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Apoptotic effect of As_2S_2 on K562 cells and its mechanism[1]

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KEY WORDS arsenicals; chronic myeloid leukemia; apoptosis; Bcr-Abl fusion proteins

ABSTRACT

AIM: To investigate the apoptotic effect of As_2S_2 on K562 cells and its mechanism. **METHODS:** The effect of As_2S_2 on proliferation of K562 cells was determined by counting the number of cells. Apoptosis was assessed by flow cytometry, DNA fragmentation analysis, and morphology observation. Expression of protein was determined by Western blot. RT-PCR was used to evaluate changes in gene expression. **RESULTS:** As_2S_2 greatly inhibited the proliferation and induced apoptosis of K562 cells in a concentration- and time-dependent manner at the concentration range of 1−5 μmol/L during 24−72 h. Viable cells were decreased to approximately 71 % of control at the concentration of 5 μmol/L after 48-h incubation, 31.4 % after 72-h incubation, and 45.4 % at 3 μmol/L after 72-h incubation. At 3 μmol/L for 72 h, 5 μmol/L for 48 h, and 5 μmol/L for 72 h, the apoptosis rate were 34.4 %, 21.8 %, and 46 % of the treated-cells, respectively. As_2S_2 decreased the Bcr-Abl fusion protein and protein tyrosine kinase (PTK) activity of c-abl and Bcr-Abl, but it did not change the transcription of bcr-abl assayed. As_2S_2 also induced apoptosis in fresh mononuclear cells derived from chronic myelogenous leukemia (CML) patients. CML Ph^+ leukemia cells were more sensitive to the apoptotic effect of As_2S_2 than Ph^- mononuclear cells ($P<0.05$). **CONCLUSION:** As_2S_2 inhibited the proliferation and induced apoptosis in K562 and fresh CML mononuclear cells. The decline of the Bcr-Abl protein and its PTK activity may play an important role in the apoptotic effect of As_2S_2. As_2S_2 may be a useful agent for the treatment of CML.

INTRODUCTION

At present, chronic myelogenous leukemia (CML) therapies are mostly consisted of using chemotherapies, interferon α (IFN- α) and bone marrow transplantation. Recently, STI571 was introduced in the treatment of CML as an effective agent [1,2], because it is a specific inhibitor of Bcr-Abl tyrosine kinase. It was reported that this agent was very effective in the treatment of CML at chronic phase, and the adverse effects were mild. However, according to the results of clinical trials, relapse of the disease usually occurs after suspending the treatment, because the malignant colony of the CML cannot be completely eradicated[3,4]. Therefore, it is very important to find other effective agents for CML therapy.

During recent years, it was demonstrated that As_2O_3 is very effective for the treatment of acute promyelocytic leukemia (APL)[5]. In 1970s, it was reported by Shanghai Leukemia Research Cooperation Group that a compound from Chinese traditional medicine named *Niuhuang JieduPian* containing As_2S_2 was

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shown effective for CML treatment.

In this study, we investigated the effects of As_2S_2 on K562 cells, a human CML cell line from a CML patient in blast crisis, as well as fresh mononuclear cells (MNC) derived from CML patients, and its mechanism.

MATERIALS AND METHODS

Reagents As_2S_2 (AlfaAesar Company) was dissolved in NaOH 1 mol/L. The final concentration was 1.5 mmol/L and HCl was used to adjust the pH to 7.35-7.45.

Cell culture Human CML cell line K562 cells were cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal calf serum. Bone marrow mononuclear cells were obtained from CML patients or idiopathic thrombocytopenic purpura (ITP) patients and were cultured in Iscove's modified Dulbecco's medium supplemented with 10 % heat-inactivated fetal calf serum (Gibco/BRL, Grand Island NY). Diagnosis of CML was performed according to the standard criteria. All cells were cultured in a humidified atmosphere of 5 % CO_2 at 37 ºC.

Cell proliferation and viability assay K562 cells 2×10^5 in logarithmic growth phase were collected and seeded in RPMI-1640 medium and were treated with As_2S_2 . At the indicated time points, viable cells were counted by Trypan blue exclusion method. Each assay was triplicated, then, a cell growth curve was drawn.

Flow cytometric analysis for apoptosis K562 cells 1×10^6 were washed with PBS and fixed in 75 % ethanol at -20 ºC overnight. Prior to analysis, cells were washed again with PBS and resuspended and treated with RNase 200 mg/L for 30 min at 37 ºC, then, cells were incubated in propidium iodide (PI) solution containing PI 20 mg/L in the darkness for 15 min. The suspension was then passed through a nylon mesh filter and analyzed on flow cytometry (Becton Dickson). Apoptosis induced by As_2S_2 in fresh mononuclear cells was detected by annexin V binding assay according to the manufacture's instruction (Clontech). All data were collected and analyzed by lysis II software (Becton Dickson). The experiments were repeated three times and the results were presented as mean±SD.

Morphological examination for apoptosis Cells (2×10^4) were prepared by cytospin centrifugation (250×*g* for 5 min at room temperature) and stained with Wright's staining.

DNA fragmentation assay Apoptosis was also confirmed by detection of fragmentation of chromosomal DNA with the classic DNA ladder method with slight modification. Briefly, cells (2×10^6) were immersed in cytolysis buffer (Tris-HCl 1 mmol/L pH 8.0, edetic acid 10 mmol/L pH 8.0, proteinase K 200 mg/L, 0.5 % SDS) and incubated for 3 h at 50 ºC. DNA was extracted with phenol-chloroform, precipitated in 1/10 volume of NaAc 2 mol/L and 2 volumes of ethanol at –20 ºC overnight, recovered by centrifugation at 1000×*g* for 30 min at 4 ºC, and then resuspended in TE buffer. RNase A was then added at a concentration of 200 mg/L, then the treated extract was incubated at 37 ºC for 30 min and electrophoresed for 2 h on a 1.2 % agarose gel.

Western blot for protein expression Cells were lysed by RIPA-buffer, homogenized by sonication, centrifuged at 10 000×*g* for 10 min and the supernatant was collected. Samples containing 100 mg of total protein were resolved by SDS-PAGE gel, transferred onto a nitrocellulose membrane by electroblotting, and probed with anti c-abl polyclonal antibody. The blot was developed by ECL chemiluminescence reaction kit (Amersham).

Immunoprecipitation and PTK activity assay C-abl and Bcr-Abl in samples containing 100 μg of total protein were obtained using immunoprecipitation. Immunoprecipitates were washed with RIPA-buffer and then resuspended in the assay buffer (Tris-HCl 50 mmol/L pH 7.4, $MgCl_2$ 40 mmol/L, sodium vanadate 50 μmol/L, DTT 2 mmol/L, $MnCl_2$ 1 mmol/L) and centrifuged. After removal of the supernatant , the precipitated protein was used directly for the protein tyrosine kinase (PTK) assay performed according to the protocol described by the kit (Boehring Mannhein). Each group contained 6 repeated tubes with blank and positive control, and the PTK activity was expressed by *OD* value measured at 405 nm (reference wavelength 490 nm).

RT-PCR for detection of *bcr-abl* gene Total cellular RNA was isolated with Trizol reagent (Gibco). RT-PCR was performed according to the protocol described by TaKaRa RT-PCR kit. Human housekeeping gene GAPDH was used to normalize for equal cDNA. The primers for *bcr-abl* were synthesized as flollows: 5'CTT CTC CCT GAC ATC CGT GG3', 5'CAT GTG GGC CAT GAG GTC CAC CAC 3'; the primers for GAPDH were synthesized as follows: 5'AGATGCTACTGGCCG CTGAA 3', 5'TGA AGG TCG GAG TCA ACG GAT TTG GT3'. The denaturing was performed for 3 min at 95 ºC, annealing for 30 min at 56 ºC, extension for 45

min at 72 ºC, and PCR for 32 cycles.

Statistical analysis All experiments were performed at least in triplicate and the results were expressed as mean±SD. Statistical analysis were performed with *t*-test using SAS6.12 software.

RESULTS

As_2S_2 inhibited the proliferation and viability of K562 cells To investigate the effect of As_2S_2 on proliferation and viability of K562 cells, the cells were treated with various concentrations of As_2S_2 ranging from 1 μmol/L to 5 μmol/L for 24–72 h. Significant concentration- and time-dependent inhibition of cell growth was observed. As shown in Fig 1, viable cells decreased to approximately 71 % of the control cells at 5 μmol/L for 48 h, 31.4 % for 72 h, and 45.4 % at 3 μmol/L for 72 h.

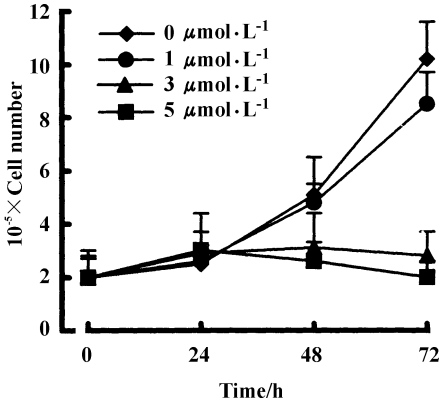

Fig 1. The effect of As_2S_2 on growth curve of K562 cells. *n*=3. Mean±SD.

As_2S_2 induced apoptosis of K562 cells To show whether the growth inhibition induced by As_2S_2 in K562 cells was caused by induction of apoptosis, the cells were stained with PI and analyzed by flow cytometry. The percentage of sub-G1 cells (apoptotic pick) in K562 cells was increased in a time- and concentration-dependent manner. At 1 μmol/L, sub-G1 pick was not observed. When the cells were treated with As_2S_2 3 μmol/L for 72 h, the percentage of sub-G1 cells was 34.4 %. At 5 μmol/L for 48 h and 72 h, the percentage of sub-G1 cells reached 21.8 % and 46 %, respectively (Fig 2). The apoptosis of the cells was confirmed by morphological examination. Typical changes of apoptosis represented by plasma membrane blebbing, chromatin condensation, and fragmentation of nuclei were shown (Fig 3).

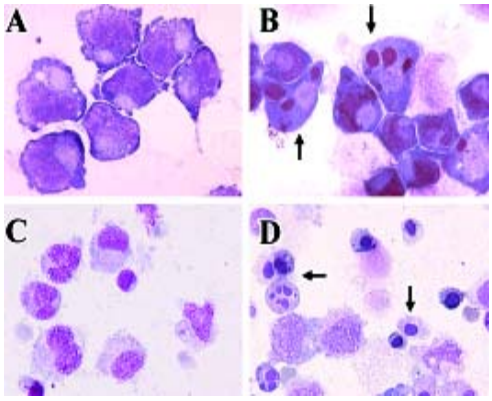

Fig 2. The percentage of sub-G1 cells in K562 cells treated with As_2S_2 at different concentrations for different times *n*=3. Mean±SD.

As_2S_2 decreased Bcr-Abl fusion protein in K562 cells without *bcr-abl* fusion gene alteration To explore the mechanism by which As_2S_2 induced apoptosis in K562 cells, we detected the changes of Bcr-Abl protein in K562 cells treated with As_2S_2 at concentration of 1 μmol/L, 3 μmol/L, and 5 μmol/L, respectively for 72 h and at 5 μmol/L for 24 h, 48 h, and 72 h, respectively using Western-blot. We found that the content of Bcr-Abl protein in treated K562 cells was decreased in a time- and concentration-dependent manner. However, the mRNA level of *bcr-abl* fusion gene was not changed in any sample assayed using RT-PCR method (Fig 4).

As_2S_2 decreased the activity of protein tyrosine

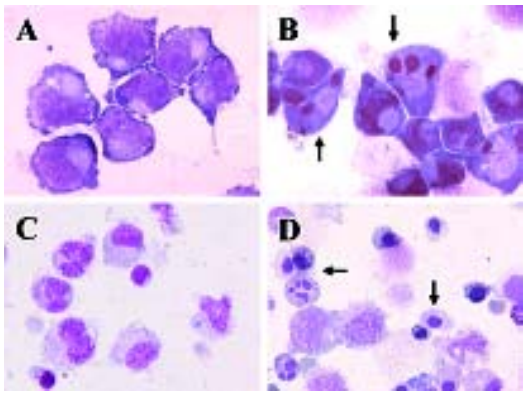

Fig 3. Morphological changes of K562 cells and CML mononuclear cells treated with As_2S_2 (Wright's staining, ×1000). A: Untreated K562 cells; B: K562 cells treated with As_2S_2 at 5 μmol/L for 72 h: C: Untreated CML mononuclear cells; D: CML mononuclear cells treated with As_2S_2 at 5 μmol/L for 72 h.

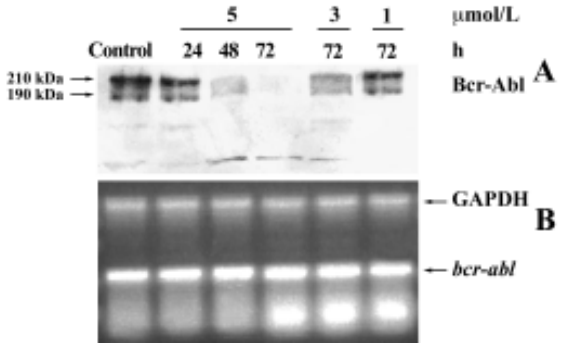

Fig 4. The changes of bcr-abl in K562 cells treated with As_2S_2. (A) Western-blot for Bcr-Abl protein content in K562 cells treated with As_2S_2 at different concentrations for different times. (B) RT-PCR for bcr-abl in K562 cells treated with As_2S_2 at different concentrations for different time using GAPDH as internal standard.

kinase of c-Abl and Bcr-Abl in K562 cells Correlating with the changes of the content of c-Abl and Bcr-Abl, the PTK activity of c-Abl and bcr-abl in K562 cells was decreased in a time- and concentration-dependent manner (Fig 5).

As_2S_2 induced the apoptosis in fresh CML mononuclear cells CML mononuclear cells treated with As_2S_2 were dual-stained with Annexin-V-FITC and PI and analyzed by flow cytometry. A concentration-dependent increase in apoptotic cells was shown when the cells were treated with As_2S_2 1 μmol/L to 5 μmol/L, and a time-dependent increase in apoptotic cells was shown when the cells were treated with As_2S_2 at 5 μmol/L for 24-72 h. At different concentration, at As_2S_2 1 μmol/L for 72 h, (3.4±0.6) % cells underwent apoptosis and (8.6±1.5) % and (23±4) % cells showed signs of apoptosis at 3 μmol/L for 72 h and 5 μmol/L for 72 h, respectively. In the different time group, (8.2±0.5) %, (9.6±1.5) %, and (23±4) % cells showed signs of apoptosis at 5 μmol/L for 24 h, 48 h, and 72 h, respectively. Apoptosis induced by As_2S_2 was verified by the morphology (Fig 3) and a typical DNA "ladder" was shown by gel electrophoresis (Fig 6).

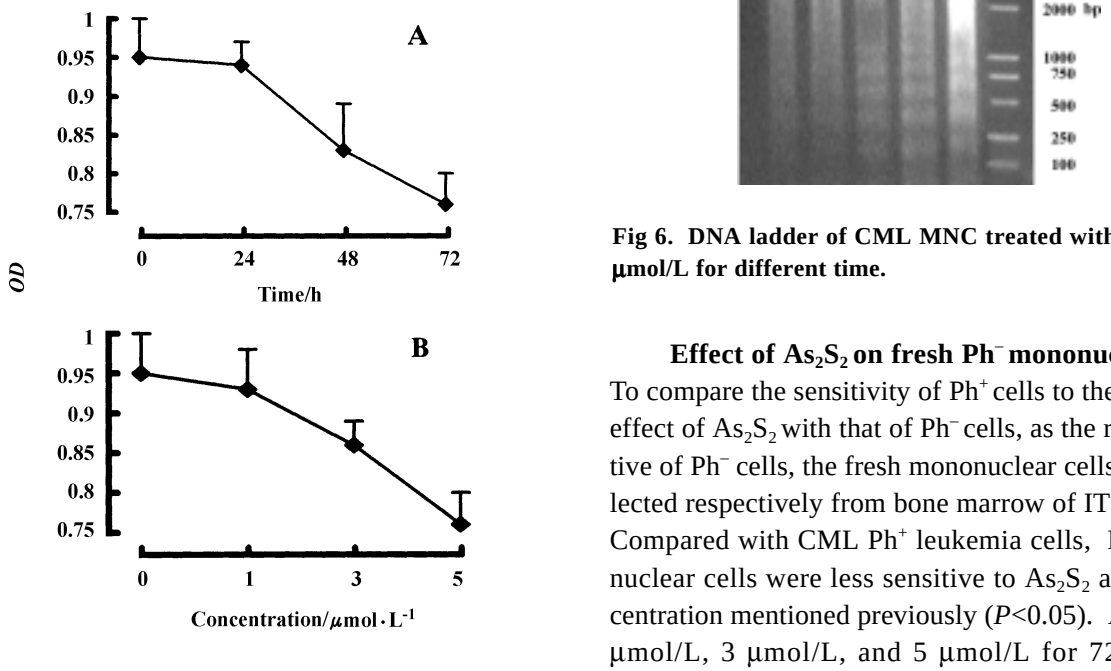

Fig 5. PTK activity of c-Abl and Bcr-Abl in K562 cells treated with As_2S_2 at different concentrations for different time. (A) PTK activity of c-Abl and Bcr-Abl in K562 cells treated with As_2S_2 5 μmol/L for different time. (B) PTK activity of c-Abl and Bcr-Abl in K562 cells treated with As_2S_2 at different concentrations for 72 h. *n*=6. Mean±SD.

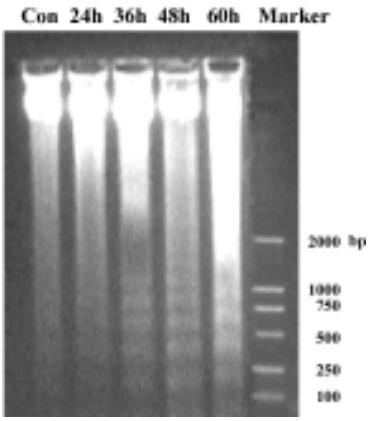

Fig 6. DNA ladder of CML MNC treated with As_2S_2 at 3 μmol/L for different time.

Effect of As_2S_2 on fresh Ph^- mononuclear cells To compare the sensitivity of Ph^+ cells to the apoptotic effect of As_2S_2 with that of Ph^- cells, as the representative of Ph^- cells, the fresh mononuclear cells were collected respectively from bone marrow of ITP patients. Compared with CML Ph^+ leukemia cells, Ph^- mononuclear cells were less sensitive to As_2S_2 at any concentration mentioned previously (P<0.05). At As_2S_2 1 μmol/L, 3 μmol/L, and 5 μmol/L for 72 h, (4.5±1.3) %, (5.5±0.8) %, and (9.5±1.5) % cells showed signs of apoptosis, respectively. At As_2S_2 5 μmol/L for 24 h, 48 h, and 72 h, (3.2±1.8) %, (6.8±2.4) %, and (9.5± 1.5) % cells were underwent apoptosis, respectively (P<0.05, Tab 1).

Tab 1. The apoptotic effect of As_2S_2 on CML Ph^+ MNC and Ph^- MNC. *n*=3. Mean±SD. $^bP<0.05$ *vs* Ph^- MNC.

	Apoptotic cells/%	
	CML Ph^+ MNC	Ph^- MNC
Control	2.9±0.3	3.0±0.4
1 μmol/L (72 h)	3.4±0.6	4.5±1.3
3 μmol/L (72 h)	8.6±1.5^b	5.5±0.8
5 μmol/L (72 h)	23±4^b	9.5±1.5
5 μmol/L (48 h)	9.6±1.5^b	6.8±2.4
5 μmol/L (24 h)	8.2±0.5^b	3.2±1.8

DISCUSSION

CML is a leukemia characterized cytogenetically by the presence of Philadelphia chromosome (Ph^+) resulting in the generation of *bcr-abl* chimeric gene that encodes Bcr-Abl protein with elevated PTK activity. It accounts for about 20 % in all leukemias in adults[6-8]. One of the clinical features of CML is the presence of excessive number of mature myeloid cells in peripheral blood. The percentage of myeloblasts and promyelocytes is generally less than 10 % and the progenitors show no greater proliferative potency than normal. So, it is presumed that CML arises from apoptosis suppression rather than abnormal proliferation and differentiation[9,10]. Bcr-Abl is considered as a crucial factor in the pathogenesis of CML. Therefore, induction of apoptosis through targeting Bcr-Abl may be an effective therapy for CML.

Recently, it was reported that NB4 cells exposed to As_4S_4 could be induced to apoptosis. Induction of apoptosis was one of the major mechanisms of therapeutic effect of As_4S_4 for APL at therapeutic concentration. In 1970s, a compound containing As_2S_2 from Chinese traditional medicine termed *Niuhuang JieduPian* was shown effective for the treatment of CML by Shanghai Leukemia Research Cooperation Group and other clinician of the northern part of China, suggesting that As_2S_2 may be effective agent for the treatment of CML.

In this study, we found that K562 cells, a CML cell line with Bcr-Abl fusion protein could be induced to apoptosis by As_2S_2. In the studies on the mechanism of As_2S_2-induced apoptosis in K562 cells, we found that c-abl and Bcr-Abl protein were reduced during the process of apoptosis in K562 cells treated with As_2S_2 at concentration from 1 μmol/L to 5 μmol/L and the changes of PTK activity of c-Abl and Bcr-Abl was corresponded with that of protein content. However, the bcr-abl gene expression was unchanged, suggesting that Bcr-Abl was down-regulated by As_2S_2 at post-transcription level and the decline of PTK activity of Bcr-Abl might resulted from the decline of c-abl and Bcr-Abl protein. Furthermore, the time when the significant decline of Bcr-Abl occured was the time when apoptosis occurred, suggesting that the decline of Bcr-Abl protein played a role in the apoptosis induced by As_2S_2 in Ph^+ cells.

Similar apoptotic effect of As_2S_2 was also observed on primary CML mononuclear cells (Ph^+), and compared with CML mononuclear cells, fresh Ph^- mononuclear cells showed less sensitivity to As_2S_2 under the same condition. These data supported our presumption about the mechanism by which As_2S_2 induces apoptosis in K562 cells. It was reported[11] that Bcr-Abl mediated As_2O_3-induced apoptosis in Ph^+ CML and Ph^+ ALL (acute lymphocytic leukemia). We consider that the effect of As_2S_2 on Ph^+ CML is analogous to that of As_2O_3.

In summary, the results of the present studies suggest that As_2S_2-induced apoptosis in K562 was probably realized through degradation of Bcr-Abl protein and it could be used as a new therapeutic agent for CML.

REFERENCES

1 Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, *et al*. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996; 2: 561-6.

2 Deininger MW, Goldman JM, Lydon N, Melo JV. The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL-positive cells. Blood 1997; 90: 3691-8.

3 Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, *et al*. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med 2001; 344: 1038-42.

4 Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, *et al*. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001; 344: 1031-7.

5 Watzinger F, Gaiger A, Karlic H, Becher R, Pillwein K, Lion T. Absence of N-ras mutations in myeloid and lymphoid blast crisis of chronic myeloid leukemia. Cancer Res 1994; 54: 3934-8.

6 Morrison VA. Chronic leukemias. CA Cancer J Clin 1994; 44: 353-77.
7 Brincker H. Population-based age-and sex-specific incidence rates in the 4 main types of leukemia. Scand J Haematol 1982; 29: 241-9.
8 Call TG, Phyliky RL, Noel P, Habermann TM, Beard CM, O'Fallon WM, *et al*. Incidence of leukemia in Olmsted County, Minnesota, 1935 through 1989, with emphasis on changes in initial stage at diagnosis. Mayo Clin Proc 1994; 69: 315-8.
9 Fialkow PJ. Cell lineages in hematopoietic neoplasia studied with glucose-6-phosphate dehydrogenase cell markers. J Cell Physiol Suppl 1982; 1: 37-43.
10 McGahon A, Bissonnette R, Schmitt M, Cotter KM, Green DR, Cotter TG. BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. Blood 1994; 83: 1179-87.
11 Puccetti E, Guller S, Orleth A, Bruggenolte D, Hoelzer D, Ottmann OG, *et al*. BCR-ABL mediates arsenic trioxide-induced apoptosis independently of its aberrant kinase activity. Cancer Res 2000; 60: 3409-13.

As_2S_2 诱导 K562 细胞凋亡及其机制[1]

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关键词 砷剂；慢性髓样白血病；细胞凋亡；Bcr-Abl 融合蛋白质类

目的：探索 As_2S_2 对 K562 细胞的作用及其机制. **方法**：As_2S_2 对 K562 细胞的生长抑制作用用细胞计数法；细胞凋亡的检测用流式细胞分析、基因组 DNA 电泳、细胞形态学观察等方法；Western-blot 方法用于蛋白表达的检测；基因表达的变化用半定量 RT-PCR 方法. **结果**：As_2S_2 浓度在 1-5 μmol/L 作用 24-72 h 即可抑制 K562 细胞生长，大于 3 μmol/L 时可诱导K562细胞凋亡. As_2S_2能降低K562细胞中Bcr-Abl 蛋白水平及 c-abl 和 Bcr-Abl PTK 活性，但不调变 *bcr-abl* 基因表达水平. As_2S_2 也能诱导慢性粒细胞性白血病（CML）患者单个核细胞凋亡，且 Ph^+ 单个核细胞比 ph^- 单个核细胞对 As_2S_2 诱导的凋亡更敏感. **结论**：As_2S_2 可通过降低 Bcr-Abl 蛋白含量而诱导 CML 细胞凋亡. As_2S_2 可能为治疗 CML 的有效药物.

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