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• LIVER CANCER •

Effects of tachyplesin on the regulation of cell cycle in human hepatocarcinoma SMMC-7721 cells

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Abstract

AIM: To investigate the effects of tachyplesin on the cell cycle regulation in human hepatcarcinoma cells.

METHODS: Effects of tachyplesin on the cell cycle in human hepatocarcinoma SMMC-7721 cells were assayed with flow cytometry. The protein levels of p53, p16, cyclin D1 and CDK4 were assayed by immunocytochemistry. The mRNA levels of $p21^{WAF1/CIP1}$ and c-myc genes were examined with *in situ* hybridization assay.

RESULTS: After tachyplesin treatment, the cell cycle arrested at G_0/G_1 phase, the protein levels of mutant p53, cyclin D1 and CDK4 and the mRNA level of c-myc gene were decreased, whereas the levels of p16 protein and $p21^{WAF1/CIP1}$ mRNA increased.

CONCLUSION: Tachyplesin might arrest the cell at G_0/G_1 phase by upregulating the levels of p16 protein and $p21^{WAF1/CIP1}$ mRNA and downregulating the levels of mutant p53, cyclin D1 and CDK4 proteins and c-myc mRNA, and induce the differentiation of human hepatocacinoma cells.

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INTRODUCTION

A variety of anticancer agents with distinctive effects have been used for treatment of malignant tumors. These agents generally induce tumor cell necrosis and apoptosis as well as differentiation. Today, induction of differentiation is a new strategy in cancer therapy[1-6]. A number of recent experiments have showed that cell-cycle-arrest may be necessary for cell differentiation. The current knowledge of cell cycle regulation have revealed that the progression of the cell cycle is governed mainly by the activation and deactivation of cyclin-dependent kinases (CDKs). In order for cell cycle arrest to differentiation, it is necessary either to downregulate positive regulation of CDKs, such as cyclins, or to activate negative regulators of CDKs, such as CDK inhibitors (CKIs)[7].

It had revealed that tachyplesin, a low molecular weight peptide, could alter the morphological and ultrastructural characteristics, inhibit the proliferation and induce the differentiation of human gastric carcinoma cells and hepatocarcinoma cells[8,9]. In this paper, we investigate the effects of tachyplesin on the regulation of cell cycle in human hepatocarcinoma SMMC-7721 cells.

MATERIALS AND METHODS

Reagents

Tachyplesin was isolated from acid extracts of Chinese horseshoe crab (*Tachypleus tridentatus*) hemocytes as described by Nakamura[10] with minor modification. RPMI-1640 medium were obtained from Gibco. Fetal calf serum was supplied by Si-Ji-Qing Biotechnology Co. (Hangzhou, China). Mouse anti-human p53, p16, cyclin D1 monoclonal antibodies and rabbit anti-human CDK4 antibody were purchased from Santa Cruz. Immunocytochemistry detection kit and in situ hybridization kit were provided by Beijing Zhongshan Biotechnology Co.

Cell culture and treatment

SMMC-7721 cells, provided by the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, were maintained in RPMI-1640 medium supplemented with 20 % heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 mg/L streptomycin and 50 mg/L kanamycin at 37 ℃, 5 % CO_2 in air atmosphere. SMMC-7721 cells were treated with culture medium containing 3.0 mg/L tachyplesin after being seeded for 24 hours.

Flow cytometry assay

SMMC-7721 cells and the cells treated with 3.0 mg/L tachyplesin for 2, 4 or 6 days were collected, rinsed in 0.1 M PBS, resuspended and fixed in 70 % ethanol at 4 ℃ overnight. Cells were centrifuged, resuspended in 100 mg/L RNase A at 37 ℃ for 30 min and in 50 mg/L propidium iodide at 4 ℃ for 30 min. Cell cycle was analyzed by flow cytometry.

Immunohistochemistry analysis

SMMC-7721 cells and the cells treated with 3.0 mg/L tachyplesin for 5 days were seeded in little penicillin bottles with coverslips for 48 hours respectively. The cells grown on coverslips were fixed with cold acetone for 10 min, rinsed twice in PBS for 15 min, immersed in 3 % hydrogen peroxide for 10 min, washed with distilled water and PBS for 15 min, blocked with 10 % normal goat serum for 10 min at room temperature, and incubated with primary antibodies at 4 ℃ overnight. After incubation with primary antibodies, coverslips were rinsed twice in PBS for 15 min, incubated with biotin-labeled secondary antibody at 37 ℃ for 10 min, rinsed twice in PBS for 15 min, and then incubated in streptavidin-peroxidase at 37 ℃ for 10 min. The antigen-antibody complex was visualized with diaminobenzidine (DAB) substrate. Negative controls were incubated in the absence of primary antibodies.

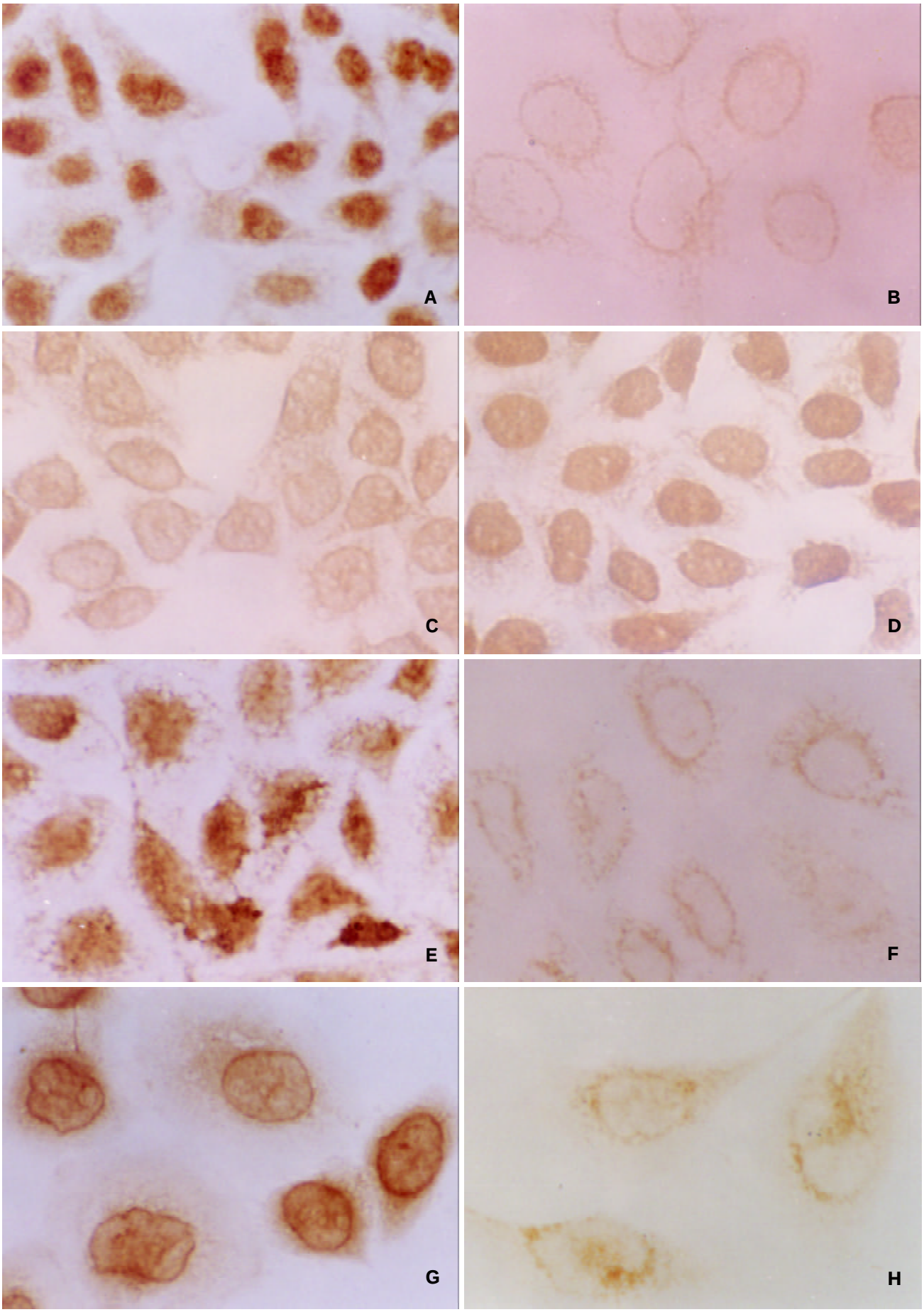

Figure 1 Immunocytochemistry analysis of the effects of tachyplesin on the protein levels of mutant p53, p16, cyclin D1, CDK4 in SMMC-7721 cells (×536). The protein levels of mutant p53 (**A**), cyclin D1 (**E**), CDK4 (**G**) were high in SMMC-7721 cells while the levels of mutant p53 (**B**), cyclin D1 (**F**), CDK4 (**H**) were decreased by tachyplesin. The level of p16 protein was low in SMMC-7721 cells (**C**) while high in the tachyplesin-treated cells (**D**).

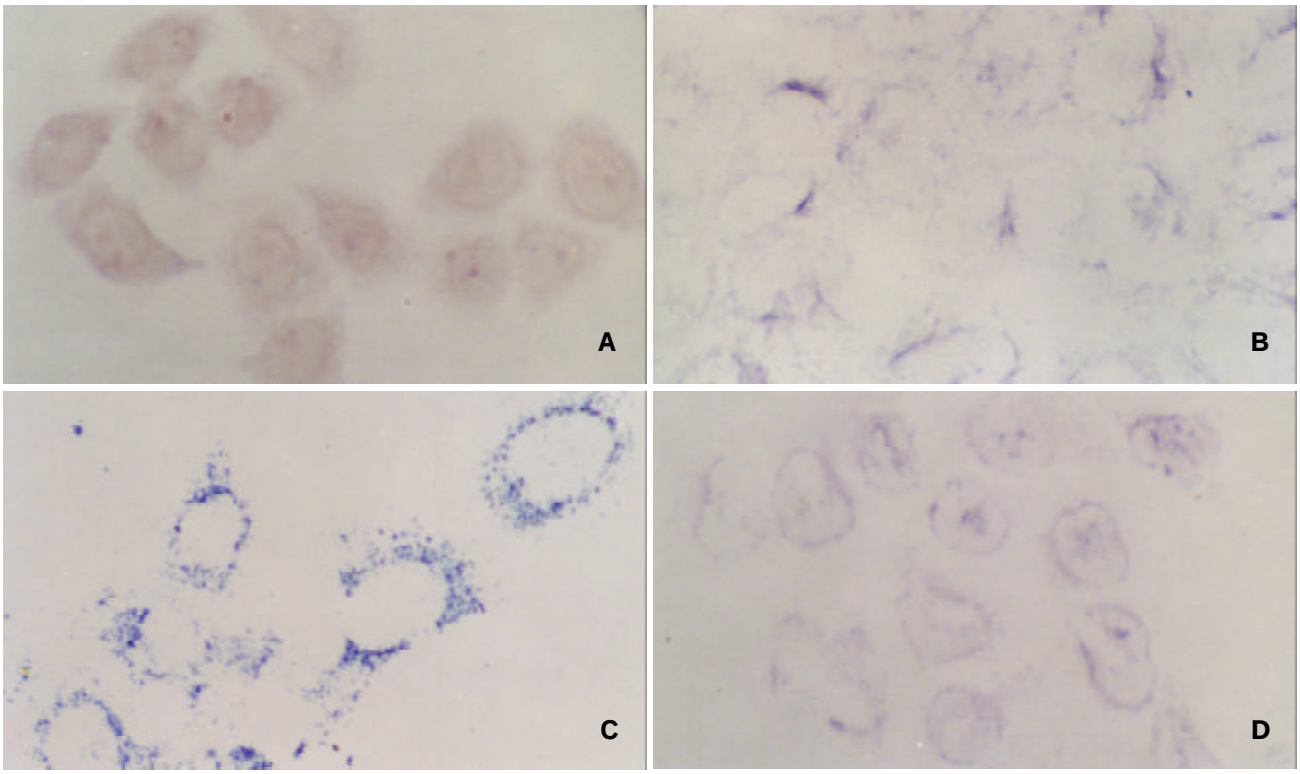

Figure 2 *In situ* hybridization analysis of $p21^{WAF1/CIP1}$ and c-myc mRNA in untreated and tachyplesin-treated SMMC-7721 cells (× 536). The level of $p21^{WAF1/CIP1}$ mRNA was low in SMMC-7721 cells (**A**) but high in the tachyplesin-treated cells (**B**). High level of c-myc mRNA was observed in SMMC-7721 cells (**C**) while the level of c-myc mRNA in the tachyplesin-treated cells down-regulated (**D**).

In situ hybridization

SMMC-7721 cells and the cells treated with 3.0 mg/L tachyplesin for 5 days were seeded in little penicillin bottles with coverslips for 48 hours respectively. *In situ* hybridization procedure was carried out as described by Spector[11] with minor modifications. The coverslips were rinsed twice in PBS, prefixed with methanol/acetone (1:1, v/v) for 10 min, incubated in 0.1 M HCl for 10 min, rinsed in 0.01 M PBS for 5 min×2, and incubated in 25 mg/L proteinase K at 37 ℃ for 20 min and in 0.2 % glycine in PBS for 5 min×3. They were postfixed in 4 % paraformaldehyde for 10 min, dehydrated, and air dried. The prehybridization was conducted at room temperature for 1 hour, followed by hybridization in a humid chamber at 42 ℃ for 16-22 hours. The hybridization solution contained prehybridization solution and 1.0 ug/mL digoxigenin-labeled $p21^{WAF1/CIP1}$ cRNA or c-myc cRNA probe. Coverslips were rinsed in 2×SSC at 37 ℃ for 15 min×2, in 1×SSC for 5 min×2, in 0.5×SSC for 5 min×2, in 0.2×SSC for 5 min×2, washed with Buffer I (100 mM Tris-HCl buffer, 150 mM NaCl, pH7.5) for 5 min, and incubated with Blocking buffer (2 % horse serum and 0.3 % Triton X-100 in Buffer I) for 1 hour, and then incubated at 37 ℃ for 1hour in alkaline phosphatase-conjugated anti-digoxigenin antibody diluted 1:500 in Blocking buffer, then rinsed in Buffer I for 15 min×3, and equilibrated in Buffer II (100 mM Tris-HCl buffer, 100 mM NaCl, 50 mM $MgCl_2$, pH9.5). The alkaline phosphatase reaction was conducted by incubation with NBT/BCIP solution for 20-30 min. The reaction was stopped with Buffer III (10 mM Tris-HCl buffer, 1 mM EDTA, pH8.0). The coverslips were then dehydrated in alcohol, cleared in xylene, and mounted on gelatin. The negative controls were processed without labeled probes.

RESULTS

Effects of tachyplesin on the cell cycle distribution of SMMC-7721 cells

Cell cycle kinetics of SMMC-7721 cells was analyzed by flow cytometry. As demonstrated in Table 1, 3.0 mg/L tachyplesin could induce an accumulation of the cells at G_0/G_1 phase on day 2, 4, 6, respectively. Compared with control group, the amount of cells at G_0/G_1 phase increased from 48.2 % to 65.6 %, while the quantity of cells in S phase decreased from 48.0 % to 24.8 % after being treated with tachyplesin for 6 days. This indicated that tachyplesin could arrest the SMMC-7721 cells at G_0/G_1 phase.

Table 1 Effects of 3.0 mg/L tachyplesin on the cell cycle kinetics of SMMC-7721 cells

	SMMC-7721	Tachyplesin 2 d	Tachyplesin 4 d	Tachyplesin 6 d
G_0/G_1	48.2 %	66.5 %	61.8 %	65.6 %
S	48.0 %	29.7 %	34.8 %	24.8 %
G_2/M	3.8 %	3.8 %	3.4 %	9.6 %

Effects of tachyplesin on p53, p16 protein levels in SMMC-7721 cells

It has revealed that p53 protein detected by immunohistochemistry is mutant p53. Immunohistochemistry showed that the level of mutant p53 protein was high in the nucleus and cytoplasm of SMMC-7721 cells. However, very low level of mutant p53 protein in the tachyplesin-treated cells was observed (Figure 1A and B). p16 protein was distributed mainly in the nucleolus of SMMC-7721 cells in low level while the level of immunohistochemistrical reaction was very high in the tachyplesin-treated cells (Figure 1 C and D).

Effects of tachyplesin on cyclin D1, CDK4 protein levels in SMMC-7721 cells

As shown in Figure 1E and F, the level of cyclin D1 protein was high in the nucleolus and cytoplasm of untreated cells while it was very weak in the cells treated with tachyplesin. It also revealed that exposure of SMMC-7721 cells to tachyplesin resulted in an obvious decrease of CDK4 protein level (Figure 1 G and H).

Effects of tachyplesin on p21$^{WAF1/CIP1}$, c-myc mRNA levels in SMMC-7721 cells

To investigate the effects of tachyplesin on the expression of tumor suppressor gene and oncogene associated with the G_0/G_1 arrest, the levels of p21$^{WAF1/CIP1}$ and c-myc mRNA were also detected. *In situ* hybridization assay showed that the level of p21$^{WAF1/CIP1}$ mRNA was low in the nuclear and cytoplasm of the untreated cells while it was high in the cytoplasm around the nucleolus in the cells treated with tachyplesin (Figure 2A and B). And high level of c-myc mRNA was observed in the cytoplasm of SMMC-7721 cells. However, the level of c-myc mRNA was down-regulated in the tachyplesin-treated cells (Figure 2 C and D).

DISCUSSION

In multi-cellular organism, the balance among cell proliferation, cell differentiation and cell death maintains a constant cell number. Cell proliferation depends on the cell' s growth and division cycles, which are governed by periodic assembly of the core cell cycle clock, composed of cyclins and cyclin-dependent kinases (CDKs)[7,12]. Distinct cyclins associate and activate different CDKs throughout the cell cycle. The activity of cyclin/CDK complexes is modulated by both activating and inhibiting phosphorylation of the CDKs, and by binding to cyclin-dependent kinases inhibitors (CKIs). In higher eukaryotic cells, signals that arrest the cycle usually act at a G_1 checkpoint. The G_1 checkpoint can be viewed as a master checkpoint of the mammalian cell cycle[7,13]. Regulation of the G_1 phase of the cell cycle is extremely complicated and involves many different families of cyclins, CDKs and CKIs. In eukaryotes, D-cyclins (D1, D2, and D3) bind CDK4 /6 to wire external signals to the cell cycle and regulate progression through mid-G_1. cyclin E binds CDK2 in late G_1 and its activity is rate-limiting for progression from G_1 to S phase[14]. The p16 tumor suppressor belongs to the INK4 family of CKIs. p16 specifically inhibit CDK4/6 by preventing binding of the activating cyclin subunits. p21$^{WAF1/CIP1}$, one CKI of CIP/KIP family, unlike p16, binds to a number of cyclin/CDK complexes such as cyclin D1/CDK4, cyclin E/CDK2 and cyclin A/CDK2, inhibits kinases activities and induces cell cycle arrest and cell differentiation[7,15-17].

In addition, some other genes are associated with G_1 checkpoint. It is known that wild-type p53 gene has anti-proliferation, anti-transforming and inducing apoptosis activities. Growth arrest induced by wild-type p53 blocks cells prior to or near the checkpoint in late G_1 phase. Mutations of p53 are observed with a high incidence in most cancer[18,19]. One of the phenotype effects of mutant p53 protein is called dominant-negative effect, which mutant p53 can override the normal inhibitory function of wild-type p53. In the meantime, c-myc gene, an early-response gene necessary for cell cycle progression (G_1-S transition), also plays an essential role in the regulation of the cell cycle and differentiation[20-24].

As revealed by many experiments, the regulation of G_1/S checkpoint was abnormal in tumor cells. G_0/G_1 arrest was a common phenomenon in the cells undergoing induction of differentiation. And many differentiation inducers could arrest tumor cells in G_0/G_1 phase and alter the expression of cell cycle regulators[25-31]. Our results showed that tachyplesin could down-regulate the levels of mutant p53, cylin D1, CDK4 proteins and c-myc mRNA, up-regulate the levels of p16 protein and p21$^{WAF1/CIP1}$ mRNA in SMMC-7721 cells, and arrest the cell in Go/G_1 phase as other inducers on tumor cells. Taken together, the results indicated that tachyplesin might decrease the kinase activity of cyclin D1/CDK4 complex by decreasing the expression of mutant p53 protein and increasing the levels of p16 and p21$^{WAF1/CIP1}$, and suppress the phosphorylation of retinoblastoma (Rb) protein and downregulate the level of c-myc mRNA, which cause a decrease of the cell in S phase significantly and arrest most of the cells at G_0/G_1 phase, and induce the cells to terminal differentiation as other inducers.

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