·Original Article in English·

Induction of apoptosis in K562 human chronic myelogenous leukemia cells by tetra-arsenic tetra-sulfide, STI571 and herbimycin

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Abstract: Objective The therapeutic effect of chronic myeloid leukemia (CML) is undesirable. In order to find a new sensitive drug for CML, this study aims at exploring the effects of tetra-arsenic tetra-sulfide (As_4S_4) on human leukemia K562 cells. **Methods** The viability of K562 cells, represented by absorbance, was measured by MTS assay. The cells morphological changes were determined by Wright's staining and Hoechst33342 assay. The cell apoptosis was evaluated by DNA agarose gel electrophoresis and the cell apoptosis rate was measured by flow cytometry. **Results** The cell viability decreased significantly being cultured with 5.0 μmol/L As_4S_4, STI571 and herbimycin for 72 hrs (with absorbances of 0.32 ±0.04, 0.49 ±0.01 and 0.69 ±0.02, respectively). As_4S_4 and STI571 had more inhibition on the K562 cells viability than herbimycin ($P < 0.01$), and there was no statistically significant difference between that of As_4S_4 and STI571. The effects of 1.0, 5.0 and 10.0 μmol/L As_4S_4 on K562 cells were time-dependent. When the concentration was lower than 1.0 μmol/L, As_4S_4 had little effect on K562 cells. After being cultured with 2.0 μmol/L As_4S_4 for 24 to 48 hrs, typical morphological changes of apoptosis appeared in the K562 cells. After being cultured with 5.0 μmol/L As_4S_4, STI571 and herbimycin for 72 hrs, the apoptosis rate of K562 cells were 68.8%, 56.7% and 35.5%, respectively. When the concentrations of As_4S_4 changed from 2.0 to 3.0 μmol/L, the apoptosis rate increased from 25.7% to 45.3%. There was no significant difference between the apoptosis rate of K562 cells induced by 5.0 and 10.0 μmol/L As_4S_4. **Conclusions** As_4S_4 with the concentration of 2.0 μmol/L could inhibit the growth of K562 cells efficiently through inducing apoptosis. **[Chin J Contemp Pediatr, 2004, 6(1): 15 - 18]**

Key words: Tetra-arsenic tetra-sulfide; STI-571; Herbimycin; K562; Apoptosis

四硫化四砷 STI571 和除莠霉素诱导 K562 细胞凋亡的研究

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[**摘　要**] **目的**　目前慢性粒细胞性白血病(CML)的化疗效果仍不理想,为寻找对 CML 敏感的药物,该研究探讨四硫化四砷、STI571 和除莠霉素对 CML 细胞株 K562 的作用。**方法**　通过 MTS 方法检测三种药物在不同浓度(0.01,0.1,1.0,2.0,5.0,10.0 μmol/L)下对 K562 细胞活力的影响。采用瑞氏染色、荧光染色和琼脂糖凝胶电泳观察药物处理后细胞形态学的变化和细胞凋亡的发生。用流式细胞仪检测细胞凋亡率。**结果**　用5.0 μmol/L 的四硫化四砷、STI571 和除莠霉素培养 3 天,K562 细胞活力明显下降,其吸光值分别为0.32 ±0.04,0.49 ±0.01,0.69 ±0.02,其中四硫化四砷和 STI571 对细胞的抑制作用强于除莠霉素($P < 0.01$),前两者间无明显差异($P > 0.05$)。1.0,5.0,10.0 μmol/L 的四硫化四砷对 K562 细胞的抑制作用呈时间依赖关系($P < 0.01$)。小于 1.0 μmol/L 的四硫化四砷对 K562 细胞活力无明显影响。四硫化四砷培养 24 至 48 小时后,K562 细胞出现染色质浓缩、核碎片及凋亡小体等典型的凋亡形态学改变。用 5.0 μmol/L 的四硫化四砷、STI571 和除莠霉素培养 72 小时后,K562 细胞的凋亡率分别为 68.8%、56.7%和 35.5%,2.0 与 3.0 μmol/L 的四硫化四砷诱导 K562 细胞凋亡率

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分别为 25.7 %和 45.3 %,5.0 与 10.0 μmol/L 的四硫化四砷诱导细胞凋亡的差异不明显。**结论** 2.0 μmol/L 的四硫化四砷能有效地通过诱导凋亡抑制 K562 细胞生长。 [**中国当代儿科杂志**,**2004**, **6**(**1**): **15 - 18**]

[**关 键 词**] 四硫化四砷;STI571;除莠霉素;K562;凋亡
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Chronic myeloid leukemia (CML), a particular subtype of leukemia, is a clonal disorder in which cells of the myeloid lineage undergo massive clonal expansion. The characteristic genetic abnormality of CML, the Philadelphia (Ph) chromosome, results from a reciprocal translocation between the long arms of chromosomes 9 and 22. At present, the only therapy for CML is allogeneic bone marrow transplantation, but the suitable donor is not easily found. Some drugs, such as hydroxyurea and interferon alfa can prolong patients' lives, but they have more adverse effects[1]. Recently, it was reported that STI571, a highly selective tyrosine kinase inhibitor to bcr-abl, had an effect on CML in vivo and vitro and arsenic trioxide (As_2O_3) and tetra-arsenic tetra-sulfide (As_4S_4), two components of realgar which was used as Chinese medicine, were very effective in treatment of acute promyelocyte leukemia (APL)[2-6]. Herbimycin is a benzoquinone ansamycin and can cause cell apoptosis. It has been shown that herbimycin is an inhibitor to nonreceptor protein tyrosine kinases and does not inhibit receptor-type tyrosine kinases[7]. In this study, the effects of As_4S_4 on apoptosis of the K562, a cell line of CML, were studied by comparing with those of STI571 and herbimycine

Materials and methods

Reagents

The As_4S_4 was provided by Dr. Lu Daopei (Institute of Hematology, Peking University), STI571 was provided by Dr. Elisabeth Buchdunger (Novartis Pharma, Basel, Switzerland). The herbimycin was from Sigma Chemical Co. (USA). The K562 cells were from Shanghai Institute of Cell Biology. The RPMI 1640 medium and fetal bovine serum were from Gibco-BRL. Stock solutions were made at concentrations of 1 to 20 mmol/L with dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA). The Annexin V/FITC kit was from Oncogene (USA). The apoptotic DNA Ladder Kit was from Roche Molecular Biochemicals (Germany).

Cell culture

The K562 were cultured at 5×10^5 cells/ml in the RPMI 1640 medium with 10 % heat-inactived fetal bovine serum in a humidified incubator which contained 5 % CO_2 and a temperature of 37 ℃, and then some exponential growing stage cells were cultured respectively with 0.01, 0.1, 1.0, 2.0, 5.0 and 10.0 μmol/L As_4S_4, STI571 and herbimycin for 24 to 96 hours.

Cell morphology assessment

After being cultured with above drugs, the cells were centrifuged on slides (800 rpm, 4 min) by cytospin (Shandon Southern Product, Cheshire, UK) and were stained with Wright's staining method and were observed then under microscope. The nuclei of the cells were stained with Hoechst33342 for fragmented DNA content. The amount of 5 μl Hoechst33342 (100 μg/ml) was added to 500 μl drug-treated cell medium. After being incubated for another 30 minutes, the cells were centrifuged on slides and then were observed under the fluorescence microscope.

Cell viability determination

The cell viability of cultured K562 was determined by the MTS [3-(4, 5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega CellTiter 96 Aqueous kit; Promega, USA). The amount of 0.5×10^5 cells per well were cultured with different concentrations of the above drugs in a 96-well tissue plate and the same number cells cultured without drugs were taken as the controls. After being cultured for 24 to 96 hours, MTS were added to the cells according to the directions and the cells were then incubated for another 90 minutes. The absorbance, which represented the cell viability, was determined at 490 nm by a spectrophotometer. Each condition and experiment was performed at least three times.

Cell apoptosis rate detection

The cell apoptosis rate was determined by the Annexin V/ FITC staining and flow cytometric analysis. After being cultured with different concentrations of As_4S_4, STI571 and herbimycine respectively, the amount of 5×10^5 cells were washed in ice-cold PBS and suspended in 0.5 ml binding buffer and 1.25 μl annexin V-FITC for 15 minutes at room temperature. After being washed in ice-cold PBS again, the cells were suspended in 0.5 ml binding buffer and 10 μl propidium iodide. Then the apoptosis rate of 2.0×10^4 cell was analyzed immediately using an FACS Calibur flow cytometer (Becton Dickinson, USA) and CellQuest 3.2 software. The experiment was repeated twice.

DNA ladder

Apoptosis was proved by DNA ladder using Apoptotic DNA Ladder Kit. It was performed according to the directions.

Statistical analysis

SPSS 10.0 software was used to perform *F* test and unpaired Student's *t* test.

Results

Effects of As_4S_4, STI571 and herbimycine on the K562 cells viability

The cell viability decreased significantly after being cultured with 5.0 μmol/L As_4S_4, STI571 and herbimycin for 72 hours (with the absorbances of 0.32 ±0.04, 0.49 ±0.01 and 0.69 ±0.02, respectively) (Figure 1). The As_4S_4 and STI571 had more inhibition on the K562 cells than herbimycin ($P < 0.01$). There was no statistical significance between As_4S_4 and STI571. At the concentration of 1.0 μmol/L, the absorbances of the three drugs were 1.08 ±0.01, 0.76 ±0.01 and 1.26 ±0.05 respectively and that of STI571 was lower than that of As_4S_4 and herbimycin ($P < 0.01$). No difference was found between those of the latter two drugs. There was no difference in the absorbances between the K562 cells cultured with 5.0 μmol/L of As_4S_4 and those cultured with 10.0 μmol/L of As_4S_4 ($P > 0.05$). The absorbances of the K562 cells cultured with 1.0, 5.0 and 10.0 μmol/L As_4S_4 respectively significantly decreased with the cultured time ($P < 0.01$) (Figure 2). When the concentration was lower than 1.0 μmol/L, the As_4S_4 had little effect on K562 cells viability.

The K562 cells morphological changes

Typical morphological changes for apoptosis, including chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies, were observed in the K562 cells cultured with 1.0, 2.0 and 5.0 μmol/L As_4S_4 respectively (Figures 3 and 4). After being cultured with 1.0 μmol/L As_4S_4 for 24 hours, the cells became round, swelling and some nuclei appeared condensation (Figures 3B and 4B). At the concentration of 2.0 μmol/L As_4S_4, the DNA was fragmented and the nuclei broke into several discrete chromatin bodies (Figures 3C, 3D, 4C, and 4D). The DNA ladder pattern of fragments of the K562 cells cultured with 2.0 μmol/L As_4S_4 for 72 hours was shown in DNA agarose gel electrophoresis.

The K562 cells apoptosis rate

When the K562 cells were cultured with less than 1.0 μmol/L As_4S_4, STI571 and Herbimycin for 72 hours respectively, the apoptosis rate was lower than 10.4 % and at the concentration of 5.0 μmol/L, the apoptosis rate of K562 cells was 68.8 %, 56.7 % and 35.5 % respectively. When the concentration of As_4S_4 changed from 2.0 to 3.0 μmol/L, the apoptosis rate increased from 25.7 % to 45.3 %. There was no difference between the apoptosis rate induced by 5.0 and 10.0 μmol/L As_4S_4.

Discussion

Researchers are focusing on developing more efficient therapeutic drugs for the treatment of CML. Realgar has been used to treat a variety of diseases, including syphilis, malaria and several other parasitic infections for more than 1 500 years in China. As_4S_4, the main composition of realgar, was regarded as having low toxicity. So far, it was not reported whether As_4S_4 was effective on malignant diseases when being used alone. This study explored the effect of As_4S_4 on the K562 cells in vitro.

The K562 cells proliferation were inhibited when they were cultured with 1.0 to 10.0 μmol/L As_4S_4,

STI571 and herbimycin respectively. The cells were not affected when the concentration of these reagents were less than 1.0 μmol/L. The 5.0 μmol/L As_4S_4 and STI571 had more inhibition on cells viability than herbimycin, while the inhibition of 1.0 μmol/L STI571 was more than that of As_4S_4. The mechanism needs to be clarified in a later study. In addition, the effects of As_4S_4 on K562 cells viability were time-dependent. The K562 cells had characteristic apoptotic morphologic changes, including cell shrinkage, chromatin condensation, internuclear DNA cleavage, and the formation of apoptotic bodies after being cultured with the three reagents. The 5.0 μmol/L As_4S_4 and STI571 induced marked apoptosis and the cell apoptosis rate induced by As_4S_4 was even a little higher than that by STI571. After the concentration of As_4S_4 was increased from 2.0 to 3.0 μmol/L, the apoptosis rate also increased significantly from 25.7% to 45.3%. After 24 hours when the K562 cells were cultured with 2.0 μmol/L As_4S_4, the nuclei obviously broke into discrete chromatin bodies. This suggested that the As_4S_4, STI571 and herbimycin could induce cell apoptosis and result in cytotoxicity and 2.0 μmol/L As_4S_4 was effective in inducing K562 cells apoptosis. As_4S_4 may be a promising reagent in the treatment of CML.

Previous studies showed that the cells with bcr-abl fusion gene were particularly resistant to apoptosis induced by a number of chemical and biological agents[8]. The bcr-abl is a constitutively activated tyrosine kinase, whose activity is essential to the CML cells. As an inhibitor of the kinase, STI571 does inhibit the expression of bcr-abl[3]. But a recent study showed that the CML cell lines apoptosis induced by As_2O_3 had nothing to do with bcr-abl[9]. The exact mechanism of As_4S_4 inducing K562 cells apoptosis is not clear and needs to be further investigated.

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(Figures are on the inside back cover)

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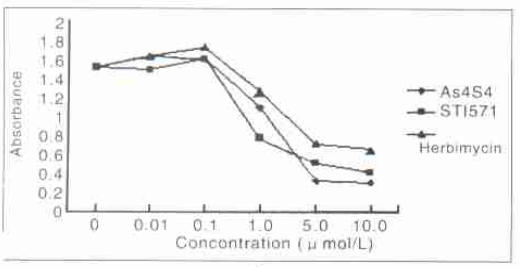
Induction of apoptosis in K562 human chronic myelogenous leukemia cells by tetra-arsenic tetra-sulfide, STI571 and herbimycin

(These figures refer to the paper on page 15)



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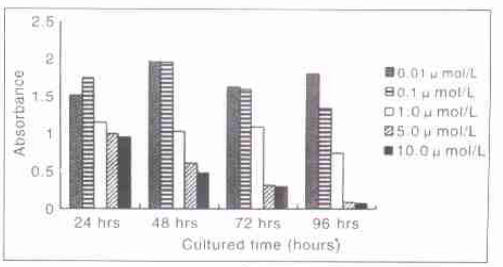

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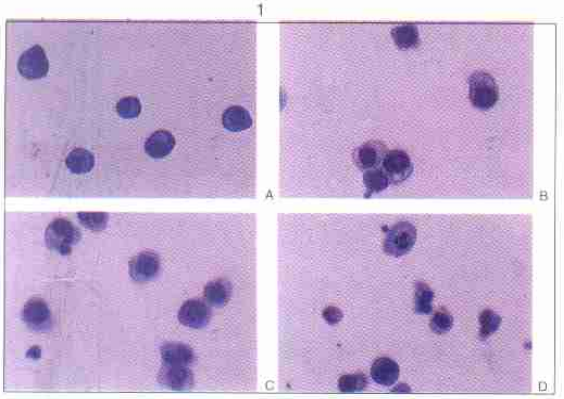

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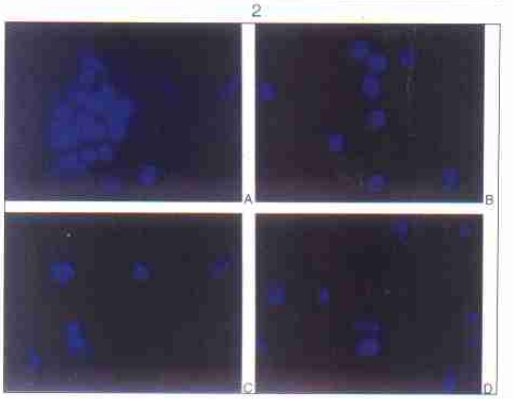

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Figure 1 Effects of different concentrations of As_4S_4, STI571 and herbimycin on cell viability

Figure 2 Effects of different cultured time with As_4S_4 on the K562 cell viability

Figure 3 The K562 cell morphological changes after being cultured with As_4S_4 (wright 400 ×)

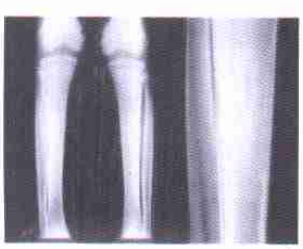
Note: A: controls without treatment. B: cells treated with 1.0 μ mol/L As_4S_4 for 24 hours. C: cells treated with 2.0 μ mol/L As_4S_4 for 24 hours. D: cells treated with 2.0 μ mol/L As_4S_4 for 48 hours

Figure 4 The K562 nuclei changes after being cultured with As_4S_4 (hoechst 3342 400 ×)

Note: A: controls without treatment. B: cells treated with 1.0 μ mol/L As_4S_4 for 48 hours. C: cells treated with 2.0 μ mol/L As_4S_4 for 24 hours. D: cells treated with 2.0 μ mol/L As_4S_4 for 48 hours

儿童肺部炎性假瘤合并肥大性骨关节病例

（正文见第 73 页）



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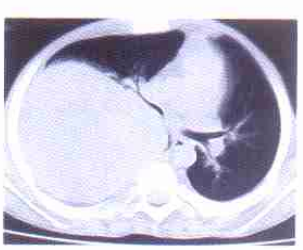

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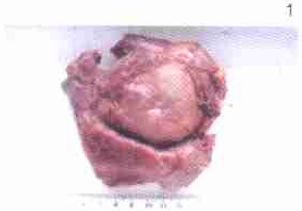

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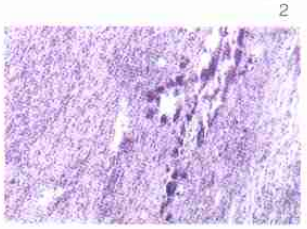

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图 1　双胫腓骨见明显分层状骨膜增生，骨皮质增厚，骨干增粗，但无弯曲变形，骨髓腔无明显狭窄

图 2　胸部 CT：右侧胸腔可见一圆形实质性肿块影，边界清，CT 值 29–41 Hu，大小约为 11.5 cm × 9.0 cm，并可见肺组织推移受压，纵隔稍偏左，支气管通畅，肋骨未见破坏

图 3　切除肿物呈暗红色，大小约为 12 × 10 × 4.5 cm，包膜完整，切面大部分实性，小部分呈囊性，可见坏死，切面灰红色，质地中等

图 4　镜检（HE 染色，10 × 10）：肿物记要由弥漫增生浸润的浆细胞构成，夹杂少量梭形细胞、淋巴细胞及嗜酸性粒细胞，部分区域可见钙化