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ORIGINAL ARTICLE

Effective Targeting Survivin, Caspase-3 and MicroRNA-16-1 Expression by Methyl-3-pentyl-6-methoxyprodigiosene Triggers Apoptosis in Colorectal Cancer Stem-Like Cells

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Abstract Over-expression of the proto-oncogene survivin in colorectal cancer stem cells (CCSCs) is thought to be one the primary causes for therapy failure. It has also been reported that tumor suppressor miR-16-1 is down-regulated in colorectal cancer (CRC) cells. Therefore, the search for new anti-proliferative agents which target survivin or miR-16-1 in CCSCs is warranted. Several studies have shown that prodigiosin isolated from cell wall of *Serratia marcescens* induces apoptosis in different kinds of cancer cells. Here, we investigated the effects of prodigiosin on HCT-116 cells that serve as a model for CRC initiating cells with stem-like cells properties. HCT-116 cells were treated with 100, 200 and 400 nM prodigiosin after which cell number, viability, growth-rate, survivin and miRNA-16-1 expression, caspase-3 activation and apoptotic rate were evaluated. Prodigiosin decreased significantly growth-rate in a dose-and time-dependent manner. After a 48 h treatment with 100, 200 and 400 nM prodigiosin, growth-rates were measured to be 84.4 ± 9.2 %, 58 ± 6.5 % and 46.3 ± 5.2 %, respectively, compared to untreated cells. We also found that treatment for 48 h with indicated concentrations of prodigiosin resulted in 41 %, 54.5 % and 63 % decrease in survivin mRNA levels and induced 32 %, 48 % and 61 % decrease in survivin protein levels as well as resulted in 128.3 ± 10 %, 178.7 ± 6.1 % and 205 ± 7.6 % increase in caspase-3 activation respectively compared to untreated cells. Prodigiosin caused a significant increase in miRNA-16-1 expression at a concentration of 100 nM and treatment with different concentrations of prodigiosin resulted in 2.2- to 3-fold increase in miRNA-16-1/survivin ratios compared to untreated cells. An increase in number of apoptotic cells ranging from 28.2 % to 86.8 % was also observed with increasing prodigiosin concentrations. Our results provide the first evidence that survivin and miRNA-16-1 as potential biomarkers could be targeted in CRC initiating cells with stem-like cells properties by prodigiosin and this compound with high pro-apoptotic capacity represents the possibility of its therapeutic application directed against CCSCs.

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies with high incidence and mortality in Iran and the world [1, 2]. Despite receiving surgical resection, chemotherapy and radiotherapy, many cancer patients develop resistance, tumor relapse, or metastatic diseases [1, 3]. Recent studies have shown that this may be due, at least in part, to

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the presence of chemotherapy-resistant colorectal cancer stem cells (CCSCs) [4]. The cancer stem cells are a subpopulation of tumor-initiating cells within a tumor possessing self-renewal ability and pluripotency and are able to sustain tumor growth [5, 6]. In 2007, O'Brian [7] and Vitiani [8] were the first to provide independent proof of the existence of CCSCs.

For several years, microorganisms have been studied for their secondary metabolites with anticancer properties [9]. In this regard, it has been shown that prodigiosin (Fig. 1; 2-methyl-3-pentyl-6-methoxyprodigiosene) as a secondary metabolite of *Serratia marcescens* exerts apoptotic effect on a diverse array of human cancer cells with low toxicity on normal cells [10, 11]. Anticancer potency of prodigiosin is attributed to its pro-apoptotic effect irrespective of p53 status [12]. Recently, it has been shown that prodigiosin exerts pro-apoptotic effects in cancer cells via regulation of apoptosis and anti-apoptosis-related genes [12, 13]. Therefore, prodigiosin is attractive candidate for evaluation of its effects on CRC cells with stem cell-like properties.

Survivin and Bcl-2, as members of inhibitor of apoptosis (IAP) gene family which are over-expressed in most human cancers but rarely in normal tissues [14, 15] along with different microRNAs [16] that function as negative regulators of target genes by directing specific mRNA cleavage or translational inhibition through RNA induced silencing complex [17, 18] play important roles in colorectal tumor biology including;
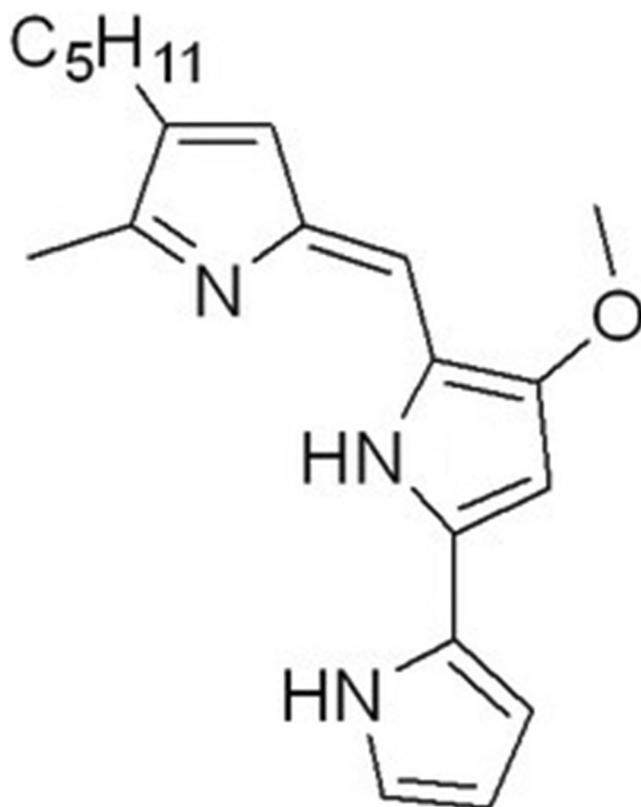
oncogenesis, progression, invasion, metastasis and angiogenesis [19–22]. In this regard, it has been shown that, miRNA-16 as a tumor suppressor miRNA is down-regulated in a variety of human cancers, including colon cancer [23] and over-expression of miR-16 induced apoptosis in colorectal cancer cells through the intrinsic apoptosis pathway [24]. Therefore, targeting survivin, Bcl-2 or mir-RNA-16-1 expression in CRC stem-like cells by a therapeutic agent may provide minimal toxicity to normal stem cells, as well as its differentiated progeny, and may open up avenues to new therapeutic strategies for CRC-directed therapy. In this regard, the cytotoxic effects of prodigiosin isolated from cell wall of *Serratia marcescens* in CRC stem-like cells have been remained unclear. Therefore, in the present study, we aim to investigate the various effects of prodigiosin on cell number and viability, cell proliferation, expression of miR-16-1, survivin and Bcl-2 genes, caspase-3 activation and apoptosis in HCT-116 cells that serve as a model for CRC initiating cells with stem-like cells properties.

Materials and Methods

Cell Line and Culture Condition

The colorectal cancer (CRC)-derived cell line HCT-116 was obtained from the National Cell Bank of Iran (Tehran). The cells were grown in RPMI-1640 medium containing 10 % (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) all from PAA Laboratories (Austria), 20 mM HEPES and 2 mM L-glutamine (Roche, Mannheim, Germany) at 37 °C in a humidified incubator with 5 % CO_2.

Preparation of Prodigiosin and Cell Treatments

Prodigiosin (Sigma, USA) was dissolved in absolute ethanol (Merck, Germany) to prepare 20 μg/ml stock solutions and stored in the dark at −80 °C until use. For each experiment, the prodigiosin was freshly prepared from the stock solution at the concentration ranging from 100 to 400 nM by serially diluting in culture medium. Control cells were cultured in the medium containing the same concentration of absolute ethanol (v/v) as the prodigiosin containing medium. The final ethanol concentration never exceeded 0.5 % (v/v).

Cell Count and Viability Assays

HCT-116 cells were seeded in 6-well plates at a density of 5×10^5 cells per well in 1.5 ml of complete medium and incubated at 37 °C for 24 h. Next, the cells were treated with various concentrations of prodigiosin (100, 200 and 400 nM) for 48 h. Subsequently, the cells were harvested and stained with trypan blue (Sigma, USA) after which the numbers of viable cells and

Fig. 1 Structure of prodigiosin isolated from cell wall of *Serratia marcescens*

the total cell counts were determined under an inverted microscope. Cell viability after each treatment procedure was expressed as percentage of the untreated control cells.

Cell Proliferation Assays

HCT-116 cells were plated in 96-well plates (5×10^3 cells/well). After 24 h incubation, the cells were treated with different concentrations of prodigiosin for 48–72 h and cell proliferation-rates were evaluated by performing WST-1 proliferation assay. Following each incubation period, the WST-1 solution (10 μl) was added to each well and the cells were incubated for an additional 4 h at 37 °C and 5 % CO_2. The absorbance in each well was then measured with a microplate reader (State Fox, USA) at 450 nm and a reference wavelength of 620 nm. Finally, the proliferation-rates were calculated as $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100$ % and the results were expressed as the percentages of untreated control cells.

To measure the total growth inhibition and the IC_{50} values of prodigiosin, dose response curves were plotted as graphs of percentage of cell proliferation rate on y-axis against the concentration of drug on x-axis. Finally, all calculations were performed by using regression analysis.

RNA Isolation, cDNA Synthesis and Reverse Transcription (RT)-PCR

Total cellular RNAs were extracted from treated and untreated cells using RNA preparation kit (Sinaclon Bioscience, Iran) and subsequently, used as a template to perform RT-PCR method to generate a first cDNA strand according to the manufacturer's instruction (Fermentas, Canada). The generated fragments were subsequently used as templates for PCR amplification of the double-stranded cDNA corresponding to a preselected region of the survivin coding sequence, using oligonucleotides survivin-F and survivin-R (Bioneer, South Korea) as forward and reverse primers, respectively (Table 1) with the following program: After an initial

Table 1 List of oligonucleotides used in the present study

Primer name	Sequence (5'-3')
Survivin-F	GCATGGGTGCCCCGACGTTG
Survivin-R	GCTCCGGCCAGAGGCCTCAA
GAPDH-F	CAAGGTCATCCATGACAACTTTG
GAPDH-R	GTCCACCACCCTGTTGCTGTAG
Bcl-2F	TGTGGATGACTGAGTACCTGAACC
Bcl-2R	CAGCCAGGAGAAATCAAACAGAG
Survivin-F1	TCCACTGCCCCA CTGAGAAC
Survivin-R1	TGGCTCCCAGCCTTCCA
GAPDH-F1	GAGTCCACTGGCGTCTTCA
GAPDH-R1	TCTTGAGGCTGTTGTCATACTTC

denaturation step at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 20 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s, were followed by a final extension at 72 °C for 10 min. For internal control, the generated cDNA was used as template for the PCR amplification of a section of the human *GAPDH* coding sequence, using oligonucleotides GAPDH-F and GAPDH-R (Bioneer, South Korea) as forward and reverse primers, respectively (Table 1). PCR products were visualized on 1 % agarose gels after ethidium bromide staining.

Quantitative Real-Time RT-PCR

Total RNAs were isolated as mentioned above. Each 2 μg sample of RNA was amplified with the Primescript™ RT reagent kit (Takara, Japan) using an oligo (dT) primer to generate 20 μl of cDNAs. Two microliters sample of the cDNA was then quantified by real-time PCR using primer pairs for survivin (F1 and R1), Bcl-2 (F and R) and GAPDH (F1 and R1) (Table 1) with SYBRGreen PCR Master mix (Takara, Japan). Amplification of the GAPDH cDNA was used as an internal and normalization control for real-time RT-PCR.

To evaluate expression of mir-16-1, each 2 μg sample of RNA was subjected to the polyadenylation reaction using poly (A) polymerase enzyme, ATP and other necessary reagents to generate poly (A) tail at 37 °C for 10 min. In the next stage, reverse transcriptase and other necessary reagents for cDNA synthesis were subsequently added to convert the poly (A) tailed miRNAs into cDNA using an oligo-dT primer to generate 20 μl of cDNAs at 43 °C for 60 min followed by 85 °C for 1 min. Two microliters sample of the cDNA was then quantified by real-time PCR using specific primers for miR-16-1 (Parsgenome Company, Iran) with SYBRGreen PCR Master mix (Takara, Japan) on an ABI PRISM 7000 (Applied Biosystems, USA) according to the following program: After an initial denaturation step at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, annealing at 62 °C for 20 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min were performed. Data analysis was carried out using the $2^{-\Delta\Delta Ct}$ relative quantification method and miR-16-1 expression was normalized against U6 snRNA.

Enzyme-Linked Immunosorbent Assay (ELISA)

Intracellular survivin protein expression level was assayed by the sandwich ELISA following the procedure provided by the manufacturer (Abcam, USA). Briefly, HCT-116 cells (5×10^5 cells/well) were cultured in the absence or in the presence of prodigiosin (100, 200 and 400 nM) for 48 h. After trypsinization, the cells were washed twice with sterile ice-cold phosphate buffered saline (PBS), and suspended in cold lysis buffer for 30 min followed by centrifugation at $12,000 \times g$ for 15 min at 4 °C. Next, the supernatant was collected and

used for survivin protein assay. Briefly, microtiter ELISA plate coated with mouse anti-human survivin monoclonal antibody. Following sample application, a biotinylated detection polyclonal antibody from goat specific for human survivin is then added followed by washing with PBS buffer. Thereafter, Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with PBS buffer. Tetramethyl benzidine (TMB) substrate was finally added and reactions were stopped after 15 min. The optical density values of the samples were recorded at 450 nm using an ELISA reader.

Apoptosis Assays

Caspase-3 Activity Assay

Caspase-3 activation was determined using colorimetric assay as manufacturer's instruction (Roche, Germany). Briefly, HCT-116 cells (5×10^5 cells/well) were cultured at the indicated concentrations of prodigiosin for 48 h. After trypsinization, the cells were washed twice with sterile PBS, and suspended in cold lysis buffer for 30 min followed by centrifugation at $12,000 \times g$ for 15 min at 4 °C. Next, the supernatant was collected and used for caspase-3 activation assay. The principle was that caspase-3 derived from cellular lysates recognizes the sequence Asp-Glu-Val-Asp (DEVD). The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (p-NA) after cleavage from the labeled substrate (DEVD-p-NA). The p-NA light emission can be quantified using a microtiter plate reader at 405 nm. Comparison of the absorbance of p-NA from an apoptotic sample with an untreated control sample allows determination of the fold increase in caspase-3 activity.

Flow Cytometric Analysis

The amount of apoptosis was assessed using an Annexin V FLOUS/propidium iodide (PI) labeling solution according to the manufacturer's instructions (Roche, Germany). Briefly, HCT-116 cells were treated with different concentrations of prodigiosin for 48 h. Thereafter, the cells were washed twice with sterile cold PBS and, after centrifugation, re-suspended in 100 μl 1× binding buffer containing FITC-Annexin V at a density of 5×10^5 cells/ml. Next, the cells were gently mixed and incubated in the dark at room temperature for 20 min. To differentiate cells with membrane damage, PI solution was added to the cell suspension prior to flow cytometric analysis using a fluorescence-activated cell sorter (FACScan, USA).

Statistical Analyses

Data represent at least means of two independent experiments. The results are expressed as mean ± standard deviation. All calculations were performed using the SPSS 15 for Windows (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of Prodigiosin on Cell Number and Viability

Results showed that prodigiosin significantly decreased cell number and viability in a dose-dependent manner (Table 2). As shown in Table 2, prodigiosin diminished cell counts by 10.4×10^5 to 6.4×10^5 cells at concentrations ranging from 100 to 400 nM compared to untreated cells. Concurrently, the HCT-116 cell viabilities were measured to be 64.4 to 34.3 % at the same concentrations ranging compared to the untreated cells (Table 2). Based on these data, prodigiosin is a potent agent in decreasing HCT-116 cell number and viability at the doses used.

Effect of Prodigiosin on Cell Proliferation

Treatment of cells with increasing concentrations of prodigiosin diminished proliferation-rate in a dose- and time-dependent manner (Table 2). As shown in Table 2, treatment of cells with increasing concentrations of prodigiosin resulted in a lower growth-rate, specifically after 72 h treatment compared to untreated cells. After a 72 h treatment, prodigiosin at a concentration of 400 nM was found to be more potent in inhibiting cell proliferation compared to other treatments at the same conditions.

Reduction in total growth inhibition of HCT-116 cells and IC_{50} values of prodigiosin was observed after 72 h treatment compared to 48 h treatment (Table 2).

Effect of Prodigiosin on the Survivin, Bcl-2 and miR-16-1 Expression

Since the role of survivin, Bcl-2 and miR-16-1 expression in prodigiosin-induced apoptosis has so far not been addressed in CCSCs, we set out to evaluate the effect of prodigiosin treatment on the expression of survivin, Bcl-2 and miR-16-1. To this end, HCT-116 cells (as a model for CCSCs), were treated with different concentrations of prodigiosin. We found that, after a 48 h treatment with prodigiosin, the high level of survivin expression in untreated HCT-116 cells decreased significantly with increasing drug doses (Fig. 2a, b). Specifically, we found that treatment with 400 nM prodigiosin resulted in 63 % decrease in the survivin mRNA level compared to untreated cells. Concomitantly, the level of Bcl-2 mRNAs did not change significantly after treatment with different concentrations of prodigiosin compared to untreated cells (Fig. 2c).

Table 2 Evaluation of cell proliferation, cell number, viability and growth-inhibition rates in prodigiosin-treated cells

Treatment	48 h			72 h		
Prodigiosin concentration (nM)	100	200	400	100	200	400
Proliferation rate (% control)	*84.4 ± 9.2	**58 ± 6.5	**46.3 ± 5.2	*85 ± 10.6	**46.2 ± 7	**33 ± 5.6
Cell count ($\times 10^5$ cells)	*8.5 ± 0.6	**7.6 ± 0.54	**6.4 ± 0.53	ND	ND	ND
Cell count (% control)	*82.3 ± 1.5	**73.3 ± 9.5	**62 ± 1.7	ND	ND	ND
Viability (% control)	**64.4 ± 2.7	**53.1 ± 2.1	**34.3 ± 2.3	ND	ND	ND
Growth-inhibition (100–400 nM)						
IC50 (nM)	352.8 ± 42			281.1 ± 19.3		
Total (nM)	746 ± 70.3			590.3 ± 58.3		

* $P < 0.05$, ** $P < 0.01$ vs. untreated control cells. *ND* not determined

We also found that, treatment of HCT-116 cells with 100 nM prodigiosin resulted in a significant increase of miR-16-1 expression compared to untreated cells (Fig. 2d).

Effect of Prodigiosin on Survivin Protein Level

To evaluate the decrease in survivin mRNA levels results in decrease in survivin protein levels in HCT-11 cells, we evaluated the intracellular survivin protein levels in prodigiosin-treated cells. As shown in Fig. 3, treatment of HCT-116 cells with different concentrations of prodigiosin resulted in low level of survivin protein and was significantly decreased with increasing prodigiosin doses. Specifically, treatment with 400 nM prodigiosin resulted in 61 % decrease in survivin protein level compared to untreated cells. A significant positive correlation was observed between survivin mRNA levels and survivin protein levels ($r = 0.99$).
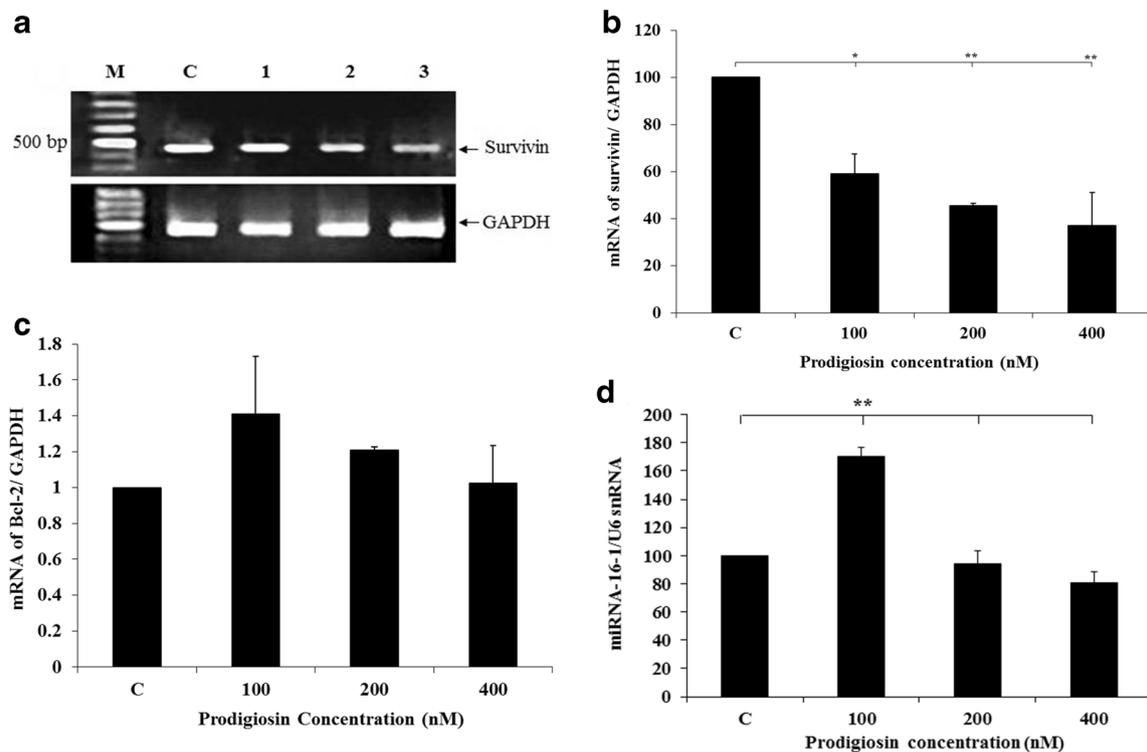

Fig. 2 Effect of prodigiosin on the survivin, Bcl-2 and miR-16-1 expression. **a** RT-PCR detection of survivin mRNA after gel electrophoresis in prodigiosin treated HCT-116 cells. Lane M: DNA size marker, Lane C: untreated cells, Lanes 1 to 3: HCT-116 cells were treated with 100, 200 and 400 nM prodigiosin respectively. **b**, **c** and **d** Quantitative real-time RT-PCR detection of survivin, Bcl-2 and miR-16-1 expression in prodigiosin treated HCT-116 cells respectively. Parallel real-time RT-PCR with GAPDH and U6 snRNA specific primers was performed to normalize the equal loading. C: untreated cells. *$P < 0.05$, **$P < 0.01$ vs. untreated cells
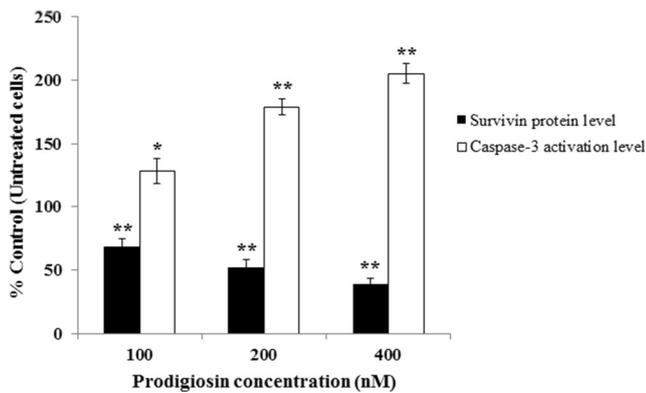
Fig. 3 Effect of prodigiosin on survivin protein and caspase-3 activation levels in HCT-116 cells after 48 h treatment. C: untreated cells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated cells

Effect of Prodigiosin on Caspase-3 Activation

As shown in Fig. 3, treatment of tumor cells with prodigiosin resulted in high level of caspase-3 activation and was significantly increased with increasing prodigiosin doses. Specifically, treatment with 400 nM prodigiosin resulted in 2.1-fold increase in caspase-3 activation compared to untreated cells.

Effect of Prodigiosin on Apoptosis Induction

To determine whether the growth-inhibitory effect observed upon treatment of HCT-116 cells with prodigiosin was due to the induction of apoptosis, the cells were treated with indicated concentrations of prodigiosin for 48 h and subsequently stained with Annexin V/PI and analyzed by means of flow cytometer. With this method, the cells stained single positive for PI were considered mostly necrotic cells, and cells stained single positive for Annexin V were considered mostly early apoptotic cells, but cells that were stained double positive could be either necrotic or apoptotic cells. As depicted in Fig. 4a-b, HCT-116 cells treated with prodigiosin at various concentrations of 100, 200, and 400 nM displayed approximately 28.2 %, 76.2 % and 86.8 % late apoptotic cells as well as 5.2 %, 5.2 % and 6.8 % necrotic cells, respectively, compared to untreated cells. Based on these data, prodigiosin is an attractive natural compound to induce apoptosis in HCT-116 cells that serve as a model for CRC initiating cells with stem-like cells properties and the above observed decreases in cell number, viability, and proliferation could be attributed to the induction of apoptotic mechanisms.

Effect of Prodigiosin on the miRNA-16-1/Survivin mRNA and Caspase-3 Protein/Survivin Protein Ratios

The ultimate vulnerability of cells to diverse apoptotic stimuli is determined by the relative ratio of various tumor suppressor and proto-oncogene genes expression. In the present study, we determined the relative ratios of tumor suppressor miRNA-16-1 to proto-oncogene survivin after 48 h treatment with different concentrations of prodigiosin. In this regard, treatment with different concentrations of prodigiosin resulted in 2.2- to 3-fold increase in miRNA-16-1/survivin ratios compared to untreated cells (Fig. 5a).

The level of pro-apoptotic marker such as caspase-3 versus the level of anti-apoptotic marker such as survivin could be important in determining the resistance of cells to apoptosis. As shown in Fig. 5b, the caspase-3 protein/survivin protein ratios were increased with increasing prodigiosin concentrations and resulted in 2.2- to 6.3-fold increase compared to untreated cells.

### Associations between Survivin Expression, Caspase-3 Activation and Cell Proliferation

In treated HCT-116 cells, expression of survivin at both the mRNA and protein levels was down-regulated by prodigiosin. We found that, there is a significant association between survivin gene expression and cell proliferation. The decrease in survivin mRNA and protein levels was accompanied by an increase in caspase-3 activation, growth inhibition and apoptosis in prodigiosin-treated cells compared to untreated cells. Significant correlations were observed between survivin protein levels with caspase-3 activation ($r = -0.96$) and cell proliferation ($r = 0.97$) as well as apoptosis rate ($r = -0.97$).

Discussion

CCSCs are thought to be responsible for tumor formation and maintenance. These cells have also been linked to the acquisition of chemotherapy resistance and to tumor recurrence [4]. Thus, novel and more efficient stem cell-based therapies, able to target CCSCs are required.

Several experimental studies showed that prodigiosin attenuates the proliferation of various kinds of cancer cells with low cytotoxicity on normal cells [12, 13, 25]. These results suggest that prodigiosin could be a promising candidate for cancer chemotherapeutic agent. However, little attentions have been paid to the effects of prodigiosin on the undifferentiated CRC cells with stem-like cells properties. In this regard, HCT-116 cells that serve as a model for CRC initiating cells with stem-like cells properties are attractive candidate. This cell line is known to be highly aggressive cells with little or no capacity to differentiate and contains a high proportion of CCSCs that have lost the capacity to differentiate [26, 27]. In view of the fact that HCT-116 cell line contains almost only CCSCs and do not significantly separate into different types of colony-forming cells, nor into different categories with respect

Fig. 4 Effect of prodigiosin on apoptosis induction in HCT-116 cells after 48 h treatment. **a** Q1) Pre-necrotic cells, Q2) Late apoptosis + necrosis, Q3) Living cells and Q4) Early apoptotic cells. C: untreated cells. **b** $^{**}P < 0.01$ vs. *untreated cells*
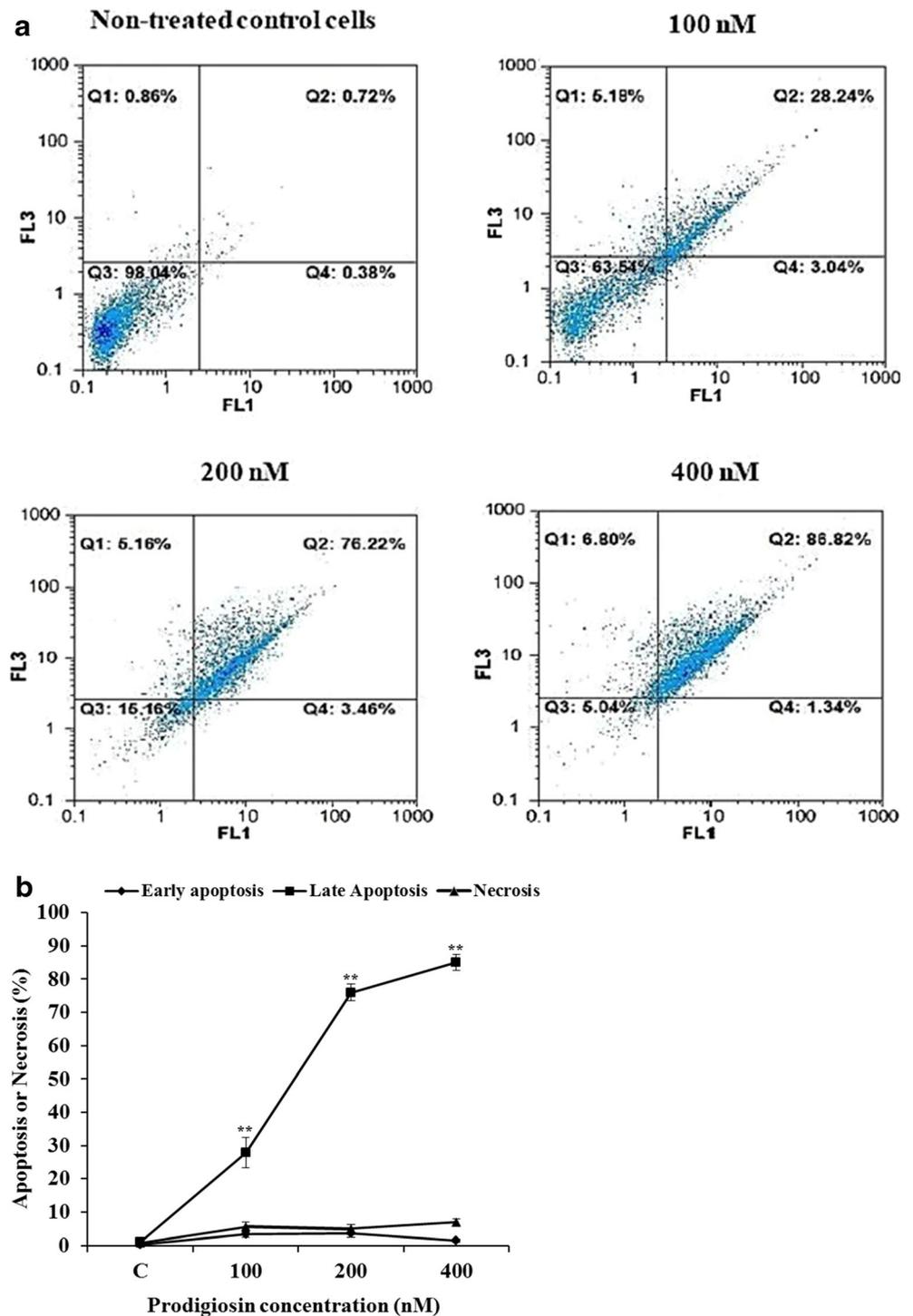

to the ability to form tumors in NOD/SCID mice [26, 27], we did not perform to isolate the cell fraction characterized by putative cancer stem cell characteristics.

In treated HCT-116 cells, expression of survivin was down-regulated by prodigiosin. It seems that inhibition of survivin expression is due to repression of transcriptional activity of the survivin gene promoter. In this regard, further experiments are required to highlight this assumption.

In this study, the treatment with different concentrations of prodigiosin for 48 h induced no statistically significant change in Bcl-2 mRNA levels compared to untreated cells. These results suggest that times longer than 48 h may be required to observe Bcl-2 mRNA changes or prodigiosin may induce apoptosis via the Bcl-2 independent pathway in HCT-116 cells.

It has been observed that high Wnt activity functionally designates the CCSCs population [28] and dysregulation of

Fig. 5 Effect of prodigiosin on miRNA-16-1/survivin mRNA (**a**) and caspase-3 protein/survivin protein (**b**) ratios in HCT-116 cells after 48 h treatment. C: untreated cells. $^{**}P < 0.01$ vs. untreated cells
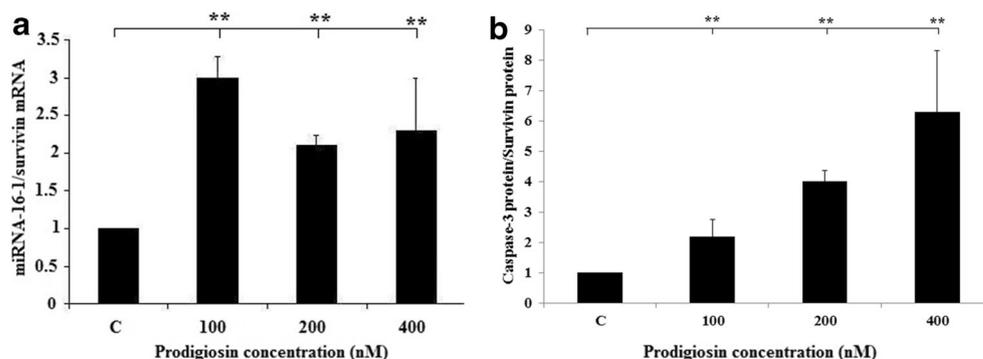
Wnt/β-catenin signaling pathway is responsible for the pathogenesis of CRC [28]. Therefore, high level of survivin expression may be due to a result of aberrant activation of Wnt/β-catenin signaling pathway in HCT-119 cells. Recently, mechanisms of resistance to 5-fluorouracil in colorectal cancer cells have been linked to the activation of the Wnt pathway by the antineoplastic drug [29]. With these in mind and based on our observation, prodigiosin could be introduced as a new therapeutic CCSCS-sensitizing agent to chemotherapeutic drugs due to its effect on decreasing survivin expression. Our observation clearly showed that prodigiosin dose dependently triggers the down-regulation of survivin which was followed by activation of caspase-3. Induction of caspase-3 activation through inhibition of the survivin expression seems to be one of the molecular pathways leading to prodigiosin apoptotic effect in HCT-116 cells which has been previously shown in HT-29 cells [13]. The enhanced apoptosis caused by survivin inhibition clearly validates survivin as a valuable drug target, raising the possibility of prodigiosin therapeutic effects as a natural chemotherapeutic agent directed against CCSCs in vivo.

P53 protein is frequently mutated in most human cancers, which is related to poor prognosis [30]. As prodigiosin-induced apoptosis in colorectal cancer cells and other malignant cells is p53 independent [13, 31], this could mean an advantage over other chemotherapeutic drugs that need functional p53 to provoke its cytotoxic effect [32].

MicroRNA-based therapeutics that can rectify the aberrant expression of survivin in CCSCs may provide great potential in CRC-directed therapy. In this regard, the role of miR-16-1 expression as a tumor suppressor had never been addressed in prodigiosin-treated colorectal cancer cells with stem-like cells properties. It has been shown that, tumor suppressor miR-16-1 is down-regulated in CRC [24]. In our study, 100 nM prodigiosin caused a significant increase in miR-16-1 expression. At 100 nM prodigiosin, high level of miR-16-1 expression was inversely proportional to survivin expression and was correlated to higher apoptosis rate compared to untreated cells. Based on our observations, miR-16-1 may serve as an attractive molecular target of prodigiosin in CRC stem-like cells.

Recently, it has been shown that, survivin mRNA is a direct target of miRNA-16-1 in CRC cells [24]. It is possible that one of the mechanisms contributing to the prodigiosin cytotoxicity is related to the interaction of miRNA-16-1 with survivin mRNA in HCT-116 cells. In our study, the miRNA-16-1/survivin ratio was associated with apoptosis in treated HCT-116 cells. In this regard, the miRNA-16-1/survivin ratio may up-regulate caspase-3 activation and modulate apoptosis in the treated cells compared to untreated cells. However, further experiments are required to answer this question in these cells.

In conclusion, given the increasing understanding that survivin and miRNA-16-1 play key roles in colorectal carcinogenesis, our results provide the first evidence for the ability of prodigiosin to increase miR-16-1 expression and decrease survivin expression in CRC stem-like cells and this compound as a strong inhibitor of survivin with high pro-apoptotic capacity represent an attractive anti-tumor agent directed against CCSCs and may provide a possible clinical application for overcoming drug treatment failure due to the presence of CCSCs.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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