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Anisomycin suppresses Jurkat T cell growth by the cell cycle-regulating proteins

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Abstract:
Background: Recent studies have shown that anisomycin significantly inhibits mammalian cell proliferation, but its mechanism remains unclear. In this study, Jurkat T cells were used to first explore a relationship between effect of anisomycin on them and alteration of cell cycle-regulating proteins.
Methods: Cell colony formation, CCK-8 assay, flow cytometry, RT-PCR and western blot were employed to evaluate correlation of ten cell cycle-regulating proteins with suppression of the cell proliferation and arrest of the cell cycle by anisomycin.
Results: Our data showed that anisomycin inhibited the colony-formation and proliferation of Jurkat T cells in a dose-dependent manner, and arrested the cells into S and G2/M phases with the production of sub-diploid cells. The levels of P21, P-P27 and P53/P-P53 reached their peaks 4 h after anisomycin treatment, presenting a positive correlation with anisomycin concentration, and P16, P-P21, P27, P57, P73/P-P73 and P-Rb changed little with the prolonged exposure time or increased concentrations of anisomycin. But the level of Rb protein was increased at 24 h after the treatment of anisomycin. The expression of an inverted CCAAT box binding protein (ICBP90) in Jurkat T cells came to decrease 12 h after the treatment of anisomycin, presenting a negative correlation with anisomycin concentration. Subsequently, the expression of P-CDK2 was also decreased at 24 h, presenting an obviously negative correlation, whereas P-CDK1 showed no differences among the differently treated Jurkat T cells. Furthermore, the level of P21 and P53 mRNA was increased with the enhanced concentrations of anisomycin.
Conclusion: The results indicate that anisomycin may activate the P53/P21/P27 signaling to decrease the expression of ICBP90, inhibit expression of P-CDK2 to block the cells into S and G2/M phases, and finally result in proliferation inhibition of Jurkat T cells.

Key words:
anisomycin, P21, P27, P53, ICBP90, CDK2, Jurkat T cell

Abbreviations: CCK-8 – Cell Counting Kit-8, CDKs – cyclin-dependent protein kinases, CKIs – cyclin-dependent kinase inhibitors, ICBP90 – inverted CCAAT box binding protein, PI – propidium iodide, PMA – phorbol-12-myristate-13-acetate

Introduction

Anisomycin, an antibiotic isolated from *Streptomyces*, can bind with the 60S ribosomal subunit and prevent peptide bond formation to result in block of peptide elongation and degradation of polyribosome, functionally inhibiting synthesis of numerous proteins and DNA [7]. Many studies show that anisomycin can activate p38, ERK1/2 or JNK in MAPK family, which is dependent on the cell types [18]. Recently, it has been discovered that anisomycin induces macrophage apoptosis in rabbit atherosclerotic plaques through p38 signalling [8], significantly influencing reactivity of lymphocytes [21, 23, 27] and inhibiting activation and proliferation of Jurkat T cells stimulated by PMA and ionomycin to induce apoptosis of Jurkat T cells [19], while its concrete mechanism remains to be clarified.

Inverted CCAAT box binding protein (ICBP 90) was newly identified in 2000. Higher expression of ICBP90 is found in proliferative tissues, but it does not appear to express in highly differentiated tissues [10]. ICBP90 expression is much higher in various cancer cells and loses the cell-cycle dependent expression pattern [16]. ICBP90 may participate in the regulation of cell proliferation and cell cycle progression [1]. Cyclin-dependent kinase inhibitors (CKIs) bind and inactivate CDK-cyclin complexes, and negatively regulate cell cycle [15]. The down-regulation of ICBP90 is an important mechanism for cell cycle arrest at G1/S transition, which is induced by the activation of p53/$p21^{Cip1/WAF1}$-dependent DNA-damage. As a cell-cycle- inhibiting factor with most comprehensive activity and an important down-stream target of P53, $P21^{WAF1/CIP1}$ involves regulation of multiple courses, such as cell proliferation, cell cycle, cell division, senescence, apoptosis and repairs of DNA-damage [14]. P53 can bind specially with DNA to maintain the stability of the genosome through blocking the cell cycle or inducing cell apoptosis in many cell-damage stimulating reactions [25]. The core of the mammalian cell cycle is triggered by the cyclin-dependent protein kinases (CDKs) [14]. CDK2 binding with cyclin E can phosphorylate H1 histone and make the two DNA strands separated, which is benefit for the replication of DNA. Operation of cell-cycles is driven by the combination and dissociation of the cyclins with CDKs. Besides, process of cell-cycles is regulated by the phosphorylation and dephosphorylation of CDKs. Since the above cell cycle-regulating proteins play important roles in controlling cell growth, it is proposed whether or not anisomycin functions to block Jurkat T cell proliferation through these proteins. Thus, the goal of this study was to provide mechanistic insight into effects of ten cell cycle-regulating proteins on the inhibition of Jurkat T cell proliferation by anisomycin through detecting cell colony formation, cell viability, cell cycle, ICBP90 and CDK-inhibiting proteins as well as cell-cycle-dependent kinases in the process of Jurkat T cell proliferation.

Materials and Methods

Cell culture

Jurkat T cells, a human T cell lymphoma cell line (China Center for Type Culture Collection Wu Han University), were cultured in RPMI 1640 medium containing 10% fetal calf serum (GibcoBRL) (i.e., complete medium) at 37°C in the presence of 5% CO_2 and the culture were renewed every 2 days for passage.

CCK-8 assay

Cell proliferation was detected by Cell Counting Kit-8 (CCK-8) assay, according to the manufacturer's instructions. Briefly, Jurkat T cells at a density of 1×10^6 cells/ml were planted into a 96-well plate (100 µl/well), and treated with anisomycin (1.0, 10.0, 20.0, 40.0, and 80.0 ng/ml) or medium as a control. The cells were incubated at 37°C in atmosphere of 5% CO_2 for 24 h. After the incubation, 10 µl of CCK-8 reagent in Cell Counting Kit-8 (Beyotime, Shanghai, China) was added to each well and the cells continued to be cultured for 3 h. After the cells were incubated for 3 h at room temperature, the absorbing value (450 nm) of each pore was analyzed by a 680-type microplate reader (Bio-Rad, Berkeley, CA, USA).

Then, the inhibition rate of the cell proliferation was calculated as follows:

$$\% \text{ inhibition rate} = \frac{\text{Mean OD of control group} - \text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

OD = optical density.

Flow cytometry

Jurkat T cells were incubated with different concentrations of anisomycin for 48 h as described above. The cells were collected (1,200 rpm, 5 min), washed once with PBS, and fixed with 70% ethanol at –20°C for at least 1.5 h. The fixed cells were washed twice with PBS, thereafter stained with 400 µl of propidium iodide (PI) (KeyGen, Nanjing, China) solution (50 mg/l PI, 1 ml/l TritonX-100, 0.1 mmol/l EDTA and 50 µg/ml RNase) for 30 min at room temperature in darkness. DNA contents were measured by flow cytometry (FACS Calibur, Becton Dickinson, USA) and analyzed using Cell Quest software.

Western blot

The cells were harvested (1,200 rpm, 5 min) and washed thrice after exposure to anisomycin for different time. After the supernatant was removed, the cells were collected and then lysed in 50 µl RIPA solution (Biocolors, Shanghai, China) supplemented with 0.5 µl PMSF on the ice for 1/2 h. The lysate was clarified by centrifugation at 4°C for 20 min at 12,000 rpm. Each sample was denatured at 100°C for 5 min in loading buffer and the sample (20 µg) were separated on 5% stacking and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes (Amersham Biosciences, Pittsburgh, PA, USA). The membranes were blocked with TBS containing 0.05% Tween 20 (TBST) and 5% non-fat milk at room temperature for 1 h. After being washed 3 times with TBST for 5 min each time, the membranes were probed with primary antibodies. The following antibodies were used: mouse monoclonal antibody for ICBP90 (1 : 4000), P16 (1 : 1000), P21 (1 : 1500), P-P21 (Thr145, 1 : 1000), P27 (1 : 1000), P-P27 (Ser10, 1 : 1000), P-CDK1 (Glu8, 1 : 1000), P-CDK2 (Thr160, 1 : 1500), P53 (1 : 1000), P-P53 (Ser20, 1 : 1500), P57 (1 : 1500), P73 (1 : 1000), P-P73 (Tyr99, 1 : 1000), RB (1 : 2000), P-RB (Ser807, 1 : 2000) and β-actin (1 : 2000) at room temperature for 2 h and washed 3 times with TBST for 5 min each time. Then, the membranes were labeled with relative second antibodies for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Rockford, IL. USA) according to the manufacturer's instruction. The band density was checked by FluorChem 8000 system (Alpha Innotech, Santa Clara, CA, USA).

Semiquantitative RT-PCR

Total RNA from Jurkat T cells was extracted using the RNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany) following manufacturer's recommended protocols. For measuring levels of P21 and P53 mRNA expression in the treated cells, a reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with a QIAGEN OneStep RT-PCR Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Twenty five µl of RT-PCR mixture contained 10 µl of RNase free H_2O, 5 µl of 5 × buffer, 2 µl of 10 mM dNTP, 100 nmol/l of each primer and 1 µg RNA. The following primers were employed in semi-quantitative RT-PCR analysis: p21 [24, 30] forward primer: 5'-AGCAGAGGAAGACCATGTGGAC-3' and p21 reverse primer: 5'-TTTCGACCCTGAGA GTCTCCAG-3' p53 forward primer: 5'-TGCGTGTGGAGTATTTG GATG-3' and p53 reverse primer: 5'-TGGTACAGTCAGAGCCAACCAG-3'; β-actin [5] forward primer: 5'-AACAGTCCGCCTAGAAGCAC-3' and β-actin reverse primer: 5'-CGTTGACATCGTAAAGACC-3'. The amplified products were size-fractionated by electrophoresis on 1.5% agarose gel, and analyzed by a FluorChem 8000 system with a software (Alpha Innotech, Santa Clara, CA, USA).

Statistical analysis

SPSS 17.0 software (SPSS Inc., IL, US) was used for statistical analysis. The results were expressed as the means ± SD of three independent experiments. Individual comparisons were made by Student's *t*-test for paired data or one-way ANOVA for multiple comparison data, and p-values less than 0.05 were considered

to be statistically different, p-values less than 0.01 to be significantly different.

Results

Anisomycin suppresses Jurkat T cell colony formation and proliferation

It is known that anisomycin dramatically inhibits the growth of T cells. Hence, we first detected its effect on the growth of Jurkat T cells. The cells were cultured with anisomycin ranging from 1.0 to 80.0 ng/ml. Under an inverted microscope, we found that anisomycin treatment resulted in distinct changes in cell morphology and density. Compared to the control, the cell colony became smaller with the increased dosage of anisomycin (Fig. 1A).

$OD_{450\ nm}$ was detected through CCK-8 assay, and inhibitory rate of anisomycin on Jurkat T cell proliferation was calculated. As shown in Figure 1B, the inhibitory rate of the cell proliferation was increased with the treatment of anisomycin in a dose-dependent manner ($r = 0.973$, $p < 0.01$), reaching over 90% at 80 ng/ml.

Anisomycin arrests the cell cycle of Jurkat T cells

DNA content from Jurkat T cells treated by the different concentrations of anisomycin was determined by flow cytometry. As shown in Figure 2, anisomycin at concentrations of 10, 20, 40, and 80 ng/ml inhibited the cells into S-phase and G2/M-phase, inducing obvious apoptosis at Sub-G1 phase. The results suggest that anisomycin can arrest the cell cycle of Jurkat T cells in a dose-dependent manner ($r = 0.954$, $p < 0.01$).

Anisomycin promotes the expression of some CKIs in Jurkat T cells

CKIs bind and inactivate CDK-cyclin complexes, and negatively regulate cell cycle. As shown above, anisomycin can affect the cell cycle of Jurkat T cells. This promotes us to find out if anisomycin functions through some of CKIs. Therefore, Jurkat T cells were treated by anisomycin for 0, 2, 4, 8, 12, 16, and 24 h. The expressions of P21, P-P27 and P53 proteins from the treated Jurkat T cells reached their peaks at 4 h (Fig. 3A, C, E, I), and were significantly up-regulated with the enhanced concentrations of anisomycin (Fig. 3B, D, F, G, J), presenting a dose-related relation. But P16, P-P21, P27, P57, P73/P-P73 and P-Rb presented

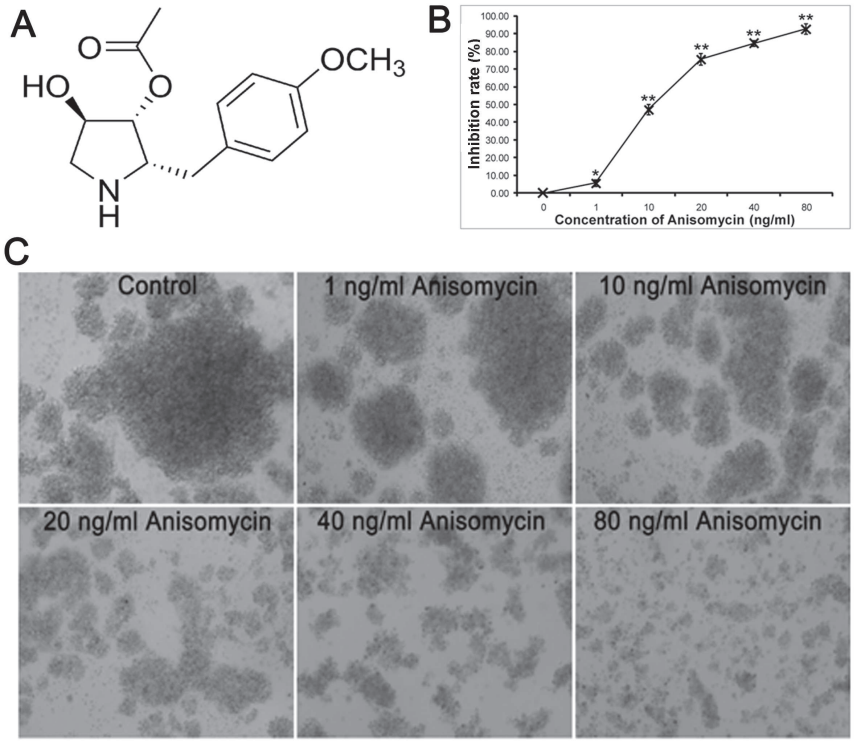

Fig. 1. Effect of anisomycin on the proliferation and viability of Jurkat T cells. The cells were seeded at 4×10^4 cells into a 96-well microplate and treated with the indicated concentrations of anisomycin for 24 h. (**A**) It shows chemical structure of anisomycin. (**B**) The cell viability was determined by CCK8 assay. (**C**) The morphology and colony formation of the cells incubated with or without anisomycin for 48 h were examined under an inverted microscope (200×). The results are from a representative of three performed experiments. The data are expressed as the means ± SD, * $p < 0.05$ and ** $p < 0.01$ *vs.* the untreated control

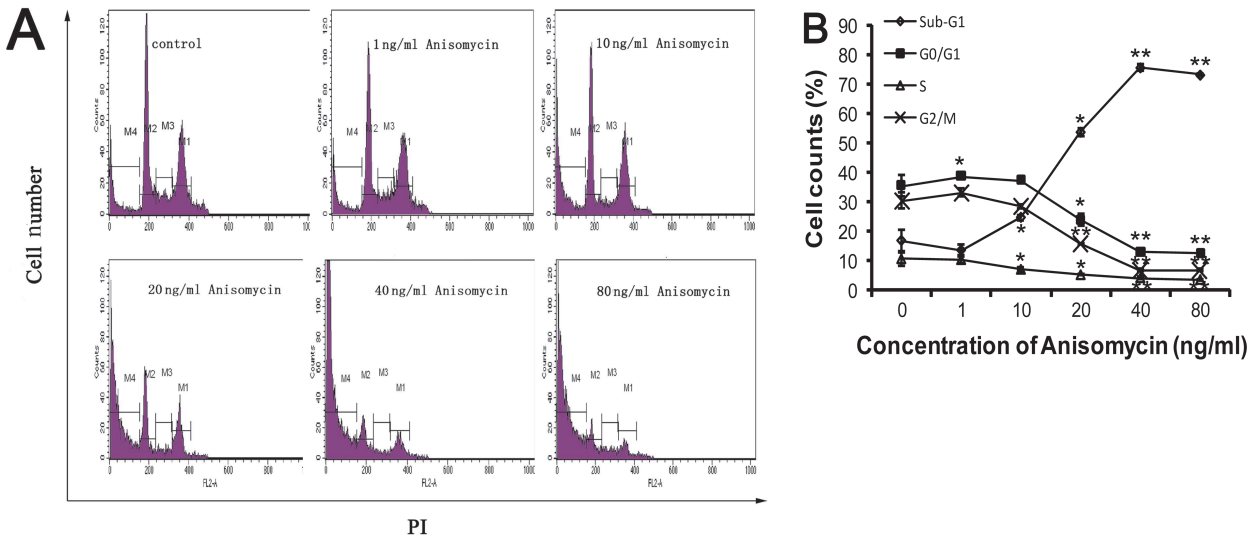

Fig. 2. Effect of anisomycin on the cell cycle progression of Jurkat T cells. The cells were treated with different concentrations of anisomycin (0–80 ng/ml) for 48 h. Then, the cells were fixed with ethanol and stained with propidium iodide, and cell cycle distribution was analyzed by flow cytometry in (**A**). The percentage of the cells in G0/G1, S and G2/M phases is shown in (**B**). The data are expressed as the means ± SD, * $p < 0.05$ and ** $p < 0.01$ *vs.* the untreated control

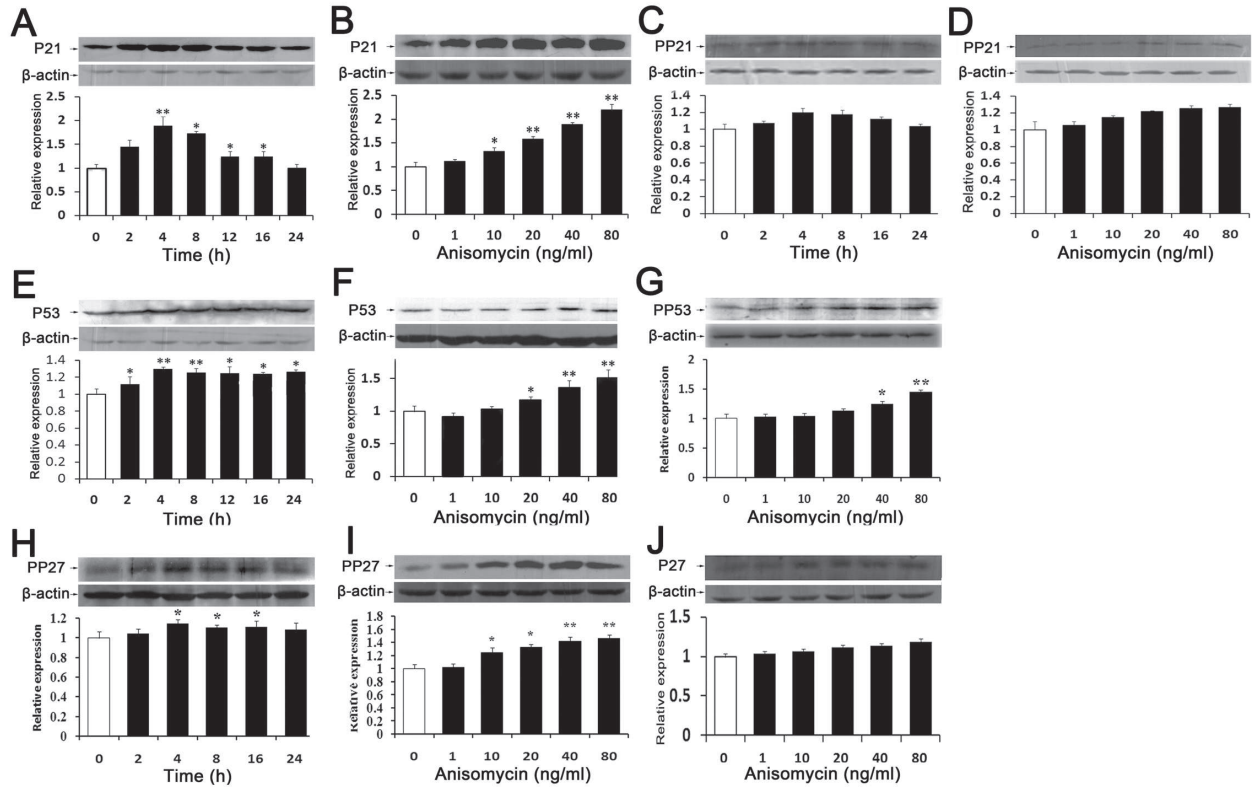

Fig. 3. Effects of anisomycin on the expression of P21, P53 and P27 proteins in Jurkat T cells. Jurkat T cells were treated with 40 ng/ml of anisomycin at the indicated time or with the different concentrations of anisomycin for 4 h. And cytoplasmic proteins of the cells were extracted for western blot. (**A**–**D**) The levels of P21/PP21 proteins were shown. (**E**–**G**) P53/PP53 proteins were determined. (**H**–**J**) P27/PP27 protein alterations were observed. Then, the blots were stripped and re-probed for β-actin control. All the results were repeated three times. The data are expressed as the means ± SD, * $p < 0.05$ and ** $p < 0.01$ *vs.* the untreated control

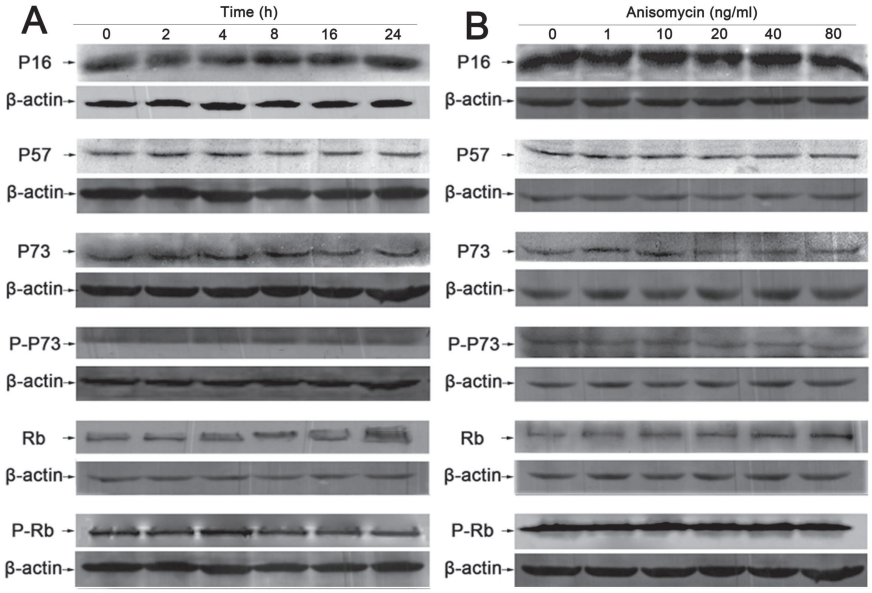

Fig. 4. Effects of anisomycin on the expression of P16, P57, P73 and Rb proteins in Jurkat T cells. Jurkat T cells were treated with 40 ng/ml of anisomycin at the indicated time or with the different concentrations of anisomycin for 4 h. Then, cytoplasmic proteins of the cells were extracted for western blot. (**A**) The levels of P16, P57, P73/PP73 and Rb/PRb proteins were determined. (**B**) The changes of P16, P57, P73/PP73 and Rb/PRb proteins were observed. The above blots were stripped and re-probed for β-actin control. All the results were repeated three times

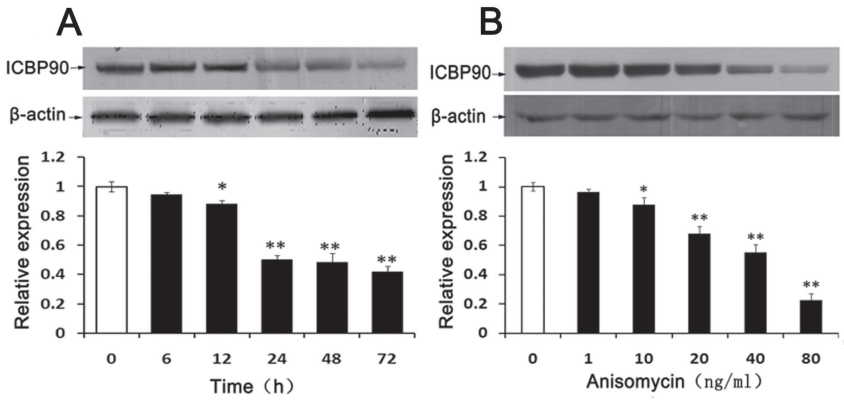

Fig. 5. Effects of anisomycin on the expression of ICBP90 in Jurkat T cells. (**A**) Jurkat T cells were treated with 40 ng/ml of anisomycin at the indicated time. (**B**) Jurkat T cells were treated with the different concentrations of anisomycin for 24 h. The cell lysate was immunoblotted with the anti-ICBP90 antibody. The above blots were stripped and re-probed for β-actin control. All of the results were repeated three times. The data are expressed as the means ± SD, * $p < 0.05$ and ** $p < 0.01$ *vs.* the untreated control

little changes with anisomycin-treated time (Fig. 4A) or with the increased concentrations of anisomycin (Fig. 4B). The level of Rb protein was increased at 24 h after the treatment of anisomycin and also done by the treatment of 80 ng/ml of anisomycin, but its phosphorylated-Rb did not change no matter what was the time or dose of anisomycin treatment.

Anisomycin inhibits the expression of ICBP90 in Jurkat T cells

Higher expression of ICBP90 protein was found in cancer cell lines (HeLa, Jurkat T and A549) than in non-cancer cell lines and ICBP90 cell-cycle-dependent expression disappeared in cancer cell lines. So we hypothesized whether anisomycin would affect the expression of ICBP90 to inhibit the proliferation of Jurkat T cells. Expression of ICBP90 extracted from the Jurkat T cells exposed to 40 ng/ml of anisomycin for 0, 6, 12, 24, 48, and 72 h was shown in Figure 5A. It came to decrease slightly 12 h after the treatment, and down to 50% of the control group at 24 h, maintaining the low level until 72 h and presenting an obvious decrease in a dose-dependent manner ($r = 0.986$, $p < 0.01$) (Fig. 5B).

Anisomycin inhibits the activation of CDK2 in Jurkat T cells

The core of the mammalian cell cycle is triggered by the cyclin-dependent protein kinases. Our results revealed that the expression of P-CDK1 from Jurkat T cells treated by anisomycin for 24 h did not change markedly with the increased doses and extended exposure time of anisomycin (Fig. 6A, B). But the expression of P-CDK2 was down-regulated by aniso-

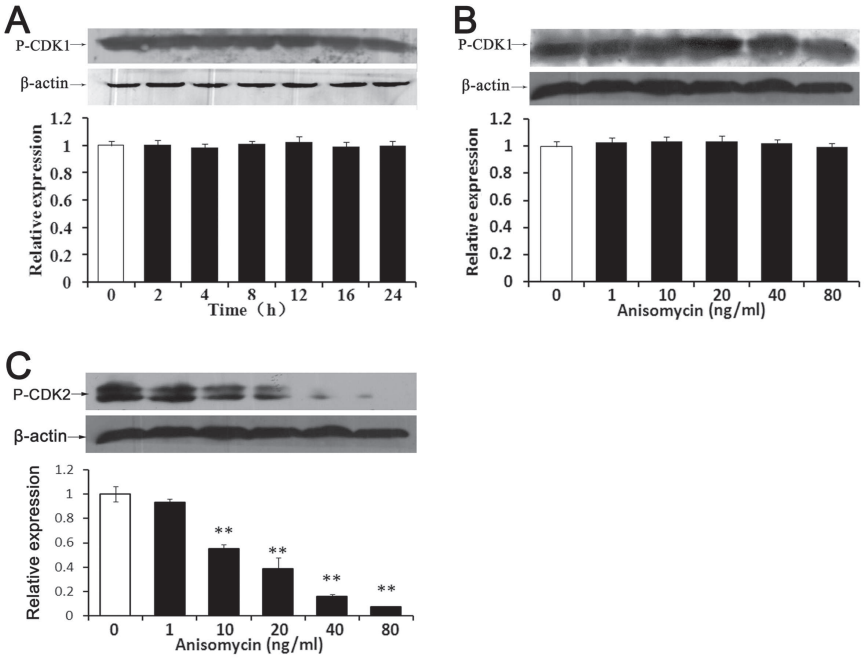

Fig. 6. Effects of anisomycin on the expression of P-CDK1 and P-CDK2 in Jurkat T cells. Jurkat T cells were treated with 40 ng/ml of anisomycin at the indicated time or with the different concentrations of anisomycin for 24 h. And cytoplasmic protein of the cells was extracted for western blot. (**A**, **B**) The level of P-CDK1 protein was shown. (**C**) P-CDK2 protein was tested. The above blots were stripped and re-probed for β-actin control. All the results were repeated three times. The data are expressed as the means ± SD, * $p < 0.05$ and ** $p < 0.01$ *vs.* the untreated control

mycin at 24 h in a dose-dependent manner. It was reduced by 84% of the control at 40 ng/ml of anisomycin, and by 10% at 80 ng/ml (Fig. 6C).

Anisomycin up-regulates the expression of P21 and P53 mRNA in Jurkat T cells

To further confirm the change of P21 and P53, the total RNA was extracted from Jurkat T cells treated with the different concentrations of anisomycin for 4 h and the level of P21 mRNA was determined through RT-PCR. The result is shown in Figure 7A, P21 mRNA expression was increased with the increased concentrations of anisomycin in a dose-dependent manner ($r = 0.966$, $p < 0.01$). P53 mRNA expression was determined after the same treatment as before (Fig. 7B). There was little difference of P53 mRNA expression in 1 ng/ml-treated group compared

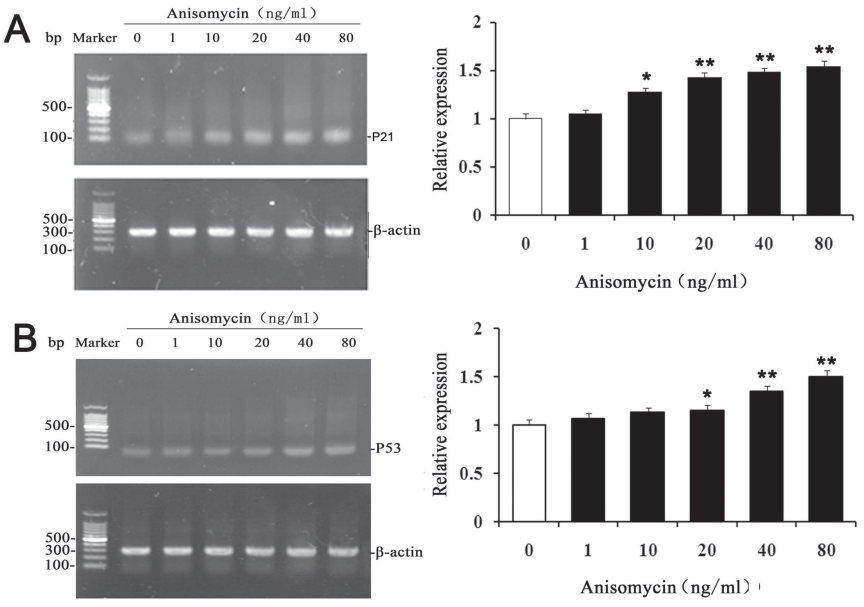

Fig. 7. Effects of anisomycin on the expression of P21 and P53 mRNA in Jurkat T cells. Jurkat T cells were treated with the different concentrations of anisomycin for 4 h. The total RNA in the cells was extracted for RT-PCR. (**A**) The level of P21 mRNA was determined. (**B**) The alteration of P53 mRNA level was observed. All the results were repeated three times. The data are expressed as the means ± SD, * $p < 0.05$ and ** $p < 0.01$ *vs.* the untreated control

with the control, whereas it was also increased obviously as the concentration increase of anisomycin in dose-dependent manner ($r = 0.937$, $p < 0.01$).

Discussion

Ribosome toxic agonist can induce apoptosis or sensitize cancer cells to produce lethal factors resulting in apoptosis [22]. Anisomycin with ribosome toxicity can inhibit cell proliferation through up-regulating promoter activity of macrophage factor-1 and strengthening stability of its mRNA in colon cancer [29]. Novel findings reveal that anisomycin induces mouse macrophage to apoptosis *via* p38 pathway, whereas not JNK or ERK1/2 pathway [8]. Anisomycin induces the expression of annexin-V mRNA in ovarian cancer *via* ERK1/2 but not p38 pathway [13] and the expression of pip92 during anisomycin-induced cell death is mediated by the JNK- and p38-dependent pathways in NIH3T3 cells [6], which suggests that anisomycin activates different MAPK signals in a dose-dependent manner to induce expression of downstream proteins inhibiting cell proliferation or inducing cell apoptosis. Our former studies show that anisomycin inhibits the proliferation of differently histological types of tumor cells and significantly promotes tumor cells to apoptosis. The current study further proves that anisomycin can inhibit Jurkat T cell proliferation in a dose-dependent manner, which is related to significantly blocking the cells into S-phage and G2/M phage.

ICBP90 is newly identified as a novel protein participating in topoisomerase IIα expression [11]. It is reported that TopoII-α expression involves cell growth [17]. ICBP90 may participate in the regulation of cell proliferation and cell cycle progression [1, 9]. Our results show that the suppression of the colony formation and cell viability in anisomycin-treated Jurkat T cells is relevant to the decrease of ICBP90 expression, and Jurkat T cells are significantly blocked into S-phage and G2/M phage. It has been reported that high-level expression of P21 can down-regulate ICBP90 through an E2F-mediated and ubiquitination-dependent protease degradation pathway. ICBP90 part co-localization with PCNA helps the cells pass G1/S point, suggesting that P21 blocks cells into S phage possibly through regulation of its down-stream target protein, ICPB90. In addition, ICPB90 knockdown by RNA interference in HeLa cells reveals that it indeed plays an important role in G1/S transition [1].

As a close transcriptional regulation factor, p53 responses to different types of cell stimuli and activates the test point target genes, such as P21, to block the cell cycle or to promote cell apoptosis. Phosphorylation of P53 (Ser20) site in the N-terminal is involved in the regulation stability and activation of P53 [20]. Brugarolas et al. early found that P21 could block cells in G1 phage through inhibiting cyclin E/CDK2 and cyclin A/CDK2 complexes [4]. Baus et al. also proved that P21 could help to block the cells in G2 phage through cyclin-A/CDK1/2 complex [2], which might be related to the evidence that P21 inactivates CDK1 to block cells into G2 phage in response to DNA-damage [14, 22]. It was revealed that interaction between P53 and P21 could inhibit interaction of cyclin-A/CDK2 complex with blocking the cell cycle through G1/S [26]. Other studies show that high phosphorylation of P27 (Ser10) is found in quiescent cells [3]. P27 can down-regulate the level of CDKs to mediate cell cycle arrest [28]. In our experiments, P53/P-P53 expressions in Jurkat T cells were increased with the elevated concentration of anisomycin. Consistent with this change, P21 expression in anisomycin-treated Jurkat T cells was also up-regulated by anisomycin in a dose-dependent manner. Although the levels of P16, P-P21, P27, P57, P73/P-P73 and Rb/P-Rb were little changed, P-P27 (Ser10) expression was prominently enhanced with the increased concentrations of anisomycin. On the contrary, the significant decrease of P-CDK2 was found with the increased doses of anisomycin. Simultaneously, it was noted that the alterations of P53/P21/P27 started early at 4 h after the treatment of anisomycin, then ICBP90 at 12 h, and P-CDK2 lately at 24 h, with the little change of P-CDK1. Moreover, the suppressed proliferation and arrested cell cycle of Jurkat T cells occurred after P-CDK1 change and were altered with the above regulating protein changes. These results suggest that anisomycin may activate the P53/P21/P27 signaling to decrease the expression of ICBP90, and inhibit expression of P-CDK2 to block the cells into S and G2/M phases, finally contributing to the proliferation inhibition and cycle arrest of Jurkat T cells.

However, in our study, although anisomycin affected the dose-dependent manner expressions of P53, PP53, PP27, and P21, it did not impact the level of

P21 phosphorylation. How can we explain this phenomenon? Dissimilar to P53 or P27, P21 has the pro- and anti-proliferative actions in cell-cycle regulation, which may be associated with the status of its phosphorylation [31]. The phosphorylation of P21 has previously been shown to be required for cyclin-B/Cdk1 activation. Hukkelhoven et al. [12] found that P21 phosphorylation facilitated accumulation of nuclear cyclin-D1/CDK 4 and promoted tumor development. He considered that its phosphorylation contributed to the conversion of CDK inhibitors from tumor suppressive roles to oncogenic roles [12]. In contrast, the un-phosphorylated P21 could inhibit interaction of cyclin-A/ CDK2 complex to cause the cell cycle arrest. Therefore, our findings further support the latest understanding for P21 action. It can be better explained that non-phosphorylated P21 functions in the cycle arrest of Jurkat T cells by anisomycin.

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