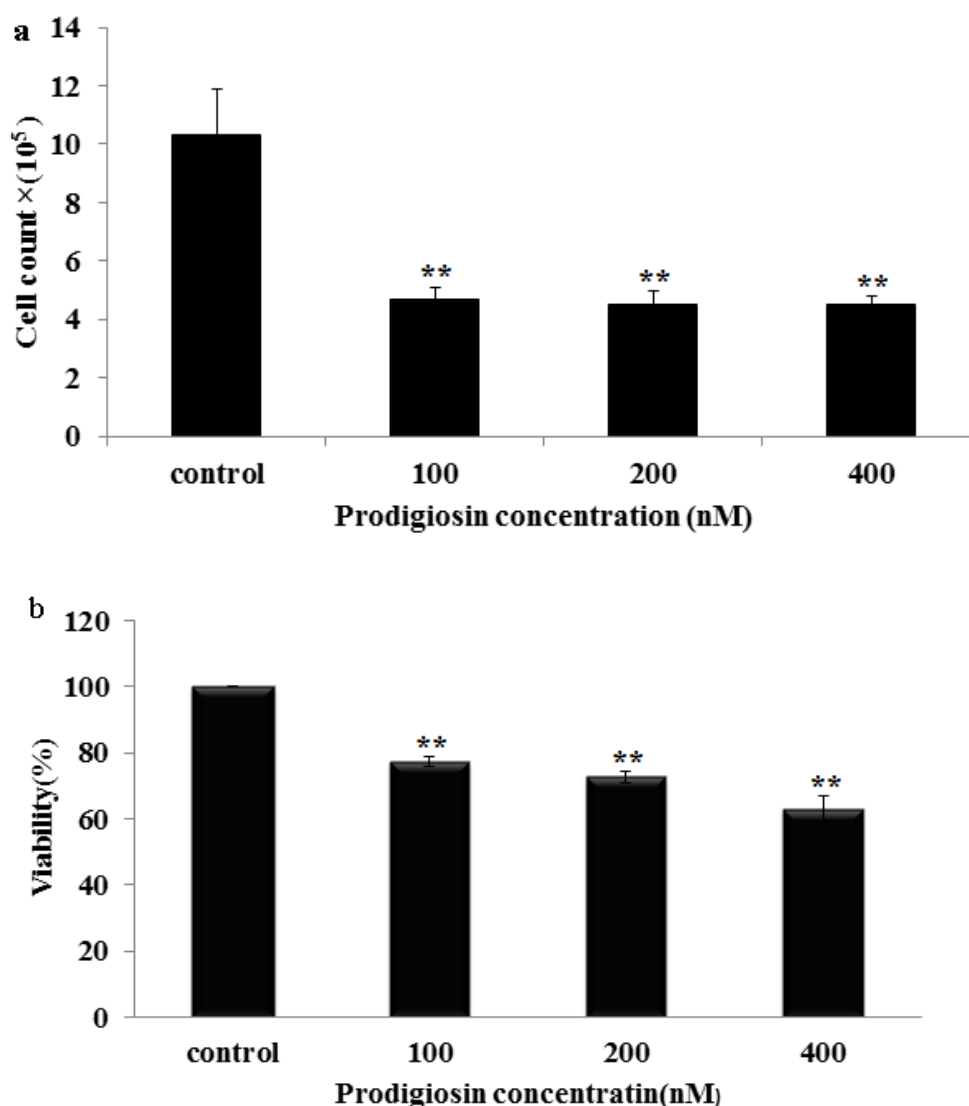


Figure 1: The effect of 48-hour treatment with prodigiosin on the number of cells and viability of CCRF-CEM cells. Cells were treated at prodigiosin concentrations of 100, 200 and nM. After 48 hours, the number of cells (Figure a) and survival rates (figure b) were calculated as compared with untreated control cells.. The results are the mean of two experiments, each with 3 to 4 repeats . A value of $p < 0.01^{**}$ was calculated as compared to untreated control cells

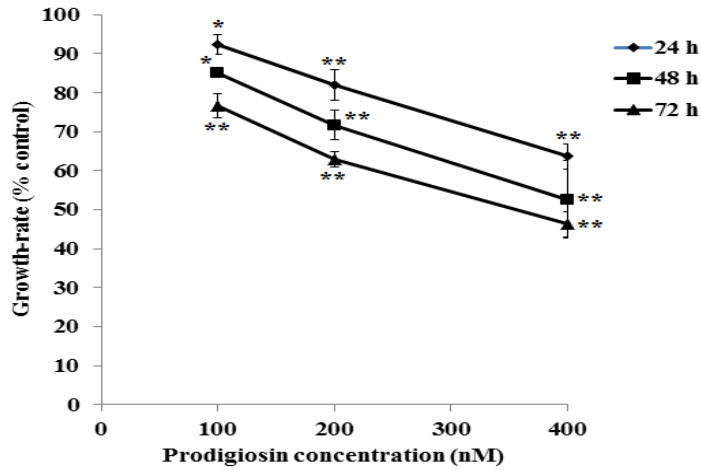


Figure 2: The growth and proliferation rate of CCRF-CEM cells treated with prodigiosin. Cells treated with above mentioned prodigiosin concentrations and the rate of cell growth and proliferation in intervals of 24, 48 and 72 hours was calculated after treatment compared with untreated control cells. The mean results are for two experiments, each with 3 repeats. A Significant values are calculated as * $P < 0.05$ and $P < ** 0.01$ compared to untreated control cells.

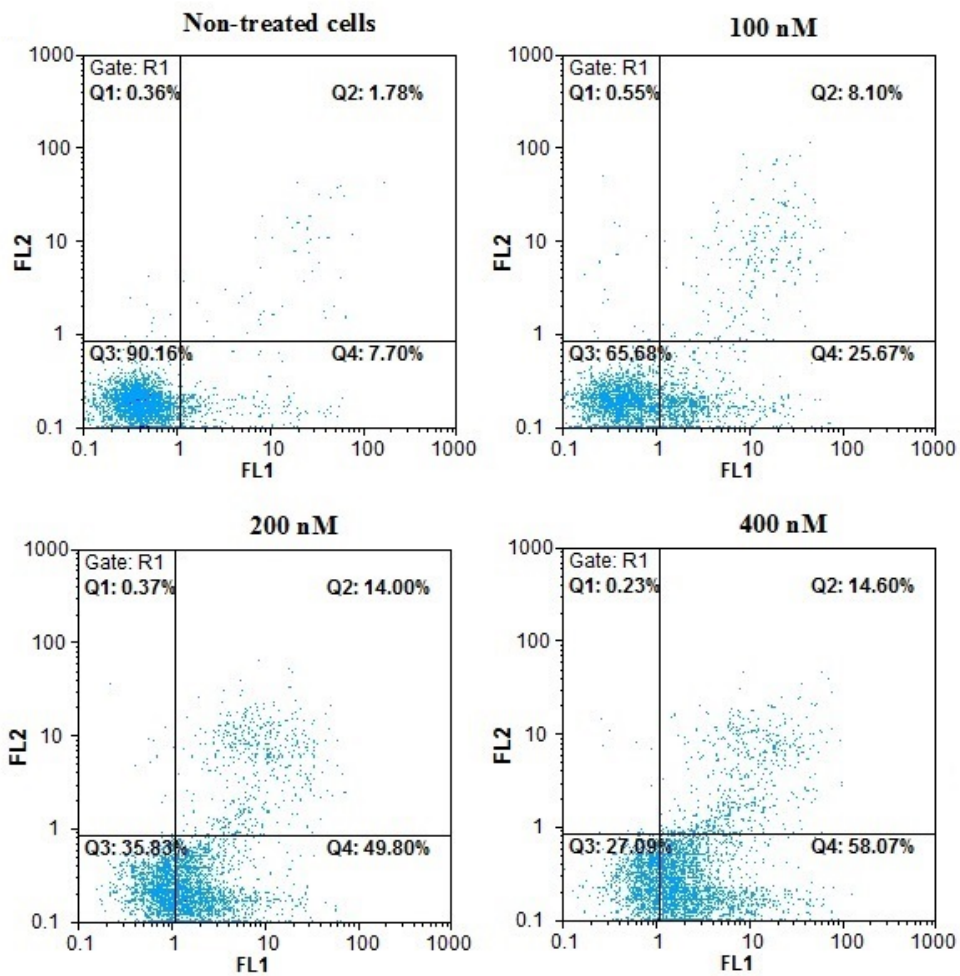


Figure 3: Flow cytometry plots of apoptosis and necrosis in CCRF-CEM cells treated with various concentrations of prodigiosin after 48 hours. Q1: necrotic cells, Q2: late apoptosis. Q3: living cells and Q4: early apoptosis

Table 1. apoptosis and necrosis in CCRF-CEM cells treated with various concentrations of prodigiosin after 48 hours

Control (untreated cells)	Results		Mean \pm SD
living cells	90.1%	92.4%	91.3% \pm 1.6
Early apoptosis	7.7%	4.8%	6.3% \pm 2.1
Late apoptosis	1.8%	2.2%	2% \pm 0.3
Necrosis	0.4%	0.42%	0.3% \pm 0.1
100nM prodigiosin			
living cells	65.7%	61.3%	63.5% \pm 3.1
Earl apoptosis	25.7%	37.1%	31% \pm 8
Late apoptosis	8.1%	2%	5% \pm 4.3
Necrosis	0.6%	0.4%	0.5% \pm 0.1
200nM prodigiosin			
living cells	35.8%	46.2%	41% \pm 7.3
Earl apoptosis	49.8%	45.5%	47.6% \pm 4.5
Late apoptosis	14%	8%	11% \pm 4%
Necrosis	0.4%	0.38%	0.4% \pm 0.1
400nM prodigiosin			
living cells	27.1%	37%	32.1% \pm 5.6
Earl apoptosis	58.1%	51.5%	54.6% \pm 5.7
Late apoptosis	14.6%	10.7%	12.4% \pm 3.1
Necrosis	0.2%	0.8%	0.5% \pm 0.1

Discussion

Despite recent advances in cancer treatment, ALL is remained as one of the deadliest ones of human life. Chemotherapy and hematopoietic stem cell transplantation is the main strategy for the treatment of acute lymphoblastic leukemia. However, these methods have limitations including resistance to chemical drugs and side effects on the tissue and healthy cells as well as the lack of appropriate response of patients to hematopoietic stem cell transplant. So researchers are interested to find drug compounds with high efficacy and fewer side effects. Recently, bacterial natural

components including prodigiosin family have attracted the attention in therapy and have become a potential source of new drugs. The results of cell counting and viability were indicative of the fact that prodigiosin has been led to a significant reduction in the number of malignant cells and in survival rates. On this basis, it can be used as a drug to inhibit the cell cycle in malignant cells. In this context, it has been shown that prodigiosin, depending on the cell type and structure, leads to cell cycle arrest in late G1-phase or G1-S phase. Induction of the cyclin-dependent kinase inhibitor of p27, Cyclin E, Cyclin D and

retinoblastoma protein phosphorylation is inhibited by End Seal prodigiosin that leads to cell cycle arrest in B and T lymphocytes.

Apoptosis is a programmed death of cells without inflammation leading the tissue homeostasis. Defects in this program can cause the cancer and its progression. Since one of the goals of anti-cancer drugs is to induce apoptosis and necrosis in the cell, this effect of prodigiosin in lymphoblastic leukemia cells is important. Related to our study, different researchers showed that the treatment of colorectal cancer cell lines (HT-29), liver (HepG2), HL60 and Jurkat, Breast (T47D) with prodigiosin leads to the induction of apoptosis in these cells, and decreases necrosis of the cells. Based on these observations, it can be concluded that prodigiosin activates similar molecular mechanisms in the different types of malignant cells. The result of its activity is to induce apoptosis rather than necrosis in these cells. Further experiments are absolutely necessary in this area to deny or prove the hypoassay mentioned above.

Since cell proliferation test has been designed based on the metabolic activity of mitochondria, reduction in cell proliferation in WST-1 may indicate this fact that this compound causes defects in mitochondria by targeting vital activities in this organ which results in reducing the proliferation of malignant cells. In a study by Montaner and colleagues on leukemia cancer cells, it was shown that the metabolic activity of living cells after 4-hour treatment with prodigiosin reduces. This reduction was prodigiosin in a dose-dependent manner. Given our results and other researchers and fact that the prodigiosin compound has not effect on normal cells [15], this compound has potential to be used in patients with acute lymphoblastic leukemia along with other chemotherapy drugs. However, there must be considered appropriate and complete experiments on animal models prior to human clinical trials.

Acknowledgments

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