Research Paper

Tanshinone IIA Induces Apoptosis of Leukemia Cancer Cells and Inhibits Tumor Growth *In Vivo* Through Mitochondrial Pathway

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To investigate the effect of Tanshinone IIA on apoptosis of leukemic cancer cells and tumor growth inhibition *in vivo*. Human leukemia cell line HL-60 cells were divided into model group, low-dose group, medium dose group and high-dose group and transfected according to the groups. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide method was used to calculate the inhibition rate of cell growth, flow cytometry was used to detect the apoptosis rate and mitochondrial membrane potential and Western blot was used to detect mitochondrial membrane potential related protein. The treatment group was treated with Tanshinone IIA and the control group was given phosphate-buffered saline+dimethyl sulfoxide intraperitoneal injection. 2 w later, the tumor size and weight were measured and weighed. The 24 h and 48 h proliferation inhibition rates of the experimental group were significantly higher than those of the model group and increased with the increase of the dose ($p<0.05$). The early apoptosis rate and late apoptosis rate of the experimental group were significantly higher than those of the model group and increased with the increase of the dose ($p<0.05$). The level of integral optical density in the experimental group was significantly lower than that in the model group and decreased with the increase of the dose ($p<0.05$). The levels of caspase-9, caspase-3 and B-cell lymphoma 2 related X protein in the experimental group were significantly higher than those in the model group, while the level of B-cell lymphoma 2 in the experimental group was significantly lower than that in the model group and there was drug dependence ($p<0.05$). The tumor size and weight of the treatment group were significantly lower than those of the control group ($p<0.05$). Tanshinone IIA can induce apoptosis and tumor growth of leukemia cancer cells *in vivo* and slow down the proliferation of cancer cells, which may be related to the regulation of mitochondrial pathway and the inhibition of mitochondrial membrane potential related protein.

Key words: Tanshinone IIA, mitochondrial pathway, leukemia, apoptosis, tumor growth, mechanism

Acute Myeloid Leukemia (AML) is a common hematologic malignancy, whose pathology mainly characterized in that marrow primitive granulocytes are more than 20 %[1]. However, the pathogenesis of AML is not fully understood at present. And many studies agree that AML is attributed to a combination of environmental, genetic and biological behaviors[2]. Chemotherapy is currently the first-line treatment for patients with AML, but there are still some patients who are not sensitive to chemotherapy, while long-term chemotherapy also foster drug resistance and unsatisfactory chemotherapy treatment[3].

Tanshinone IIA (TAT) is the main active ingredient of *Salvia miltiorrhiza*, one of the Lamiaceae plants, which has antioxidant, anti-vasodilatory, anti-liver fibrosis and anti-liver injury functions and is widely applied in clinical cardiovascular and inflammatory diseases[4]. Recent studies have revealed the apoptotic, proliferative and migratory activities of TAT in various cancer cells. Zhang *et al.*[5] showed that TAT may inhibit the Wingless-related integration site (Wnt)/beta (β)-catenin signaling pathway and reduce the expression of Glycogen Synthase Kinase-3β (GSK-3β), Axin and Adenomatous Polyposis Coli (APC) proteins, thus inhibiting the invasive metastasis of hepatocellular carcinoma stem cells. Currently, there are few studies related to TAT for the treatment of patients with AML.

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Accepted 26 May 2022
Revised 04 December 2021
Received 20 February 2021
Indian J Pharm Sci 2022;84(3):654-659

The experiment in the study selects human leukemia cell line HL-60 cells as the observation subjects and aims to analyze the effect of TAT on apoptosis of leukemia cancer cells and tumor growth inhibition *in vivo* and related mechanism thereof.

MATERIALS AND METHODS

General material:

Experimental cell: Human leukemia cell line HL-60, which is provided by Wuhan Boster Biological Technology Co., Ltd.

Experimental animal: Clean healthy female Nonobese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice, 6±1 w, 19±1 g, which are provided by Shanghai SLAC Laboratory Animal Co., Ltd. Mice were placed in an animal room at 20°-23°, 40 %-70 % humidity, with 12 h day and night alternation and given a laboratory diet with free access to water. And the experiments were performed after rearing 1 w. The experimental operations of the animals in this experiment were in accordance with the relevant standards of the Regulations for the Administration of Laboratory Animals. All experimental operations on experimental animals were in accordance with the relevant standards of the Regulations for the Administration of Affairs Concerning Ex-Experimental Animals.

Experimental drug: TAT (purity ≥98 %), which is provided by Chengdu Must Bio-Technology Co., Ltd.

Methods:

Cell culture and grouping: HL-60 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, where the medium was placed at 37° and 5 % carbon dioxide saturated humidity to culture and subculture. Cells grown in log phase were taken and randomly divided into model group, low-dose group, medium-dose group and high-dose group.

Mice subcutaneous transplantation and treatment with drugs: There were 40 NOD/SCID mice selected to establish subcutaneous implantation experiment. HL-60 cells in logarithmic growth phase were taken and their concentration was adjusted to 1×10^7 cells/ml to mix with Matrigel™ basement membrane matrix, then the mixture was injected into the left side of axilla of each mouse by 1 ml syringe. Upon observation of tumor development to 100 mm^3, the mice were randomly divided into control and treatment groups, in which the treatment group was given 50 mg/kg TAT intraperitoneal injection and the control group was given the same amount of Phosphate-Buffered Saline+Dimethyl Sulfoxide (PBS+DMSO) intraperitoneal injection and both groups were treated for 2 w.

Observation index:

MTT assay to detect the cell proliferation ability for each group: HL-60 cells were cultured in RPMI-1640 medium, then the cell concentration was adjusted to 1×10^5 cells/ml and TAT at indicated concentrations (20, 40, 60) μmol/l and DMSO were given according to the group and centrifuged after 24 h and 48 h of transfection respectively. Subsequently, 200 μl of DMSO was added to each well and the absorbance value at 490 nm was measured by enzyme standardization, followed by calculation of the growth inhibition rate of each group of cells.

Flow cytometry to detect the apoptosis rate of each group: HL-60 cells were transfected in groups and washed with PBS and then the cells were resuspended, followed by the addition of 5 μl of AnnexinV-FIFC and mixed well. After that, 5 μl of Propidium iodide (PI) was added and mixed well, with photophobic reaction for 10 min. The early apoptosis rate and late apoptosis rate of each group of cells were detected by flow cytometry.

Flow cytometry to analyze mitochondrial membrane potential changes: HL-60 cells were transfected in groups, centrifuged and washed with PBS, and then 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazoylcarbocyanine iodide (JC-1) working solution was prepared to resuspend cells. Cells were incubated for 15 min within dark, centrifuged and washed for cell smear and visualized by microscopy. Image Pro Plus image analysis software was used to detect the ratio of red and green Integral Optical Density (IOD) value.

Western blot assay to detect mitochondrial membrane potential-related proteins: HL-60 cells were transfected in groups, washed with PBS and followed by lysis in ice bath for 20 min with the addition of cell lysis solution. Then the supernatant was taken to quantify the proteins of each group by Bicinchoninic Acid (BCA) assay, the proteins were electrotransferred to Polyvinylidene Difluoride (PVDF) membrane by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), blocked with skim milk for 1 h, incubated with primary antibody overnight and blocked with secondary antibody for 1 h. BIO-RAD chemical imaging system was used to develop and image the relative content of each group of caspase-9, caspase-3, B-lymphoma-2 (Bcl-2) and Bcl-2 related

X protein (BAX), with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as the internal reference.

Tumor size detection of mice in groups: Mice were executed by cervical vertebrae luxation after intraperitoneal injection of the drug for 2 w and tumor tissues were taken from each group of mice to measure and weigh the tumor size and weight.

Statistical methods:

In this study, the data were analyzed statistically by Statistical Package for the Social Sciences (SPSS) 20.0 software package. The measured data such as 24 h proliferation inhibition rate, 48 h proliferation inhibition rate, early apoptosis rate, late apoptosis rate, IOD, caspase-9, caspase-3, BAX, Bcl-2, tumor size and weight were all conformed to normal distribution and expressed by (x±s) and the comparison among multiple groups was performed by one-way Analysis of Variance (ANOVA) and Student Newman Keuls-q test were used for pairwise comparison; and the statistical results were statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The 24 h proliferation inhibition rate and 48 h proliferation inhibition rate of cells in the experimental group are significantly higher than those in the model group, which are elevated with the increase of dose and the difference is statistically significant ($p<0.05$) as shown in Table 1.

The early apoptosis rate and late apoptosis rate of cells in the experimental group are significantly higher than those in the model group, which are elevated with the increase of dose and the difference is statistically significant ($p<0.05$) as shown in Table 2.

The mitochondrial IOD of cells in the experimental group is significantly lower than that in the model group, which decreases with the increase of dose and the difference is statistically significant ($p<0.05$) as shown in Table 3.

The levels of caspase-9, caspase-3 and BAX in the cells of the experimental group are significantly higher than those of the model group, while the level of Bcl-2 is significantly lower than that of the model group, drug dependence exists and the differences are statistically significant ($p<0.05$) as shown in Table 4.

The tumor size and weight of mice in the treatment group are significantly lower than those in the control group and the difference is statistically significant ($p<0.05$) as shown in Table 5.

TABLE 1: COMPARISON OF CELL PROLIFERATION INHIBITION RATE IN GROUPS (x±s)

Group	24 h inhibition rate (%)	48 h inhibition rate (%)
Model group	0.62±0.03	0.11±0.01
Experimental group	2 d	2 d
Low-dose group	1.03±0.24[a]	1.06±0.14[a]
Medium-dose group	2.06±0.46[ab]	1.25±0.15[ab]
High-dose group	3.41±0.25[abc]	3.06±0.23[abc]
F	372.29	1277.03
p	<0.001	<0.001

Note: [a]$p<0.05$, compared with the model group; [b]$p<0.05$, compared with the low-dose group and [c]$p<0.05$, compared with the middle-dose group

TABLE 2: COMPARISON OF APOPTOTIC ABILITY IN GROUPS (x±s)

Group	Early apoptosis rate (%)	Late apoptosis rate (%)
Model group	0	0
Experimental group		
Low-dose group	79.26±0.26[a]	1.06±0.14[a]
Medium-dose group	88.65±0.45[ab]	1.25±0.15[ab]
High-dose group	93.64±0.58[abc]	3.06±0.23[abc]
F	7088.77	5273.81
p	<0.001	<0.001

Note: [a]$p<0.05$, compared with the model group; [b]$p<0.05$, compared with the low-dose group and [c]$p<0.05$, compared with the middle-dose group

TABLE 3: CHANGES ON THE MITOCHONDRIAL MEMBRANE POTENTIAL OF THE CELLS IN GROUPS (x±s)

Group	IOD
Model group	1.32±0.16
Experimental group	<0.001
Low-dose group	0.98±0.16[a]
Medium-dose group	0.71±0.12[ab]
High-dose group	0.49±0.25[abc]
F	80.04
p	<0.001

Note: [a]p<0.05, compared with the model group; [b]p<0.05, compared with the low-dose group and [c]p<0.05, compared with the middle-dose group

TABLE 4: COMPARISON OF MITOCHONDRIAL MEMBRANE POTENTIAL-RELATED PROTEIN EXPRESSION LEVELS IN THE GROUPS OF CELLS (x±s)

Group	Caspase-9	Caspase-3	BAX	Bcl-2
Model group	0.45±0.03	0.52±0.12	0.85±0.12	2.64±0.16
Experimental group	<0.001	<0.001	<0.001	<0.001
Low-dose group	1.36±0.06[a]	0.87±0.26[a]	1.06±0.18[a]	2.31±0.15[a]
Medium-dose group	1.68±0.15[ab]	1.23±0.21[ab]	1.49±0.25[ab]	2.01±0.12[ab]
High-dose group	2.03±0.21[abc]	1.46±0.25[abc]	2.16±0.45[abc]	1.46±0.85[abc]
F	516.76	72.14	72.14	25.59
p	<0.001	<0.001	<0.001	<0.001

Note: [a]p<0.05, compared with the model group; [b]p<0.05, compared with the low-dose group and [c]p<0.05, compared with the middle-dose group

TABLE 5: ANALYSIS OF CHANGES IN TUMOR SIZE AND WEIGHT OF MICE IN GROUPS (x±s)

Group	Tumor size (mm^3)	Tumor weight (g)
Control group	1652.38±236.52	4.36±1.02
Treatment group	563.85±125.36	2.46±0.86
t	18.185	6.368
p	<0.001	<0.001

AML is one of the most common types of leukemia in adults and currently chemotherapy is still the mayor treatment for AML. However, the treatment effect is poor and there are still about 70 % of patients who obtain remission eventually relapse and evolve into refractory leukemia, leading to treatment failure and death[6-8].

Therefore, scholars at home and abroad have focused on developing an effective and safe treatment for AML. TAT is the most abundant active ingredient in the Chinese herbal medicine *Salvia miltiorrhiza*, which is widely used in clinical practice[9]. Chen *et al.*[10] studied that TAT could contribute to anti-atherosclerotic effects *via* promoting the activation of M2-type cells to secrete anti-inflammatory factors and inhibiting the activation of M1-type cells to secrete pro-inflammatory factors. Recent studies have revealed that TAT has greater potential for biomedical applications and serves as a regulator of biological activities in malignant tumors such as breast, rectal, gastric and bladder cancers.

Wenna *et al.*[11] revealed that TAT could enhance the sensitivity of breast cancer cells to Adriamycin chemotherapy by regulating the APC/β-catenin signaling pathway, with certain adjuvant therapeutic effects. Zhou *et al.*[12] concluded that TAT could lower the level of Hypoxia-Inducible Factor 1-alpha (HIF-1α) in human colon cancer cells to inhibit cellular pro-angiogenic factor expression and suppress the

angiogenesis of Human Umbilical Vein Endothelial Cells (HUVECs). Liu *et al.*[13] showed that TAT could inhibit gastric cancer cell proliferation and induce apoptosis by reducing COX-2 and NF-κB pathway-related protein levels.

In this experiment, the 24 h proliferation inhibition rate and 48 h proliferation inhibition rate of cells in the experimental group are significantly higher than those in the model group, which are higher with the increase of the dose. And the early apoptosis rate and late apoptosis rate in the experimental group are significantly higher than those in the model group, which are higher with the increase of the dose.

It is indicated that TAT could induce apoptosis and inhibit cell proliferation in leukemic cancer cells. In this experiment, we further establish a tumor growth mouse model *in vivo* and find that the tumor size and weight of mice in the treatment group are significantly lower than those in the control group, further indicating that TAT has an inhibitory effect on the development of leukemia, which is similar to the findings of Bh *et al.*[14].

Apoptosis is a form of programmed cell death comprising the death receptor pathway and the mitochondrial pathway[15]. Mitochondria are the power house within eukaryotic cells as a significant regulator in cell survival, proliferation, apoptosis, metastasis and other activities in a variety of malignant tumor cells including lung, breast and prostate cancers.

In the mitochondrial pathway, the Bcl-2/BAX ratio act as a regulator and a reduced ratio level can lead to mitochondrial dysfunction. Then mitochondria release large amounts of cytochrome c, which activates caspase-9, which further activates various caspases such as caspase-3, causing a caspase cascade reaction and ultimately inducing apoptosis[16,17]. Shen *et al.*[18] studied that both arsenic trioxide and quercetin could induce apoptosis in leukemic cells by the intervention of altered mitochondrial structure and thus activating the induced mitochondrial apoptosis pathway.

In this experiment, the IOD level of cells in the experimental group is significantly lower than that in the model group, which decreases with the increase of dose. The levels of caspase-9, caspase-3 and BAX in the experimental group are significantly higher than those in the model group, while the levels of Bcl-2 are significantly lower than those in the model group, with drug dependence. It is indicated that TAT can induce apoptosis, inhibit cell proliferation and delay the development of leukemia. Moreover, its mechanism may be associated to the regulation of mitochondrial pathway by TAT.

In summary, TAT can induce apoptosis and tumor growth of leukemia cancer cells *in vivo* and slow down the proliferation of cancer cells, which may be related to the regulation of mitochondrial pathway and the inhibition of mitochondrial membrane potential related protein.

Conflict of interests:

The authors declared no conflicts of interest.

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