Actinomycin D inhibiting K562 cell apoptosis elicited by salvicine but not decreasing its cytotoxicity¹

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ABSTRACT

AIM: To study the effects of actinomycin D (Act D) on the cytotoxicity and apoptosis elicited by salvicine in human leukemia K562 cells. METHODS: Growth inhibition of K562 cells was measured by the microculture tetrozolium (MTT) assay. Cell apoptosis was evaluated by fluorescence microscopy, DNA agarose gel electrophoresis, and flow cytometry. RESULTS: Following exposure of K-562 cells to salvicine plus Act D for 24 h, Act D at the concentrations of 0.04, 0.4, and 4 µmol/L potentiated the cytotoxicity of salvicine 6.25 µmol/L to some degree. The mean growth inhibitory rates went from 8 % up to 69 %, 71 %, and 70 %, respectively. However, the same enhancement of Act D did not continue to emerge at the higher concentrations than salvicine 6.25 µmol/L. Act D enhanced, or at least, did not decrease the cytotoxicity of salvicine against K562 cells. Fluorescence microscopy, DNA agarose gel electrophoresis, and flow cytometry revealed that Act D concentration-dependently inhibited the induction of apoptosis by salvicine in the same cell line. CONCLUSION: The combination of salvicine and Act D in a proper range of concentrations is able to enhance the cytotoxicity of salvicine against K562 cells though inhibiting apoptosis. The other mechanisms of cell death except apoptosis may be implicated in the process.

INTRODUCTION

Salvicine is a novel diterpenoid quinone compound synthesized by the structural modification of a natural product lead isolated from a Chinese medicinal plant Salvia prionitis Hance (Labiatae)[1]. It possesses potent anticancer activities, both in vitro and in vivo, especially against various solid human malignant tumors[2]. Topoisomerase II (TOPO II) is the primary cellular target of salvicine[3]. The inhibitory activity of this compound as a TOPO II poison is derived from its ability to stabilize DNA strand breaks, either through interactions with the enzyme alone or the DNA-enzyme complex[4]. Moreover, we have demonstrated its significant growth inhibitory capacity on MDR tumor cell lines with downregulation of mdr1 gene expression[5]. Now, salvicine has entered clinical trials in China.

It is well established that almost all chemothera-
Pharmacological agents exert anticancer activities by inducing apoptosis. Apoptosis, a process of active cell death, requires the expression of some related genes. Actinomycin D (Act D), as an antineoplastic drug, displays its cytotoxicity and induction of apoptosis against tumor cells. On the other hand, Act D is an inhibitor of RNA synthesis, which can alleviate or block the apoptotic process and decrease the cytotoxicity induced by several stimuli such as the dihydrofolate reductase inhibitor aminopterin and the prostaglandin derivative 15-deoxy-delta 12,14-prostaglandin J2. However, a surprising manifestation has also been observed that Act D promotes induction of apoptosis by some specific stimuli, for example, tumor necrosis factor-related apoptosis-inducing ligand and the death receptor CD95.

Our previous work revealed that salvicine induced apoptosis in human leukemia HL-60, K-562, multidrug-resistant K-562/A02, breast cancer MCF-7, and gastric cancer SGC-7901 cells. Induction of c-myc promoter-specific DNA damage by the agent is an early event leading to apoptosis in HL-60 cells. Salvicine-induced apoptosis is associated with the downregulation of P-glycoprotein expression in multidrug-resistant K562/A02 cells and c-jun activation plays a central role in this process. In view of the above particularity of Act D, we further investigated its effect on the cytotoxicity and apoptotic induction of salvicine in cultured K562 cells in this study.

**MATERIALS AND METHODS**

**Preparation of drugs** Tangerine coloured crystalloid salvicine was provided by the Department of Phytochemistry of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. A stock solution of salvicine 10 mmol/L was made in 50% dimethylsulfoxide and 50% normal saline. Act D was purchased from Shanghai Xinya Pharmaceutical Factory. Stock solution of Act D 0.16 mmol/L was prepared by normal saline. All stock solutions were aliquoted and stored at -20 °C, thawed just before the test and diluted with complete medium. The final dimethylsulfoxide concentration did not exceed 0.5%.

**Cell line and culture** The human chronic myelogenous leukemia cell line K562 was obtained from American Type Culture Collection (ATCC) and grown in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated bovine serum, L-glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, and HEPES 10 mmol/L, pH 7.4. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

**Measurement of cell growth inhibition** Growth inhibitory effect of K562 cells with various treatments was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co) assay with some minor modifications. Briefly, K562 cells at exponential phase were seeded into 96-well plates. The cell density was 8×10⁶/L that was selected based on the results of preliminary tests to maintain the control cells in an exponential phase of growth during the period of the experiment. Then cells were exposed to different concentrations of salvicine, Act D, and salvicine combined with Act D for 24 h, respectively, and each treatment was tested in triplicate wells. At the end of exposure, 20 µL of MTT 5 g/L was added to each well and the plates were incubated at 37 °C for 4 h. Then “triplex solution (10% SDS-5% isobutanol-HCl 12 mmol/L)” was added and the plates were incubated at 37 °C for 12-20 h. Media and Me₂SO control wells, in which salvicine was absent, were included in all the experiments. The A value was read on a plate reader at a wavelength of 570 nm. The inhibition of cell growth was calculated by the equation:

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\text{Growth inhibitory rate} = \left[ 1 - \left( \frac{A_{570 \text{ treated}}}{A_{570 \text{ control}}} \right) \right] \times 100\%.
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**Fluorescence microscopy** Untreated and treated K562 cells were harvested by centrifugation, resuspended in the medium, and dripped onto a glass slide. The cells on a glass slide were stained with DNA binding fluorochrome 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, Boehringer, Mannheim, Germany) after fixation with 95% ethanol. The nuclear morphology was observed under an Olympus IX 70 UV light fluorescence microscope.

**DNA agarose gel electrophoresis** The total cellular DNA was extracted from K562 cells untreated and treated with drugs for 24 h by the method described by
Slin and Stafford with some minor modifications\[20\]. Briefly, cells were washed in phosphate-buffered saline (PBS) and lysed overnight at 37 °C in lysis buffer containing Tris-HCl 10 mmol/L (pH 8.0), edetic acid 10 mmol/L, 0.4 % sodium dodecysulfate, and proteinase K 100 mg/L. After complete digestion, an equal volume of saturated phenol was added to the cell lysates and mixed fully. Samples were then centrifuged at 3340×g for 5 min. Chloroform was added to the supernatant isolated from the previous step, mixed fully and centrifuged as above. Supernatant was mixed with 2.5-fold volume of absolute ethanol and NaCl at 0.2 mol/L final concentration for DNA precipitation. The DNA pellets were obtained by centrifugation at 13 362×g for 10 min and then air-dried, dissolved in TE buffer (Tris-HCl 10 mmol/L, pH 7.8, edetic acid 1 mmol/L) containing RNase 0.5 g/L (Sigma Chemical Co) at 37 °C for 30 min. Electrophoresis was performed on 1.5 % agarose gel in TBE buffer [Tris 90 mmol/L, pH 8.0, boric acid 90 mmol/L, and edetic acid 2 mmol/L]. At the end of electrophoresis, the gel was stained in ethidium bromide (EB) 0.5 mg/L for 30 min. DNA fragments were visualized under UV fluorescence.

**Flow cytometry** Cellular DNA content was quantified by flow cytometry via determination of propidium iodide (PI)\[21\]. K562 cells untreated and treated with drugs for 24 h were collected, washed in phosphate-buffered saline and fixed in 70 % ethanol (4 ºC for 12 h or longer, usually overnight). The cells after fixation were washed in PBS and cell pellets obtained by centrifugation were stained with PI staining solution containing PI 50 mg/L, RNase 10 mg/L, 0.5 % (v/v) Triton X-100, and 0.1 % (w/v) trisodium citrate at room temperature in the dark for 30 min. DNA content was determined with a Becton-Dickinson FACSCalibur flow cytometer. The cell populations in sub-G\(_1\) area (the position where apoptotic cells are located) were quantified from a standard count of 10 000 cells using CELLQUEST, ModFIT LT for mac V1.01, BD. The experiment was repeated 2 times.

**RESULTS**

**Act D enhances the cytotoxicity of salvicine in K562 cells** Act D did not decrease the cytotoxicity of salvicine at all tested concentrations in K562 cells (Tab 1). Following exposure of K562 cells to salvicine plus Act D for 24 h, Act D at the concentrations of 0.04, 0.4, and 4 µmol/L potentiated the cytotoxicity of salvicine 6.25 µmol/L to some degree. The mean growth inhibitory rates went from 8 % up to 69 %, 71 %, and 70 %, respectively. However, the same enhancement by Act D did not continue to emerge at the higher concentrations than salvicine 6.25 µmol/L. At salvicine 12.5 µmol/L, the treatment still gave a higher growth inhibitory rate with combination of the two agents than that with any one of the two alone, but a lower rate than the summation of the growth inhibitory rates with the two separately. At salvicine 25 µmol/L or above, the combination did not bring about a significant increase or decrease in the inhibitory rates. The results revealed that the cytotoxic enhancement by Act D was dependent on the dose of salvicine in this system.

**Act D inhibited the apoptosis induced by salvicine in K562 cells** The difference of degrees of apoptosis induced by salvicine alone or in combination with Act D was further examined (Tab 2). DAPI staining showed that treatment with salvicine 25 µmol/L alone for 24 h led to extensive nuclear condensation, characteristic of apoptosis (Fig 1B), significantly different from the homogeneous nuclear seen in untreated K562 cells (Fig 1A). Contrarily, the combination of Act D allevi-
ated the nuclear condensation induced by salvicine and brought the nuclear shape back to nearly normal state as the concentration of Act D increased from 0.4 to 4 µmol/L (Fig 1C, D). The same appearance was also observed in K562 cells treated with salvicine 50 µmol/L plus Act D.

DNA agarose gel electrophoresis also showed that Act D inhibited salvicine-induced leukemia cell apoptosis. K562 cells presented the typical ‘DNA ladder’ pattern of apoptosis after treatment with salvicine 25 µmol/L alone for 24 h. Combination treatment with Act D made the ladder gradually abating as the concentration of Act D increased and finally disappearing at Act D 4 µmol/L (Fig 2). The similar results also appeared in the treatment of K562 cells with salvicine 50 µmol/L plus Act D.

The quantitation of apoptosis by flow cytometry further confirmed the concentration-effect relationship observed from the above examinations of morphology and DNA fragmentation. Act D concentration-dependently lowered the apoptotic percentage induced by salvicine 25 or 50 µmol/L in K562 cells. The apoptotic rate was decreased from 18 % by salvicine 25 µmol/L alone to 16 %, 12 %, and 8 % by combination with Act D 0.04, 0.4, and 4 µmol/L, respectively. In salvicine 50 µmol/L-treated group, the apoptotic rate was 60 % and it was declined to 34 %, 22 %, and 12.5 % individually by the same combination with Act D. The results from the morphology and DNA agarose gel electrophoresis demonstrated that Act D significantly inhibited apoptosis induced by salvicine in K562 cells (Fig 3).
Fig 3. Quantitative detection of apoptotic K562 cells (cells in the sub-G1 phase) by flow cytometry. (A) DNA histograms of apoptotic cells: (a) control, (b) salvinine 25 µmol/L, (c) salvinine 25 µmol/L plus Act D 0.04 µmol/L, (d) salvinine 25 µmol/L plus Act D 0.4 µmol/L, (e) salvinine 25 µmol/L plus Act D 4 µmol/L. (B) The dose-dependent effects of Act D inhibiting salvinine-induced apoptosis in K562 cells. n=2. Mean±SD.
DISCUSSION

Combination chemotherapy is one of the most important principles for systemic therapy of cancer because of tumor cell heterogeneity and its implication for drug resistance. One of the basic purposes of combination chemotherapy is to enhance the therapeutic effects by synergism or potentiation between drugs in combination. The data from novel anticancer agents in combination with conventional drugs in cultured tumor cells will facilitate their proper clinical use. In this study, our results demonstrated that Act D could enhance the cytotoxicity of salvicine at relatively low doses against K562 cells. However, Act D at the same concentrations inhibited the apoptotic induction by salvicine in this system.

Act D, as an inhibitor of RNA synthesis, blocking the apoptotic induction by salvicine is reasonable and understandable, which suggests the requirement of new RNA production in this process. In fact, the treatment with salvicine can indeed bring out a significant increase in the expression of both c-fos and c-jun genes[15]. In addition to inhibiting RNA synthesis, Act D intercalates DNA and produces double-strand DNA breaks as a topoisomerase II poison. DNA breaks can also occur through the generation of free radicals[21-24]. These features may explain the complexity of its activities: Act D by itself enough induces tumor cell apoptosis[8,9] although it can suppress RNA synthesis during the process, and its inhibitory effect is believed to be the main mechanism of its anticancer activity. In this study, Act D inhibits the apoptosis induced by salvicine probably via repressing RNA synthesis. Its impact on the cytotoxicity of salvicine in K562 cells suggests that other mechanisms seem to contribute to cell death except apoptosis, such as necrosis.

Our results present a new phenomenon that the combination of salvicine and Act D in a proper range of concentrations enhances the cytotoxicity of salvicine against leukemia K562 cells although apoptosis is inhibited. The other mode(s) of cell death except apoptosis may play some roles in the process. The exact mechanism underlying the phenomenon is to be clarified. And the generality of this manifestation also needs further investigation.

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