JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Alteronol inhibits proliferation in HeLa cells through inducing a G1-phase arrest

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Keywords
alteronol; cell cycle; G1 arrest; HeLa

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Received May 11, 2011
Accepted September 22, 2011

doi: 10.1111/j.2042-7158.2011.01375.x

Abstract

Objectives Alteronol is a novel compound purified from fermentation products of a microorganism in the bark of the yew tree. The study was designed to evaluate the anticancer effects of alteronol.

Methods Human cervical carcinoma cell line HeLa was cultured *in vitro*. The cell viability was evaluated by using sulforhodamine B assay. The cell cycle distribution was analysed by flow cytometry. The level of cyclin D1 protein was evaluated using Western blot analysis. The changes in cyclinD1, CDK4 and p21 were detected by ELISA assay and the changes in G1-related regulators were detected by RT-PCR assay.

Key findings Our data showed that alteronol inhibited the proliferation of HeLa cells and induced G1 phase arrest. Downregulation of the mRNA levels of CDK2, CDK4 and cyclin D1 and upregulation of p21 in alteronol-treated cells were observed.

Conclusions Downregulation of the mRNA levels of CDK2, CDK4 and cyclin D1 and upregulation of p21 might be a possible mechanism for the inhibition of proliferation induced by alteronol in HeLa cells.

Introduction

Paclitaxel has been isolated in 1971 from the inner bark of the western yew tree (*Taxus brevifolia*),[1] and is used in treating several malignancies including ovarian, breast and head and neck cancers, and refractory ovarian and breast cancers.[2] However, the yield of paclitaxel from bark is low, approximately 0.01% (w/w), and bark stripping destroys scarce plant material; scientists are still searching for new compounds in the yew tree. We have obtained a novel microorganism mutation strain named *Alternaria alternata* var. *monosporus* from the bark of a yew tree in Kunming, China (PCT/SG05/00324). In the fermentation products of this novel strain, we isolated and purified several new compounds, such as alternol and alteronol. Because they are similar in origin to paclitaxel, we hypothesize that these compounds may also have an antitumour effect. Previously, we reported that alternol is able to inhibit gastric carcinoma cell proliferation through G2/M cell cycle arrest.[3] In the study, we reported the proliferative inhibition of alteronol on Hela cells. Alternol and alteronol (Figure 1) have a similar structure, so we hypothesized that alteronol would inhibit cervical carcinoma cancer cell growth *in vitro*, and further aimed to delineate the mechanism involved.

In our previous study, we found that alteronol inhibits the proliferation of several tumour cells *in vitro*.[4] Consequently, we chose HeLa as a model to investigate the proliferation inhibition mechanisms of alteronol. Recent studies have showed that progression through the G1 phase and the G1-S transition involves sequential assembly and activation of G1 cyclins and cyclin-dependent kinases (CDKs).[5–7] G1 phase progression and G1/S transition are believed to be regulated by CDK4 (and CDK6), which assemble with cyclins D in mid-G1, and CDK2, which combine later with cyclin E. CDK activity is tightly regulated by low-molecular-weight CDK inhibitors (CKIs),[8,9] such as p21 proteins.

To clarify the mechanism of the anti-proliferative effect of alteronol, we investigated the effect of alteronol on the cell cycle distribution and the expression of cell cycle regulating proteins in HeLa cell line for the first time.

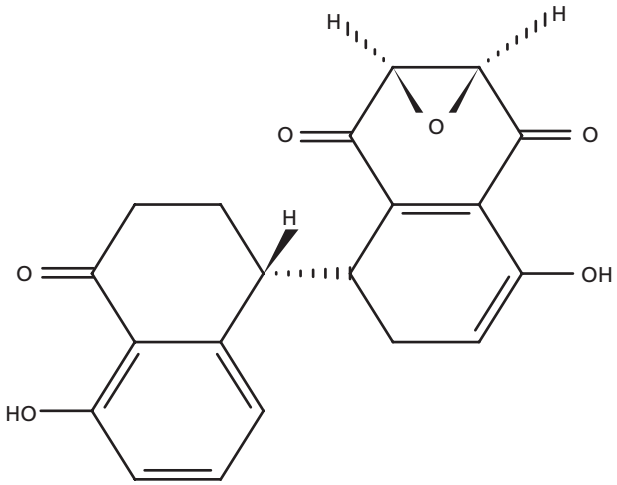

Figure 1 The structure of alteronol.

Materials and Methods

Cell culture and reagents

A human cervical carcinoma HeLa cell line was obtained from the China Center for Type Culture Collection (Wuhan, China). HeLa cells were cultured in DMEM medium (Gibco Invitrogen, Grand Island, USA). All culture media were supplemented with 10% fetal bovine serum (FBS) (Sijiqing Biotech Company, Hangzhou, China), penicillin (125 units/ml) and streptomycin (125 μg/ml) were obtained from Shandong Sunrise Pharmaceutical Co., Ltd (Shandong, China), and all cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Alteronol was provided by Strand Biotech Company Limited (Shantou, China). Propidium iodide and RNase A were purchased from Sigma (St Louis, USA). Antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

Cell proliferation and viability assays

Sulforhodamine B (SRB) assays were performed as described.[10] HeLa cells were seeded at 5×10^4 cells per well in 96-well plates and allowed to adhere overnight. Drug or vehicle control was diluted in DMEM and used to treat cells for 48 h. Cells were fixed onto the plates by addition of 10% cold trichloroacetic acid (final concentration) for 1 h at 4°C. Cellular protein was stained by addition of 0.4% SRB (Sigma) in 1% acetic acid and incubation at room temperature for 30 min. Unbound SRB was removed by washing with 1% acetic acid and plates were air-dried. Bound SRB was resolubilized in 10 mmol/l unbuffered Tris base and absorbance was determined on a microplate reader (Thermo) at 490 nm. Test results were normalized against initial plating density and drug-free controls. Data were obtained from triplicate wells per condition and representative of at least three independent experiments.

Cell cycle analysis

Cells were incubated until there was a monolayer of 70–80% confluence. After treatment with or without alteronol, cells were harvested and fixed in 70% ethanol for 30 min on ice. After washing with phosphate-buffered saline (PBS), cells were labelled with propidium iodide (0.05 mg/ml) in the presence of RNase A (0.5 mg/ml) and incubated at room temperature in the dark for 30 min. DNA contents were analysed using a flow cytometer (Partec, Munster, Germany). Propidium iodide incorporated into DNA was excited at 488 nm and detected at 650 nm.[11]

Western blot analysis

Western blot assays were carried out as described previously.[12,13] HeLa Cells were incubated with increasing concentrations of alteronol (1.5, 3.0, 4.5 μg/ml) for 24 h. Western blot analysis using total protein extracts from cultured cells was performed as previously described.[12,13] Proteins were size fractionated by 10% gel electrophoresis and the nitrocellulose membranes were probed overnight at 4°C with a 1 : 200 dilution of mouse anti-human cyclinD1 antibody (Santa Cruz Biotechnology). Then, membranes were exposed to appropriate peroxidase-coupled secondary antibodies for 2 h at room temperature and proteins were visualized with enhanced chemiluminescence (ECL, thermo).

Enzyme-linked immunosorbent assay

CyclinD1, CDK2 and p21 ELISA was performed by using ELISA Kits from Assay Designs as per the manufacture's protocol (Sangon Biotechnology Co. Ltd., Shanghai, China).

Semi-quantitative reverse transcription-polymerase chain reaction

For semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA from HeLa cells were extracted using Trizol reagent (Sangon Biotechnology Co. Ltd.) according to the instructions. After extraction 3 μl of total RNA were reverse transcribed to cDNA using reverse transcription reagents (Fermentas China Co. Ltd., Shenzhen, China) in a 20 μl volume. The PCR primers were designed by Sangon Biotechnology Co. Ltd. The PCR mixture contained 3 μl genomic DNA template, 0.2 μmol/l of each primer, 0.8 mmol/l of each deoxynucleoside triphosphate, 1.5 U Taq polymerase (Fermentas Co.) and reaction buffer in a total volume of 25 μl. PCR was performed for 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at propotional temperature and extension for 45 s at 72°C, with initial

Table 1 Primers used for semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene	Sequence of primer	Annealing temperature (°C)
CyclinD1	5-CGT GGC CTC TAA GAT GAA GG-3 5-CCA CTT GAG CTT GTT CAC CA-3	57
CyclinD2	5-TAC CTT CCG CAG TGC TCC TA-3 5-TCA CAG ACC TCC AGC ATC CA-3	55
CyclinE2	5-TTG GCT ATG CTG GAG GAA GT-3 5-CCT GGT GGT TTT TCA GTG CT-3	58
CyclinA2	5-CCT GCA AAC TGC AAA GTT GA-3 5-AAA GGC AGC TCC AGC AAT AA-3	55
p21[14]	5-GGA AGA CCA TGT GGA CCT GT-3 5-AAT CTG TCA TGC TGG TCT GC-3	58
CDK2	5-CAGCCGTGACCTACATCG-3 5-AAAGCCAAGGAAAGGACAG-3	60
CDK4[15]	5-CGGAAGGCAGAGATTCGCTTAT-3 5-CCAGCCTGAAGCTAAGAGTAGCTGT-3	60
GAPDH	5-ACCACAGTCCATGCCATCAC-3 5-TCCACCACCCTGTTGCTGTA-3	57

denaturation at 94°C for 5 min and a final extension at 72°C for 10 min. PCR products were run in 2% agarose gels (Invitrogen) along with 100 base-pair (bp) ladder markers. Amplified products were visualized by staining with GoldenView and were analysed using a gel scanner.

The primers sequences were as follows (Table 1):

Statistical methods

Data obtained from different experiments are presented as means ± SEM from at least three independent experiments and evaluated by analysis of variance followed by the Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

Results

Alteronol could decrease the viabilities of HeLa cells

To investigate the effect of alteronol on the growth of HeLa cells, HeLa cells incubated with alteronol for 48 h were analyzed by SRB assay. In the presence of alteronol, cell growth was reduced significantly in a dose dependent manner (Figure 2). After 48 h of treatment, the inhibition rate was 11.04 ± 2.68%, 40.62 ± 5.42% and 58.7 ± 3.51% for 1.5 μg/ml, 3.0 μg/ml and 4.5 μg/ml alteronol, respectively.

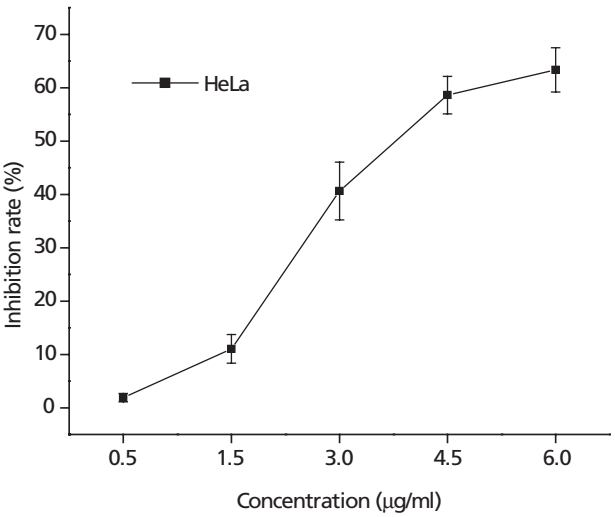

Figure 2 Effect of alteronol on the proliferation in HeLa cell lines using the sulforhodamine B (SRB) assay. HeLa cells were treated with or without alteronol for 48 h. Values represent mean values ± SD, $n = 3$ experiments, each experiment contains 3 cultures.

Alteronol induced G1 arrest in HeLa cells

According to cell cycle distribution analysis by flow cytometry (Figure 3), there were marked and consistent changes in the cell cycle at 24 h. The number of cells in the G1 phase increased in HeLa cells with a concomitant decrease in treated cells in the S phase. In 4.5 μg/ml alteronol-treated cells, the percentage of cells in the G1 phase was 73.1%, and in control cells, it was only 45.9%. The percentage of cells in the S phase decreased from 39.2% to 7.1%.

Alteronol downregulates cyclin D1 and pRb proteins in HeLa cells

To investigate the mechanism behind the induction of G1 phase arrest by alteronol, Western blot analysis was performed to detect changes in the expression of cyclin D1 (Figure 4). The amount of the cyclin D1 protein markedly decreased in response to treatment with alteronol at 24 h.

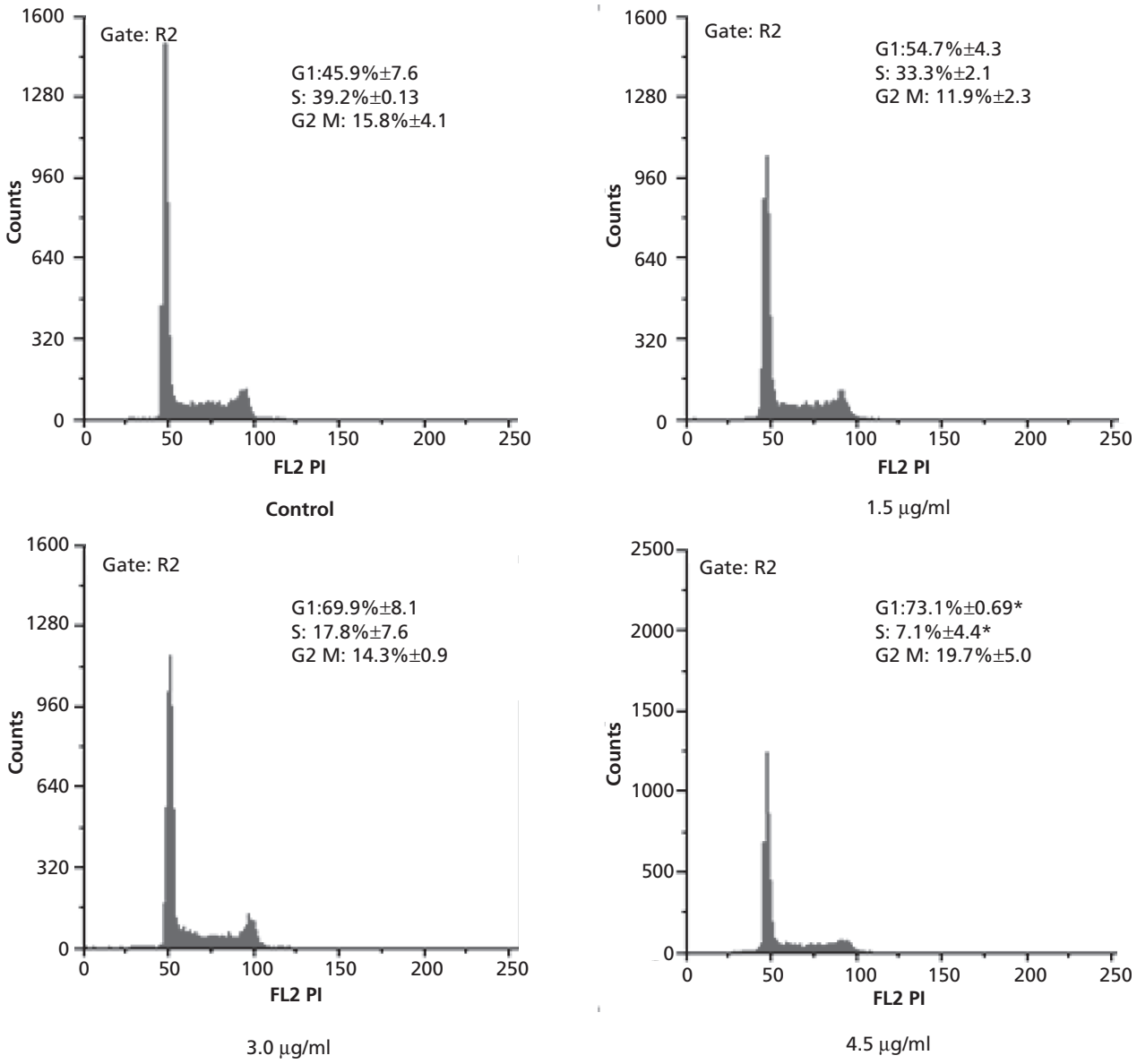

Figure 3 Cell cycle distribution analysis by flow cytometry. HeLa cells were exposed to (1.5, 3.0, 4.5 μg/ml) alteronol for 48 h, washed and then harvested. The cells were then fixed and stained with propidium iodide and DNA contents were analysed by flow cytometry. Representative examples are shown of three separate experiments. *$P < 0.05$ with respective control. Values represent mean values ± SD, $n = 3$ experiments, each experiment contains 3 cultures.

The changes in cyclin D1, CDK4 and p21 at the protein level in HeLa cells

The expressions of cyclin D1, CDK4 and p21were studied by ELISA assay (Table 2) in HeLa cells incubated with alteronol (1.5, 3.0 and 4.5 μg/ml). This revealed a significant decrease in cyclin D1 and CDK4 protein levels. Subsequently, we examined the expression of the cell cycle inhibitors on p21. A dose-dependent increase of p21 was observed after exposure to alteronol.

The changes in the G1-related regulators at mRNA levels in HeLa

To investigate mechanisms involved in the regulation of G1 arrest in HeLa cells, the expression of related regulators was studied by RT-PCR at the mRNA level. From Table 3, it can be seen that alteronol caused a remarkable reduction in cyclin D1and CDK2 in the mRNA level, whereas it had little effect in the mRNA level of CDK4, cyclin D2 and cyclin E2, while cyclin A2 was unchanged. In contrast to the CDK4 mRNA

level, CDK4 protein level (Table 2) appears to decrease, suggesting that the decreased CDK4 protein levels in HeLa cells were due to either downregulated CDK4 protein degradation or decreased CDK4 mRNA translation.

In Table 3, the mRNA levels of p21 increased in a dose-dependent fashion after 48 h of alteronol treatment.

Discussion

In this study, we first examined the anti-proliferative effect of alteronol on human cervical carcinoma HeLa cells at concentrations ranging from 0.5 to 6.0 μg/ml for 48 h. Alteronol significantly decreased the proliferation of HeLa cells in a dose-dependent manner.

To better understand the mechanism of inhibition of cell proliferation, we analysed the cell cycle of HeLa cells treated with different concentrations of alteronol at 24 h (Figure 3). The cell population in the G1 phase increased upon treatment with 4.5 μg/ml of alteronol, from 45.9% to 73.1% compared with the control, and that in the S phase decreased from 39.2% to 7.1%.

Cell cycle progression is tightly regulated through a complex network of positive and negative cell cycle regulatory molecules, such as cyclin-dependent kinases (CDKs), CDK inhibitors (CKIs), and cyclins.[16,17] The cyclin D1/cdk4 complex mediates progression of the cell cycle in early G1 phase[18,19] and inactivates the retinoblastoma protein (pRb), a tumour suppressor, by phosphorylation.[9,20]

Lee[17] found that acteoside could induce G0/G1 phase arrest via decreasing cyclin D1 and CDK2 in human promyelocytic HL-60 leukaemia cells. In our study, the mRNA and protein levels of cyclinD1 significantly decreased after alteronol treatment of HeLa cells at 24 h and as shown in Table 3 alteronol caused a remarkable reduction in CDK2 mRNA level, whereas it had little effect on the mRNA level of CDK4 and cyclin D2.

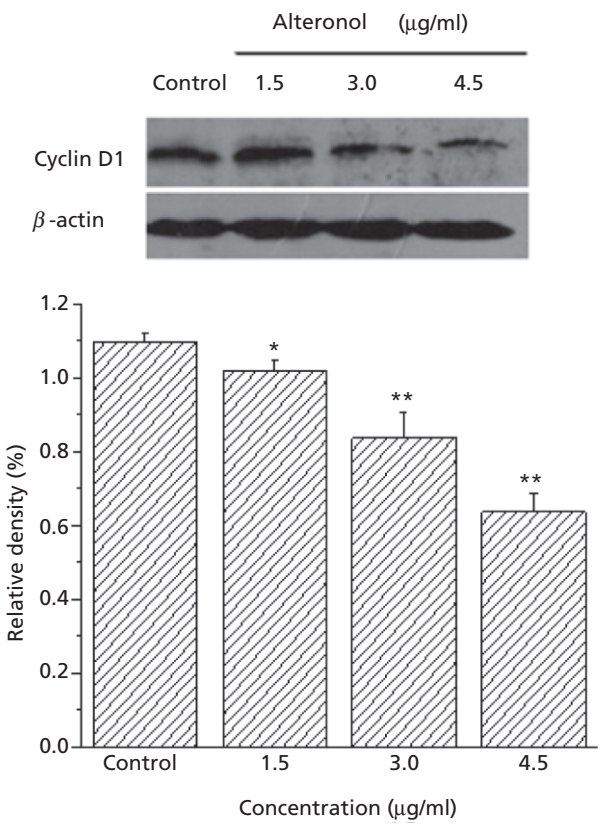

Figure 4 The effect of alteronol on the protein levels of cyclin D1 in HeLa cells. Protein extracts were harvested from HeLa cells exposed to alteronol (1.5, 3.0, 4.5 μg/ml) for 48 h and subjected to Western blot analysis using the specific antibodies for the cyclin D1 proteins. Data are the means ± SD, $n = 3$ experiments, each experiment contains 3 cultures. $*P < 0.05$, $**P < 0.01$ significantly different from the control.

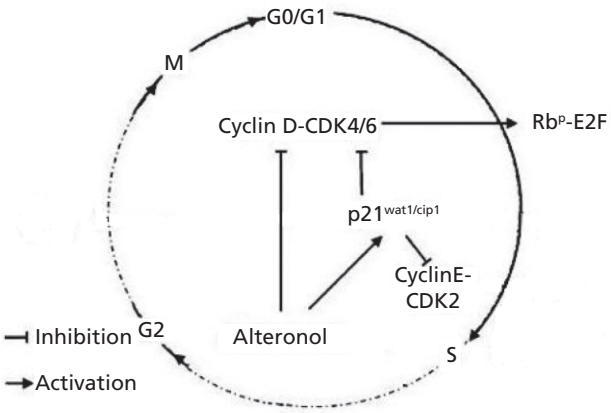

Figure 5 Regulation of the G1-S transition by the cycln–CDK pathway during alteronol treatment.

Table 2 The expression of cyclin D1, CDK4 and p21 in HeLa cells after treatment with alteronol

Protein	Protein expression (ng/ml)			
	Control	1.5 μg/ml	3 μg/ml	4.5 μg/ml
Cyclin D1	0.273 ± 0.007	0.256 ± 0.006	0.242 ± 0.005*	0.227 ± 0.008*
CDK4	0.243 ± 0.015	0.008 ± 0.008	0.206 ± 0.002	0.162 ± 0.003*
P21	0.178 ± 0.004	0.192 ± 0.004	0.203 ± 0.009	0.241 ± 0.003**

Protein changes were determined by ELISA in HeLa cells exposed to alteronol (1.5, 3.0 and 4.5 μg/ml) for 48 h. $*P < 0.05$, $**P < 0.01$ with respective control. Values represent mean values ± SD, $n = 3$ experiments, each experiment contains 3 cultures.

Table 3 The changes in cyclin D1, cyclin D2, cyclin E2, cyclin A2, CDK2, CDK4 and p21 mRNA level in HeLa cells after treatment with alteronol

	Relative density			
Gene	Control	1.5 μg/ml	3 μg/ml	4.5 μg/ml
Cyclin D1	0.741 ± 0.003	0.713 ± 0.008	0.664 ± 0.003*	0.641 ± 0.002*
Cyclin D2	0.741 ± 0.006	0.702 ± 0.003	0.713 ± 0.007	0.70 ± 0.015
Cyclin E2	0.746 ± 0.007	0.728 ± 0.003	0.729 ± 0.019	0.71 ± 0.024
Cyclin A2	1.065 ± 0.005	1.072 ± 0.016	1.063 ± 0.013	1.076 ± 0.007
CDK2	1.02 ± 0.011	0.92 ± 0.007*	0.773 ± 0.012*	0.579 ± 0.004*
CDK4	0.903 ± 0.006	0.907 ± 0.017	0.856 ± 0.008	0.863 ± 0.005
P21	0.897 ± 0.037	0.954 ± 0.022	0.966 ± 0.028	1.04 ± 0.026*

HeLa cells were exposed to alteronol (1.5, 3.0 and 4.5 μg/ml) for 48 h and the expression of was studied by reverse transcription polymerase chain reaction (RT-PCR) at the mRNA level Values represent mean values ± SD, $n = 3$ experiments, each experiment contains 3 cultures). *$P < 0.01$ compared with respective control.

Cyclin E-CDK2 complex is the rate-limiting step for entry into the S phase of the cell cycle,[21] whereas cyclin A accumulates later during S phase (Figure 5).[14,19,22,23] Our data here shows that the mRNA levels of cyclin E2 were slightly reduced after 24 h in HeLa cells exposed to alteronol, while cyclin A2 was unchanged (Table 3).

Furthermore, CDK inhibitor, p21, functions as a regulator of the G1/S phase checkpoint. The Cyclin-CDK complexes are negatively regulated by p21.[21,24,25] p21 is known to inhibit the cell cycle progression by causing inhibition of cyclin-CDK2 and cyclin-CDK4 complexes.[20,21,26] Zi *et al.*[27] found that silymarin can induce G1 arrest through an increase in p21 in MDA-MB 468 cells. As shown in Figure 5, we found that alteronol upregulated p21 protein levels, while the mRNA of p21 were increase as assessed by RT-PCR (Table 3) in HeLa cells.

Conclusions

In summary, we provide the evidence that alteronol caused a decrease in cell cycle regulatory cyclin D1 and CDK4 and an increase in the CDK inhibitor p21 levels after 48 h treatment. Collectively, these are possible factors that could contribute to the induction of G1 phase arrest in HeLa cells after alteronol incubation (Figure 5). Such findings suggest that further research should be undertaken to identify possible preferential targets and that alteronol may be used as a lead compound one day for anticancer therapy.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This study was supported by the Development of Major New Drugs of China (No. 2009ZX09103).

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