

JTE-522, a selective COX-2 inhibitor, inhibits cell proliferation and induces apoptosis in RL95-2 cells¹

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KEY WORDS non-steroidal anti-inflammatory agents; apoptosis; JTE-522; endometrial neoplasms

ABSTRACT

AIM: To investigate whether JTE-522 [4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide], a selective COX-2 inhibitor, can induce apoptosis and inhibit cell proliferation in human endometrial cancer cell line RL95-2 cells and to explore the molecular mechanisms. **METHODS:** [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), DNA ladder, enzyme-linked immunosorbent assay (ELISA), flow cytometry, RT-PCR, and Western blot analysis were employed to investigate effect of JTE-522 on human endometrial cancer cell line RL95-2 cells and the related molecular mechanisms. **RESULTS:** JTE-522 inhibited the growth of RL95-2 cells and induced the apoptosis. Furthermore, it arrested G₀/G₁ phase and inhibited S phase in RL95-2 cells. JTE-522 inhibited the expressions of COX-2 mRNA, phosphorylated Rb, and CDK4 proteins, while increased the levels of p53, p21, cyclin D₁ proteins, and the activity of caspase-3 in RL95-2 cells. **CONCLUSION:** JTE-522 inhibits cell proliferation and induces apoptosis in RL95-2 cells, which may be associated with the activation of caspase-3-like proteases, down-regulation of the expression of COX-2 mRNA, phosphorylated Rb, and CDK4 proteins, and up-regulation of the expressions of p53, p21, and cyclin D₁ proteins.

INTRODUCTION

Studies on epidemiology and animal models of colon carcinogenesis have indicated that non-steroidal anti-inflammatory drugs (NSAID) have anti-colorectal cancer activity^[1]. However, the exact molecular events leading to NSAID-mediated anticancer activity are still unclear. Multiple lines of evidence had demonstrated that NSAID inhibited both cyclooxygenase-1 (COX-1) and COX-2. Recent studies have reported a 40% - 50% lower colorectal cancer risk in people who are continuously taking aspirin or other NSAID, and the inhibitory effects of NSAID on tumorigenesis of the colon were observed in many animal experimental models^[2]. NSAID inhibit COX-1 so that a range of normal physiologic functions are affected, which frequently results in untoward gastrointestinal side effects such as ulceration and bleeding. This limits their clinical use and has led to the development of more selective COX inhibitors. Selective COX-2 inhibitors may be more effective and safer cancer chemopreventive agents than classical NSAID. It has been reported that selective inhibitors of COX-2 decrease tumor formation in experimental animals, and these compounds induce apoptosis and inhibit cell growth in several types of cancer cells^[3]. JTE-522 [4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide] was a novel selective COX-2 inhibitor. *In vitro* COX activity assays using isolated COX-1 and COX-2 enzymes from sheep seminal vesicle microsome and placenta, respectively, have confirmed that JTE-522 selectively inhibits COX-2. JTE-522 has anti-inflammatory effects and can not cause severe gastric lesions at oral doses of 300 mg/kg^[4]. These properties of JTE-522 may allow it to be administered as a prolonged prevention agent. Recently, it has been reported that selective inhibition of COX-2 by JTE-522 in mice reduced hematogenous metastasis of human colorectal cancer cells that had high levels of COX-2

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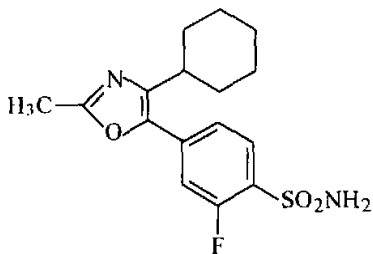
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expression^[5]. In addition, some investigators believe that a certain type of endometrial cancer has several features in common with colon cancer and may share some pathogenetic mechanisms. For example, colorectal cancer and endometrial cancer have similar histologic appearances. Microsatellite instability in colorectal cancer also has been found in endometrial cancer^[6]. In women of families afflicted with hereditary nonpolyposis colorectal cancer of the Lynch syndrome variant, endometrial carcinoma is the most common noncolorectal malignancy reported. With this in mind, we examined whether JTE-522 could inhibit the growth of endometrial cancer cells and induce apoptosis similar to the other selective COX-2 inhibitors did in colorectal cancer cells *in vitro* and *in vivo*, and also explored potential mechanisms by which JTE-522 might mediate growth inhibition and apoptosis. Based on the results of these studies, it will be important to establish whether selective inhibitors of COX-2 are useful for the prevention and treatment of human endometrial cancer.



JTE-522 [4-(4-cyclohexyl-2-methyl-5-yl)-2-fluorobenzenesulfonamide]

MATERIALS AND METHODS

Cell line and reagents Human endometrial cancer cell line RL95-2 was provided by Cancer Institute, Sun Yat-Sen University of Medical Sciences (China). Cells were grown in RPMI-1640 medium supplemented with 15 % new born bovine serum, penicillin G (100 kU/L), and kanamycin (0.1 g/L) at 37 °C in a 5 % CO₂-95 % air atmosphere. Antibodies of p53, p21, phosphorylated-Rb, cyclin D₁, CDK4, and beta actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Purified JTE-522 was obtained from Japan Tobacco Co (Tokyo, Japan). [3-(4,5)-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Gibco Co; caspase-1 and caspase-3 protease activity was measured using a commercially

available Apopain assay kit (BioRad, France). All other chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA).

MTT assay Cell growth was measured by a modified MTT assay^[7]. About 2×10^5 cells/well were plated in 96-well microtiter plates and incubated overnight. Cells were then treated with various concentrations of JTE-522 for 72 h. Then 10 μ L stock MTT (0.5 g/L) was added to each well, and the cells were further incubated at 37 °C for 4 h. The supernatant was removed, and 100 μ L of HCl 0.04 mol/L in isopropanol was added to each well to solubilize the formazan produced. The absorbance at wavelength of 570 nm was measured by a microELISA reader (Sigma). The negative control well contained medium only. The ratios of the absorbance of treated cells relative to those of the control wells were calculated and expressed as percentage of growth inhibition.

Ladder detection assay After induction of apoptosis, cells (7×10^6 per sample, both attached and detached cells) were lysed with 150 μ L hypotonic lysis buffer (edetic acid 10 mmol/L, 0.5 % Triton X-100, Tris-HCl, pH 7.4) for 15 min on ice and were precipitated with 2.5 % polyethylene glycol and NaCl 1 mol/L for 15 min at 4 °C. After centrifugation at $16\,000 \times g$ for 10 min at room temperature, the supernatant was incubated in the presence of proteinase K (0.3 g/L) at 37 °C for 1 h and precipitated with isopropanol at -20 °C. After centrifugation, each pellet was dissolved in 10 μ L of Tris-edetic acid (pH 7.6) and electrophoresed on a 1.5 % agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Detection of apoptotic DNA fragmentation

RL95-2 cells were grown in 96-well culture plates, and incubated with various dose of JTE-522 for 6 h. Apoptotic DNA fragmentation was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Sigma). This assay is based on a quantitative sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments. Briefly, the cells were incubated in 200 μ L of lysis buffer provided in the kit, the lysates were centrifuged, and 20 μ L of the supernatant containing cytoplasmic histone-associated DNA fragments was reacted overnight at 4 °C in streptavidin-coated microtiter wells with 80 μ L of the immunoreagent mix containing biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After washing,

the immunocomplex-bound peroxidase was probed with 2, 2'-azino-di [3-ethylbenzthiazoline sulfonate] for spectrophotometric detection at 405 nm.

Flow cytometry For DNA content analysis, cells were treated with different concentrations of JTE-522 for 24 h. Cells 1×10^6 were harvested, pelleted, washed with phosphate-buffered saline (PBS), and resuspended in PBS containing propidium iodide (PI) 20 mg/L and ribonuclease A 1 g/L. Fixed cells 1×10^6 were examined under per experimental condition by flow cytometry, and percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub- G_1). Cell cycle analysis was performed using the same experimental conditions and distributions using the CellFit program. All measurements were carried out under the same instrumental settings.

Measurement of caspase 1 and caspase 3 like proteases activities RL95-2 cells were lysed in a lysis buffer on ice for 30 min and centrifuged at $15\ 000 \times g$ for 15 min. The activities of caspase 1 and 3 like proteases was measured according to the method described by Chae⁽⁸⁾.

Reverse transcription-polymerase chain reaction (RT-PCR) for COX-2 Total RNA was extracted from cells using TRIzolTM. RT-PCR for COX-2 and beta-actin mRNA was performed as described⁽⁹⁾. Primers for beta-actin were: sense 5' ATCTGGCACCACACCTTCTACAATGAGCTGCG-3', antisense 5' CGT-CATACTCCTGCTTGCTGATCCACATCTGC-3'.

Western blot analysis The cells were lysed in lysis buffer [HEPES 25 mmol/L, 1.5 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, NaCl 0.5 mol/L, edetic acid 5 mmol/L, NaF 50 mmol/L, sodium vanadate 0.1 mmol/L, phenylmethylsulfonyl fluoride (PMSF) 1 mmol/L, and leupeptin 0.1 g/L, pH 7.8] at 4 °C with sonication. The lysates were centrifuged at $15\ 000 \times g$ for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (Tris-HCl 42 mmol/L, 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol, and 0.002 % bromophenol blue) was added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated sequentially with anti-p53, p21, phosphorylated-Rb, cyclin D₁, and CDK4 antibodies and then with peroxidase-conjugated secondary antibodies in the second reaction. Detection was performed with enhanced chemiluminescence reagent. The results on Western

analysis represented the average of three individual experiments.

Statistical analysis The data were mean values of at least three different experiments and expressed as $\bar{x} \pm s$. The Student's *t*-test was used to compare data. $P < 0.05$ is considered to be statistically significant.

RESULTS

Effect of JTE-522 on cell proliferation The effects of JTE-522 on growth of RL95-2 endometrial cancer cells 72 h after treatment are shown in Fig 1. JTE-522 induced a concentration-dependent inhibition of RL95-2 cell growth in the range of 0.1 - 1 mmol/L. Greater than 50 % mean inhibition was achieved at JTE-522 0.5 mmol/L and almost complete suppression was observed at 1 mmol/L.

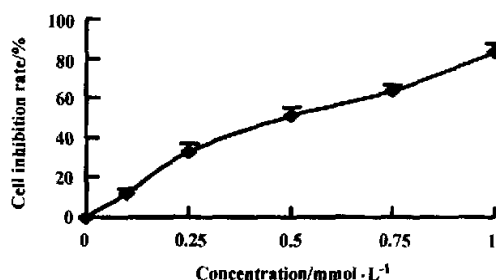


Fig 1. Effect of JTE-522 on cell growth in RL95-2 cells. $n = 3$. $\bar{x} \pm s$.

Effect of JTE-522 on apoptosis and cell cycle The effect of JTE-522 at concentrations from 0.1 mmol/L to 1 mmol/L on apoptosis DNA fragmentation in RL95-2-cells is shown in Fig 2, JTE-522 was found to significantly induce DNA fragmentation in a concentration-dependent manner. The results were gotten in DNA ladder assay (Fig 3). Agarose gel electrophoresis exhibited DNA ladder formation in exposed RL95-2 cells. The DNA ladder was clearly embodied by treatment with JTE-522 for 48 h. Obviously, the profiles of the DNA histograms were striking different from untreated RL95-2 cells, a sub G_1 peak and the apoptotic index was increased from 2.4 % \pm 2.1 % to 54.5 % \pm 3.2 %. The percentages of cells in each cell cycle phase at 24 h after addition of JTE-522 are presented in Tab 1. In keeping with its effect on cell proliferation and apoptosis, JTE-522 increased the proportion of cells in G_1 , decreased the proportion of cells in the S phase in a

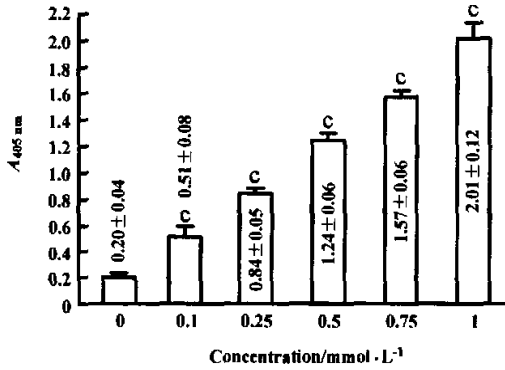


Fig 2. Effect of JTE-522 on DNA fragmentation in RL95-2 cells. *n* = 3. $\bar{x} \pm s$. **P* < 0.01 vs control.

M 1 2 3 4 5 6

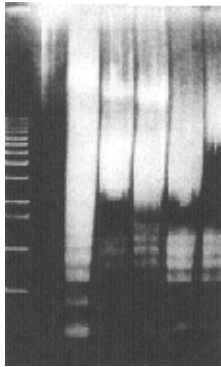


Fig 3. DNA ladder pattern formation of RL95-2 cells. M: DNA markers; lane 1: control; lane 2 - 6: RL95-2 cells treated with 0.1, 0.25, 0.5, 0.75, and 1 mmol/L of JTE-522. Results are representative of three independent determinations.

Tab 1. Effect of JTE-522 on cell cycle in RL95-2 cells. *n* = 3. $\bar{x} \pm s$. ^b*P* < 0.05, ^c*P* < 0.01 vs control.

Concentration /mmole · L ⁻¹	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
0	61.5 ± 2.1	29.1 ± 1.2	10.2 ± 1.4
0.1	67.2 ± 2.3 ^b	21.4 ± 2.1 ^c	11.3 ± 2.5
0.25	73.7 ± 0.9 ^c	15.6 ± 1.8 ^c	10.9 ± 2.1
0.5	79.2 ± 1.7 ^c	9.0 ± 1.5 ^c	12.1 ± 1.3
0.75	90 ± 6 ^c	6.1 ± 0.5 ^c	10.3 ± 0.8
1	83.4 ± 2.6 ^c	0.0 ± 0.0 ^c	11.2 ± 0.8

concentration-dependent manner in RL95-2 cells.

Effect of JTE-522 on the activity of caspase-1 and 3 like protease Caspase-3 like protease activity

was increased by JTE-522 treatment in time-dependent manner, while caspase-1 like protease activity was not that. Caspase-3 like protease activity increased in 3 h, achieved a peak at 18 h, and then decreased (Fig 4). These results indicate that DEVD-dependent protease activity may be implicated in JTE-522-induced apoptosis of RL95-2 cells.

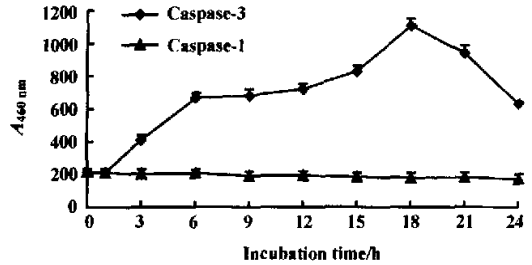
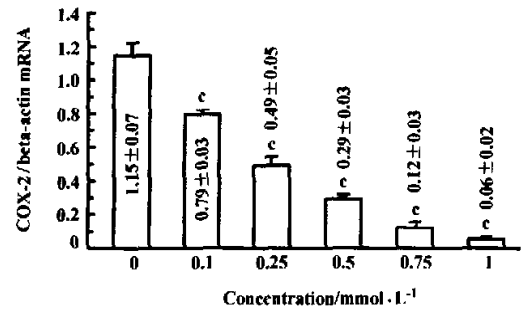


Fig 4. Effect of JTE522 on the activities of caspase-1 and 3 in RL95-2 cells for indicated time period. *n* = 5. $\bar{x} \pm s$.

Effect of JTE-522 on COX-2 mRNA expression Expression of COX-2 mRNA in RL95-2 cells incubated for 6 h with JTE-522 is shown in Fig 5. COX-2



M 1 2 3 4 5 6

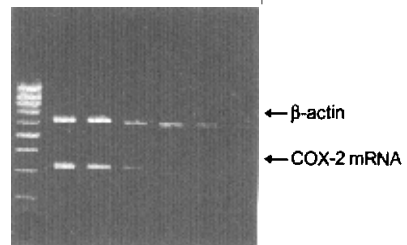


Fig 5. Expression of COX-2 mRNA in RL95-2 cells treated with JTE-522 of various concentrations. M: DNA markers; lane 1: control; lane 2 - 6: RL95-2 cells treated with 0.1, 0.25, 0.5, 0.75, and 1 mmol/L of JTE-522, respectively. *n* = 3. $\bar{x} \pm s$. **P* < 0.01 vs control.

mRNA was detected in large amounts in the untreated cells. In contrast, significantly depressed expression was found by treatment with various concentrations of JTE-522.

Effect of JTE-522 on the expression of p53, p21, phosphorylated Rb (ppRb), cyclin D₁, and CDK4 proteins The expression of p53, p21, and cyclin D₁ proteins was upregulated after treatment with JTE-522 for 6 h. On the contrary, JTE-522 decreased the expression of CDK4 proteins, and a significant reduction in the amount of phosphorylated Rb protein was observed at all concentrations. These changes were in a concentration-dependent fashion and in keeping with the anti-proliferative action of JTE-522 in RL95-2 cells (Fig 6).

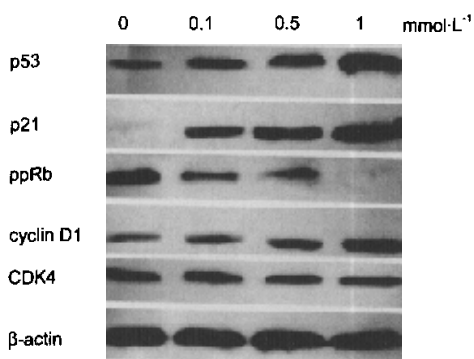


Fig 6. p53, p21, phosphorylated Rb, cyclin D₁, and CDK4 proteins levels in RL95-2 cells treated with JTE522 for 6 h. The result presented is typical of three separate experiments.

DISCUSSION

Colorectal and endometrial cancers have many features in common. Both have identifiable histopathologic precursor lesions. The histopathologic progression from adenoma to carcinoma in colon cancer also is seen in the atypical endometrial hyperplasia-carcinoma sequence. Certain alterations in oncogenes and tumor suppressor genes, such as *ki-ras* and p53, in colorectal cancer also are seen in endometrial cancer^[10]. Based on those similarities, we postulated that NSAID also might inhibit growth of endometrial carcinoma cell lines in a way similar to that described for colorectal cancer. Our data showed that JTE-522 inhibited cell growth and induced apoptosis in human endometrial cancer cell line RL95-2 cells. This is the first study

demonstrating an anticancer activity of JTE-522 in human endometrial cancer.

Several mechanisms of growth inhibition by NSAID on colon cancer cell lines have been suggested including reduction of the rate of cellular proliferation and induction of apoptosis. In this study, JTE-522 exerted two profound effects on the human endometrial cancer cell line RL95-2 cells: (a) it inhibited their proliferation, and (b) it reduced their survival by inducing apoptosis. In keeping with these effects, JTE-522 increased the proportion of cells in G₁, decreased the proportion of cells in the S phase in a concentration-dependent manner in RL95-2 cells, suggesting that the growth inhibitory effect of JTE-522 may be mediated in part by induction of apoptosis.

Although there are probably multiple signals leading to apoptosis, recent work suggests that the IL-1 β converting enzyme (ICE)-like cysteine protease family is essential for apoptotic cell death induced by several stimuli^[11]. Therefore, we measured the activities of CPP32-like and ICE-like protease using AC-DEVD-MCA and AC-YVAD-MCA as substrates, respectively. As shown in Fig 4, caspase-1 can not be processed by JTE-522 in RL95-2 cells. Thus, an YVAD-inhibitable caspase-1-like protease is probably not the target for JTE-522. These results indicate that DEVD-dependent protease activity may be responsible for apoptotic characteristics induced by JTE-522.

Multiple lines of evidence suggest that COX-2 expression is upregulated in several types of human cancers, including the colon, lung, breast, stomach, and pancreatic cancer^[12,13]. These novel findings suggest that COX-2 may play a role in tumor angiogenesis and inhibitors of this pathway may affect tumor growth by inhibiting the expression of COX-2. Consistent with previous studies in colon cancer, our results revealed that the expression of COX-2 mRNA in RL95-2 cells was high, whereas significantly reduced by treatment with different concentrations of JTE-522. These findings indicate that decreasing COX-2 expression may be one of the mechanisms of the anticancer activity of JTE-522 in human endometrial cancer cells.

Previous studies showed that NSAID affected two critical parameters that determined the final number of cells in a cell culture system; cell cycle progression and cell death rates by apoptosis. Our data revealed that JTE-522 increased the proportion of cells in the G₀/G₁ phases of the cell cycle, while it decreased the percentage of cells in the S phase. This finding indicates either (a)

an inhibition of progression through the G₁ phase or an inhibition of the transition from G₁ into the S phase, or (b) that the cells exited from the cell cycle and entered the G₀ phase. Based on the results, we postulated that JTE-522 would down-regulate the phosphorylation of Rb protein. This hypothesis was based on substantial evidence of the importance of Rb phosphorylation in cell cycle progression, both in the initiation of G₁ and in maintaining the cellular machinery necessary for progression through S phase^[14]. JTE-522 induced a rapid and profound reduction in the amount of phosphorylated Rb protein. The pathway that mediates cellular responses to stress is regulated by the p53 gene. As shown in Fig 6, a concentration-dependent increase in cellular levels of p53 was detected in response to JTE-522. The effect of p53 on cell cycle regulation acts partly through its downstream molecule p21^[15-17]. So cellular levels of p21, a member of Cip/Kip family of CKIs, were examined. In parallel with the pattern of p53 expression, levels of p21 increased significantly in a concentration-dependent fashion, consistent with a cause and effect relationship between p53 and p21. Based on these findings, we predicted that levels of cyclin D₁ would be reduced, whereas levels of CDK4 would be increased. However, the expression of cyclin D₁ was increased, while the expression of CDK4 was reduced. We currently have no explanation for these patterns of changes. But we believe that further studies need to be carried out using synchronized cells in order to improve the specificity and sensitivity of these molecular analyses.

In conclusion, The selective COX-2 inhibitor, JTE-522 inhibited cell growth and induced apoptosis in RL95-2 cells, which was associated with activation of caspase-3 and down-regulation of the expression of COX-2 mRNA, phosphorylated Rb, and CDK4 proteins, and up-regulation of the expression of p53 and p21 proteins. However, the potential role of JTE-522, alone or in combination with other agents, in the treatment of endometrial cancer needs to be further investigated. In addition, studies of the exact mechanisms of inhibition and apoptosis of JTE-522 would contribute to establish its clinical application in the treatment of endometrial cancer.

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一种选择性环氧合酶-2抑制剂 JTE-522 抑制 RL95-2 细胞的增殖及诱导其凋亡¹

R96 A

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关键词 非甾体类消炎药; 细胞凋亡; JTE-522;

子宫内膜肿瘤

目的: 探讨环氧合酶-2 抑制剂 JTE-522 是否对子宫内膜癌细胞株 RL95-2 细胞有抑制增殖和诱导凋亡的作用及其分子机理. **方法:** 应用体外噻唑兰法、琼脂糖凝胶电泳、酶联免疫试验、流式细胞术、RT-PCR 及 Western blot 等方法研究 JTE-522 对 RL95-2 细胞增殖和凋亡的作用及其分子机理. **结果:** JTE522 抑制 RL95-2 细胞的增殖、诱导其凋亡、引起 G₀/G₁ 期阻滞和 S 期抑制, 并伴有 COX-2 mRNA、磷酸化 Rb、CDK4 蛋白表达的抑制及 p53, p21 和 cyclin D₁ 蛋白表达水平的上调. 另外, 细胞经 JTE-522 处理后, 还可见 caspase-3 活性的增加. **结论:** JTE-522 抑制 RL95-2 细胞的增殖及诱导其凋亡, 可能与 COX-2 mRNA, 磷酸化 Rb、CDK4 蛋白水平的下降及 p53, p21 和 cyclin D₁ 蛋白表达的上调有关, 还可能与 caspase-3 的激活有关.

(责任编辑 韩向晖)