Synergic effect of 3′-azido-3′-deoxythymidine and arsenic trioxide in suppressing hepatoma cells

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The aim of this study was to investigate the synergic antitumor effects of arsenic trioxide (As_2O_3) and 3′-azido-3′-deoxythymidine (AZT) on hepatoma cells and explore the possible molecular basis of these effects. These results showed that AZT enhanced the inhibitory effect of As_2O_3 on HepG2 and SMMC-7721 cell growth. The IC_{50} of As_2O_3 in combination with AZT was lower than that of As_2O_3 alone. A concentration-dependent synergic effect of As_2O_3 and AZT (CI <1) was observed in all the tested combinations of these compounds. These results also showed that the combination of As_2O_3 and AZT dramatically and significantly increased the number of apoptotic cells in HepG2 and SMMC-7721 cells. Studies *in vivo* showed that the combination of As_2O_3 and AZT was statistically superior to either As_2O_3 or AZT alone in the treatment of tumor-bearing mice. As_2O_3 (1 mg/kg) containing AZT (50 mg/kg) inhibits proliferation of implanted hepatoma 22 by 56.35%. These results suggest that treating hepatoma with a combinination of As_2O_3 and AZT offers the advantages of reduced toxic side effects and improved therapeutic efficacy. To understand the mechanism through which As_2O_3 and AZT suppress tumors, we studied the effects of these compounds, both separately, and in combination, on telomerase and caspase-3 activity. The results showed that the growth inhibitory and apoptotic effects of As_2O_3 and AZT on human hepatoma cells could be related to the inhibition of telomerase and the activation of caspase 3. *Anti-Cancer Drugs* 22:435–443 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: arsenic trioxide, 3′-azido-3′-deoxythymidine, cancer treatment, caspase 3, oncology, telomerase

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors with almost 1 million patients dying from this neoplasm annually [1]. Although surgical intervention, when possible, can prolong survival rates and even cure some patients, unresectable hepatoma continues to pose a major clinical challenge.

The chemotherapeutic regimens currently in use for the treatment of HCC are either not effective enough to destroy the cancer cells or, cause significant side effects. Therefore, the development of new drugs and new combinations of existing drugs is desirable.

It has been shown that telomerase activity plays a key role in the development of HCC [2]. Telomerase is a cellular RNA-dependent DNA polymerase that serves to maintain the tandem arrays of telomeric TTAGGG repeats, specific DNA structures at the ends of chromosomes that prevent chromosome ends from being recognized as double-strand DNA breaks. Telomerase protects chromosomes from destabilizing agents, reversibly represses the transcription of neighboring genes, and plays a role in chromosome positioning in the nucleus [3,4]. Furthermore, telomerase is involved in multiple cellular processes, including cell differentiation, proliferation, and the inhibition of apoptosis. Tumorigenesis, drug resistance, and possibly DNA repair are also related to telomerase [5–8]. Its activity is upregulated in the vast majority of human tumors compared with normal somatic tissues [9]. Thus, telomerase is regarded as a promising target for the treatment of cancer [10].

Like many antitumor drugs, 3′-azido-3′-deoxythymidine (AZT), a powerful inhibitor of reverse transcriptase, seems to have a potential tumorigenic effect under certain circumstances [11,12]. When AZT has been used in phase I and II clinical trials, either alone, or in combination with other drugs in the treatment of gastrointestinal cancers, some cases of tumor regression have been reported [13,14].

Arsenic trioxide (As_2O_3), a newly found apoptosis inducer, has also been reported to have antiproliferative and apoptotic activities in some solid tumors, including human hepatoma and breast cancer [15–18]. However, this compound has not been widely used because of its toxicity and the resistance of cancer cells to it [19–23]. Therefore, this study was to investigate the effectiveness of a combination of AZT and As_2O_3 in the suppression of hepatoma cells in an attempt to find a better synergic

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combination therapy for hepatoma cells. This study describes the results of experiments designed to test the concentration-dependent synergic effect of this novel drug therapy, including its molecular basis. To our knowledge, this is the first report on the effects of the combination of AZT and As_2O_3 for hepatoma cells.

Materials and methods

Chemicals and reagents

As_2O_3, AZT, streptomycin, annexin V, propidium iodide (PI), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, Michigan, USA). Other cell culture supplies were purchased from GIBCO Laboratories (Grand Island, New York, USA). As_2O_3 and AZT were dissolved in phosphate-buffered saline (PBS), respectively, and the working concentrations were made by serial dilution of the stock solution in Dulbecco's modified Eagle's medium before use.

Animals and cell lines

BALB/c mice, 5–6 weeks of age and weighing 18–22 g, were obtained from the Animal Research center, Gansu College of Traditional Chinese Medicine, PR China and were raised in a clean room with controlled temperature ($22 \pm 1^{\circ}C$) and humidity (55%). The animals were free to drink tap water. All animal studies were done in accordance with institutional animal ethics approvals and guidelines. Human HCC HepG2, human liver cancer SMMC-7721, and Murine hepatoma H22 cell lines were purchased from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China.

Cell culture

The HepG2 and SMMC-7721 cells were cultured in Dulbecco's modified Eagle's medium with high glucose content containing 10% heat-inactivated fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.292 mg/ml of glutamine. The cells were incubated in complete medium at 37°C in a humidified atmosphere with 5% CO_2 in the air.

For the experiments, the cells were harvested from preconfluent cultures after two rinses with versene and a 10-min incubation with trypsin. The harvested cells were resuspended in a fresh medium. The cells were seeded in 96-well microtiter plates and allowed to attach for 20–24 h.

Cell proliferation assay

Drug treatment was initiated after the cells had begun attaching to the growth surface. The effects of As_2O_3 and AZT on HepG2 and SMMC-7721 cell survival were determined using the MTT reduction assay described earlier [22], with minor modifications. In brief, 5×10^3 cells were plated in 96-well plates in 100 μl of a regular medium per well. After 24 h growth, the cells were treated with As_2O_3 (0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 μmol/l) and AZT (5.0, 10.0, 20.0, 40.0, 80.0, and 160.0 μmol/l) for 24, 48, and 72 h. Untreated control and treated cells were incubated with 10 μl of MTT (5 mg/ml in serum-free medium) 4 h before the end of the incubation period. Fifty microlitre of lysis solution (10%SDS in 0.01 mol/l of HCl) was then added and another 24-h culture was made. The cell survival fraction was measured at absorbance (A) 490 nm on a microplate reader (Bio-RAD, Ultramark Microplate System, USA). Assays were carried out in triplicate. The inhibition rate was calculated as follows:

$$\text{inhibition rate}\,(\%) = 1 - \left(\frac{\text{Mean}\,A_{490}\,\text{of experiment}}{A_{490}\,\text{of control}}\right) \times 100\%.$$

IC_{50} and IC_{70} values were determined by the Forecast function using CurveExpert version1.37 (Published by Daniel G. Hyams).

Evaluation of interaction

HepG2 and SMMC-7721 cells were treated with the combination of 5.0, 10.0, and 15.0 μmol/l of AZT (approximately 1/4, 2/4, and 3/4 of IC_{50}) and As_2O_3 (0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 μmol/l), respectively, for 72 h. The sequential process was similar to that of the cell proliferation assay as described above.

Synergy determination

The synergic effect between AZT and As_2O_3 was analyzed using the combination index method [23]. Concentration effect curves calculated for each drug, both separately, and in combination, were applied to determine the amount of each drug, either separately, or in combination, required to achieve a given level of effect. The combination index (CI) was calculated as follows.

$$CI = IC_{A,B}/IC_A + IC_{B,A}/IC_B$$

IC_A and IC_B are the concentrations of A and B needed to produce a given level of cytotoxicity when used separately, whereas $IC_{A,B}$ and $IC_{B,A}$ are the concentrations needed to produce the same effect when used in combination. A CI value of 1 indicates an additive interaction, values less than 1 indicate a synergic action, and values greater than 1 indicate antagonistic interaction [24].

Quantitation of apoptotic cells

Detection of apoptotic cells was carried out by annexin V-fluorescein isothiocyanate /PI staining. The cells were plated onto six-well plates (1×10^5 cells/well) and grown overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As_2O_3 (2 μmol/l), AZT (10 and 15 μmol/l), or As_2O_3 + AZT (10 or 15 μmol/l) for 72 h. At the end of the treatment, the cells were harvested, washed once in PBS and labeled with annexin V-fluorescein isothiocyanate and PI (2 mg/ml) according to the manufacturer's protocol. The samples were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, California, USA) and analyzed with

CellQuest Software (Becton Dickinson). The amount of early apoptosis was determined as the percentage of annexin V^+/PI^-.

Determination of telomerase activity

Telomerase activity was measured by a Telomeric Repeat Amplification Protocol (TRAP) assay using a TRAP-ELISA kit (Sino-American biological company, Shanghai, PR China). Cell extracts were obtained from 2×10^5 cells lysed with 200 µl lysis buffer, incubated on ice for 30 min, and centrifuged at 16 000 *g* at 4°C for 20 min. Protein concentration was determined by the Coomassie protein assay. Each TRAP reaction contained 1 mg of total protein. The Telomerase substrate primer was elongated by telomerase at 25°C for 30 min, and then incubated at 94°C for 5 min to induce telomerase inactivation. The reaction mixture was then amplified by 30 cycles of polymerase chain reactions at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s, and finally at 72°C for 10 min. The polymerase chain reactions products of 5 µl each were analyzed according to the manufacturer's instructions. The absorbance (*A*) of telomerase was measured at a wavelength of 450 nm. All experiments were repeated at least three times.

Analysis of intracellular caspase-3 activity

Intracellular caspase-3 activity was measured using a caspase-3 cellular assay kit. Briefly, cells were treated with AZT and (or) As_2O_3 for 72 h, collected by centrifugation, washed twice with PBS and resuspended in cell lysis buffer (50 mmol/l of HEPES, pH 7.4, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 mmol/l of dithiothreitol, and 0.1 mmol/l of EDTA). The cells were then centrifuged again at 10 000*g* at 4°C for 10 min and the supernatant was used in the following assay. The supernatant (10 µl) was mixed with 80 µl assay buffer (50 mmol/l of HEPES, pH 7.4, 100 mmol/l of NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mmol/l of dithiothreitol, 1 mmol/l of EDTA and 10% glycerol) in each well of a microplate and incubated at 37°C for 10 min. Finally, a 10-µl Ac-DEVD-pNA substrate was added. The absorbance at 405 nm was measured using a microplate reader (Bio-RAD, Ultramark Microplate System, Hercules, California, USA). The results were expressed as absorbance (A_{405}).

The effect of As_2O_3 or/and AZT on growth of hepatoma 22 cells in tumor-bearing mice

Hepatoma 22 (H22) cells were allowed to replicate for two generations in the abdomen of the mice. The cell solution during the logarithmic growth phase had a density of 1.0×10^7 cells/ml. Forty BALB/c mice were then implanted subcutaneously in the right groin with 0.2 ml of H22 cells. After 7 days, when tumors of 2–3 mm in diameter formed in the right groin of these mice, they were randomly divided into four groups, 10 mice in each group. The mice in the four experimental groups were, respectively, administered with 0.9% NS, As_2O_3 (1 mg/kg), AZT (50 mg/kg), and As_2O_3 (1 mg/kg) containing AZT (50 mg/kg) intraperitoneally, once a day, from day 1 to day 14. On day 14, all mice were killed and the tumors were cut and weighed.

Statistical analysis

Data were expressed as means ± standard deviation (SD), and statistical comparisons were made using analysis of variance followed by Student's *t* test.

Results

The effects of As_2O_3 and AZT on HepG2 and SMMC-7721 cell survival

Cell proliferation was evaluated using the MTT reduction assay after treatment for 24, 48, and 72 h with six different concentrations of either As_2O_3 or AZT. The inhibition of cell growth was significant compared with control. The results indicate a dose-dependent and time-dependent increase in the inhibition of cell proliferation (Fig. 1). The IC_{50} values for As_2O_3 in HepG2 cells at 24, 48, and 72 h were 38.87, 23.25, and 8.65 µmol/l, respectively, and the IC_{50} values for As_2O_3 in SMMC-7721 cells at 24, 48, and 72 h were 28.56, 24.49, and 7.61 µmol/l, respectively. For AZT, the IC_{50} values in HepG2 cells at 24, 48, and 72 h were greater than 1000, greater than 800, and 20.40 µmol/l, and the IC_{50} values in SMMC-7721cells at 24, 48 and 72 h were greater than 1000, greater than 800, and 18.19 µmol/l, respectively. For further experiments, a 72-h time point was chosen.

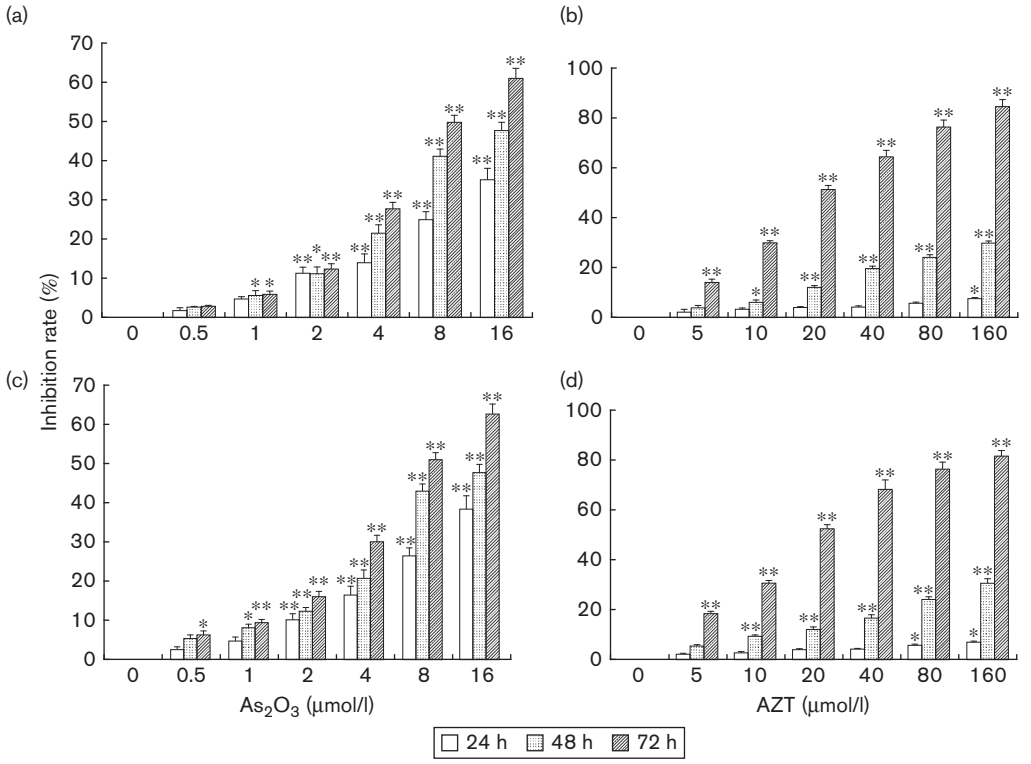
The combined effects of As_2O_3 and AZT

As both As_2O_3 and AZT were effective in inhibiting cell proliferation, we next examined the interactive effects of As_2O_3 and AZT on cell growth inhibition at 72 h. The cells were simultaneously treated with 5.0, 10.0, and 15.0 µmol/l AZT combined with six different concentrations of As_2O_3 for 72 h. As shown in Fig. 2, AZT enhanced the inhibitory effect of As_2O_3 on HepG2 and SMMC-7721 cell growth. The IC_{50} of As_2O_3 in combination with AZT was lower than that of As_2O_3 alone. Tables 1 and 2 summarize the IC_{50} values.

Analysis of synergy between As_2O_3 and AZT

The cells were treated with As_2O_3, with or without AZT for 72 h. Tables 1 and 2 summarize the resultant IC_{50} values. The CI values were below 1.0, indicating synergy between As_2O_3 and AZT. As shown in Tables 1 and 2, the synergic effect ($CI < 1$) was observed in all of the examined combinations of As_2O_3 and AZT. The synergy level (equal to the inverse value of combination index) was greater at higher drug effect levels.

Fig. 1



Effects of arsenic trioxide (As_2O_3) and 3′-azido-3′-deoxythymidine (AZT) on growth inhibition in hepatocellular carcinoma HepG2 and human liver cancer SMMC-7721 cell lines: HepG2 (a and b), SMMC-7721 (c and d). Compared with control of the same time point. $^*P<0.05$, $^{**}P<0.01$.

The effects of As_2O_3 or/and AZT on HepG2 and SMMC-7721 cell apoptosis

Considering that the true apoptotic cells should be those cells which exclude PI and exhibit phosphatidylserine on the outside of the plasma membrane, we only count the early apoptotic cells. Exposure of the cells to the combination of As_2O_3 (2 μmol/l) and AZT (10 μmol/l) for 72 h synergically induced apoptosis in HepG2 (21.27 ± 3.83%) and SMMC-7721 (17.39 ± 3.35%), respectively, compared with exposure of the cells to either agent alone (Tables 3 and 4). A similar effect was observed when they were treated in the absence (control) or presence of As_2O_3 (2 μmol/l), AZT (15 μmol/l), or As_2O_3 + AZT for 72 h, with HepG2 being 27.65 ± 3.14%, SMMC-7721 being 23.19 ± 3.18%, respectively (Tables 3 and 4). All these findings indicated that AZT could synergically potentiate As_2O_3 to induce apoptosis in both of the cell lines.

Effect on telomerase activity in HepG2 and SMMC-7721 cells

As shown in Fig. 3, treatment with AZT for 72 h resulted in a significant inhibition of telomerase activity. Low-dose As_2O_3 had little inhibitory effect on telomerase activity at both HepG2 and SMMC-7721 cells. However, As_2O_3 + 10.0 μmol/l of AZT significantly increased the inhibition of telomerase activity compared with 10.0 μmol/l of AZT alone. The inhibition of telomerase activity by AZT and As_2O_3 + 10.0 μmol/l of AZT was concentration dependent.

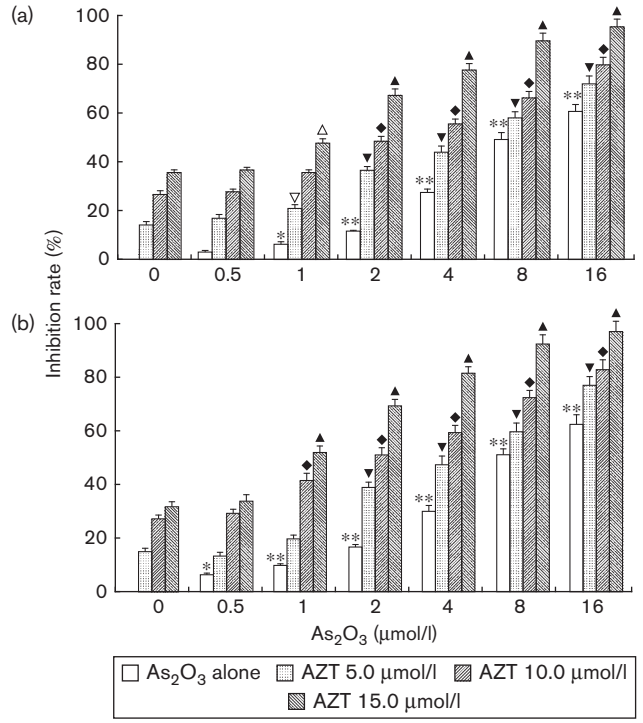
Effect on caspase-3 activity in HepG2 and SMMC-7721 cells

The caspase-3 activity of HepG2 cells significantly increased after treatment with As_2O_3. However, treatment with As_2O_3 + 10.0 μmol/l of AZT resulted in significantly higher caspase-3 activity compared with treatment with As_2O_3 alone. These results suggest that the combination of AZT and As_2O_3 had a synergic effect in increasing caspase-3 activity in human hepatoma cells. The induction of caspase-3 activity by As_2O_3 or As_2O_3 + AZT was concentration dependent (Fig. 4).

Inhibition effect of As_2O_3 containing AZT on growth of H22 cells *in vivo*

These in-vivo studies showed that the combination of As_2O_3 and AZT was statistically superior to either As_2O_3 or AZT alone in the treatment of tumor-bearing mice. As_2O_3 (1 mg/kg) combined with AZT (50 mg/kg) inhibits the proliferation of implanted H22 by 56.35% (Table 5).

Fig. 2



The combined effects of arsenic trioxide (As_2O_3) and 3′-azido-3′-deoxythymidine (AZT) on growth inhibition in hepatocellular carcinoma HepG2 and human liver cancer SMMC-7721 cell lines: HepG2 (a), SMMC-7721 (b). Cells were treated with As_2O_3, AZT, and As_2O_3 + AZT for 72 h, respectively. The IC_{50} values are shown in Tables 1 and 2. Compared with As_2O_3 control, $^*P<0.05$, $^{**}P<0.01$; compared with AZT (5 μmol/l) alone, $^{\triangledown}P<0.05$, $^{\blacktriangledown}P<0.01$; compared with AZT (10 μmol/l) alone, $^{\blacklozenge}P<0.01$; compared with AZT (15 μmol/l) alone, $^{\triangle}P<0.05$, $^{\blacktriangle}P<0.01$.

Table 1 Synergic effect of different combinations of AZT and As_2O_3 on the growth of HepG2 cells at 72 h

AZT (μmol/l)	As_2O_3 IC_{50} or IC_{70} (μmol/l)	Combination index (CI)	Synergy fold
50% effect			
0.0	8.65	Not applicable	Not applicable
5.0	5.33	0.86	1.16
10.0	2.61	0.79	1.27
15.0	1.06	0.86	1.16
70% effect			
0.0	25.35	Not applicable	Not applicable
5.0	14.67	0.67	1.49
10.0	10.30	0.57	1.75
15.0	2.42	0.36	2.78

A combination index of less than 1 indicates synergy, and its inverse value indicates the extent of synergy. Results at two effect levels, that is, 50 and 70%, are shown. IC_{50} and IC_{70} values of AZT were 20.40 and 55.80 μmol/l.
As_2O_3, arsenic trioxide; AZT, 3′-azido-3′-deoxythymidine.

Discussion

As_2O_3 has proven to be a highly effective agent in acute promyelocytic leukemia therapy [25–31]. More recent in-vitro experiments indicated that As_2O_3 might also be effective on solid tumors such as lung cancer, pancreatic cancer, and breast cancer etc [32–34]. However, higher concentrations of As_2O_3 were required to show the anticancer effect on malignant solid tumors compared with hematopoietic cancers. As high concentrations of As_2O_3 have been associated with clinical risks [35], the use of lower doses of As_2O_3, in combination with other agents, may be a better option for treating malignant solid tumors.

Table 2 Synergic effect of different combinations of AZT and As_2O_3 on the growth of SMMC-7721 cells at 72 h

AZT (μmol/l)	As_2O_3 IC_{50} or IC_{70} (μmol/l)	Combination index (CI)	Synergy fold
50% effect			
0.0	7.73	Not applicable	Not applicable
5.0	4.72	0.89	1.12
10.0	1.99	0.81	1.23
15.0	1.09	0.97	1.03
70% effect			
0.0	26.49	Not applicable	Not applicable
5.0	12.91	0.59	1.69
10.0	7.32	0.49	2.04
15.0	2.05	0.40	2.50

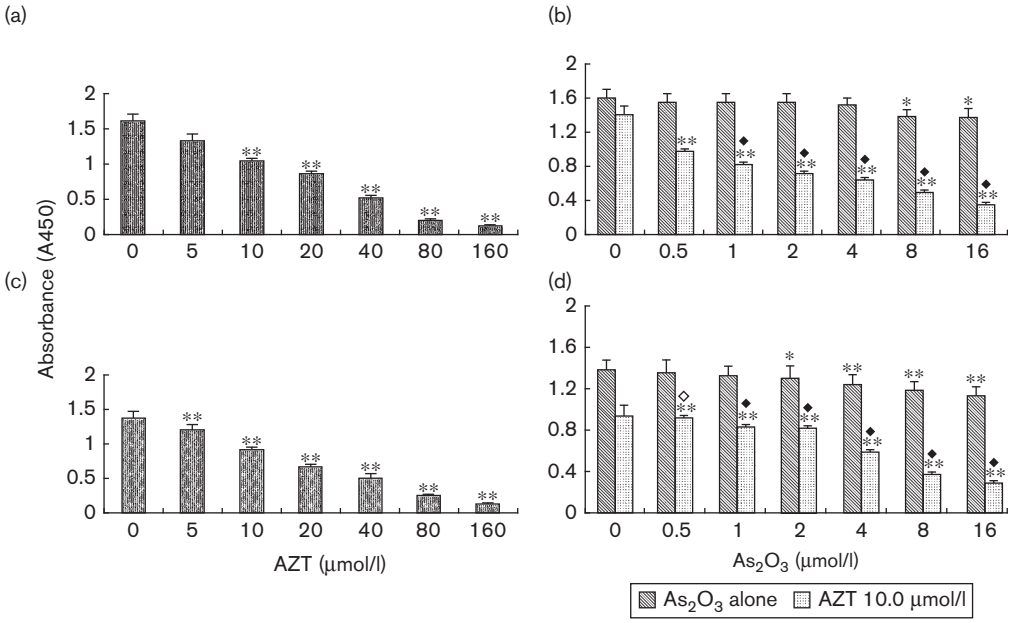
A combination index of less than 1 indicates synergy, and its inverse value indicates the extent of synergy. Results at two effect levels, that is, 50 and 70% are shown. IC_{50} and IC_{70} values of AZT were 18.19 and 46.51 μmol/l.
As_2O_3, arsenic trioxide; AZT, 3′-azido-3′-deoxythymidine.

The selective expression of telomerase in tumor cells makes it an attractive therapeutic target. AZT can inhibit telomerase activity and proliferation of cancer cells [36,37]. Some researchers reported that the effect of tumor chemotherapy was more obvious when AZT was combined with drugs for chemotherapy. AZT may act as a synergist of drug for chemotherapy [38,39].

For AZT in this study, the IC_{50} values in HepG2 cells at 24, 48 and 72 h were greater than 1000, greater than 800, and 20.40 μmol/l, and the IC_{50} values in SMMC-7721cells at 24, 48 and 72 h were greater than 1000, greater than 800, and 18.19 μmol/l, respectively. Therefore, 5.0, 10.0, and 15.0 μmol/l of AZT (about 1/4, 2/4 and 3/4 of IC_{50}) were chosen for these studies because they are well below the maximum plasma concentrations observed (600 μmol/l) after the administration of 1.5–7 g/m^2 of AZT over 2 h in patients with advanced cancer [40]. Our results indicate a concentration-dependent synergic effect ($CI < 1$) between As_2O_3 and AZT in all the combinations of these drugs that were examined, which increased with the level of drug effect. Furthermore, the addition of 10.0 μmol/l of AZT reduced significantly the IC_{50} values of As_2O_3 in HepG2 (from 8.65 to 2.61 μmol/l) and SMMC-7721 (from 7.73 to 1.99 μmol/l) cells. As the effective dosage of As_2O_3 in clinical application is 20 mg/m^2, this means that less than 6.0 mg/m^2 of As_2O_3 given in combination with AZT is equally as effective as 20 mg/m^2 of As_2O_3 given in isolation.

Apoptosis is important for the development and homeostasis of multicellular organisms. Specific therapies have been designed to enhance the susceptibility of human cancers to apoptosis. It has been reported that inhibition

Fig. 3



Effect on telomerase activity in hepatocellular carcinoma HepG2 and human liver cancer SMMC-7721 cell lines: HepG2 (a and b), SMMC-7721 (c and d). Compared with control, $*P<0.05$, $**P<0.01$; compared with the same dose of arsenic trioxide (As_2O_3) alone, $P<0.05$, $\blacklozenge P<0.01$. AZT, 3′-azido-3′-deoxythymidine.

Table 3 Effect of As_2O_3 and AZT on apoptosis of HepG2 cells for 72 h (Mean ± SD)

Group	Dose (μmol/l)	Percentage of apoptotic cells (%)
Control	–	2.04 ± 0.22
As_2O_3	2.0	5.27 ± 1.17
AZT	10.0	7.17 ± 1.46**
AZT	15.0	12.58 ± 2.08**
As_2O_3 + AZT	2 + 10.0	21.27 ± 3.83**,***,†
As_2O_3 + AZT	2 + 15.0	27.65 ± 3.14**,***,‡

As_2O_3, arsenic trioxide; AZT, 3′-azido-3′-deoxythymidine; SD, standard deviation. Compared with control, $**P<0.01$; compared with As_2O_3 alone, $***P<0.01$; compared with AZT (10 μmol/l), $^{\dagger}P<0.01$; compared with AZT (15 μmol/l), $^{\ddagger}P<0.01$.

Table 4 Effect of As_2O_3 and AZT on apoptosis of SMMC-7721 cells for 72 h (Mean ± SD)

Group	Dose (μmol/l)	Percentage of apoptotic cells (%)
Control	–	2.19 ± 0.16
As_2O_3	2.0	6.04 ± 0.89*
AZT	10.0	6.35 ± 1.77*
AZT	15.0	11.49 ± 2.65**
As_2O_3 + AZT	2 + 10.0	17.39 ± 3.35*,***,†
As_2O_3 + AZT	2 + 15.0	23.19 ± 3.18*,**,‡

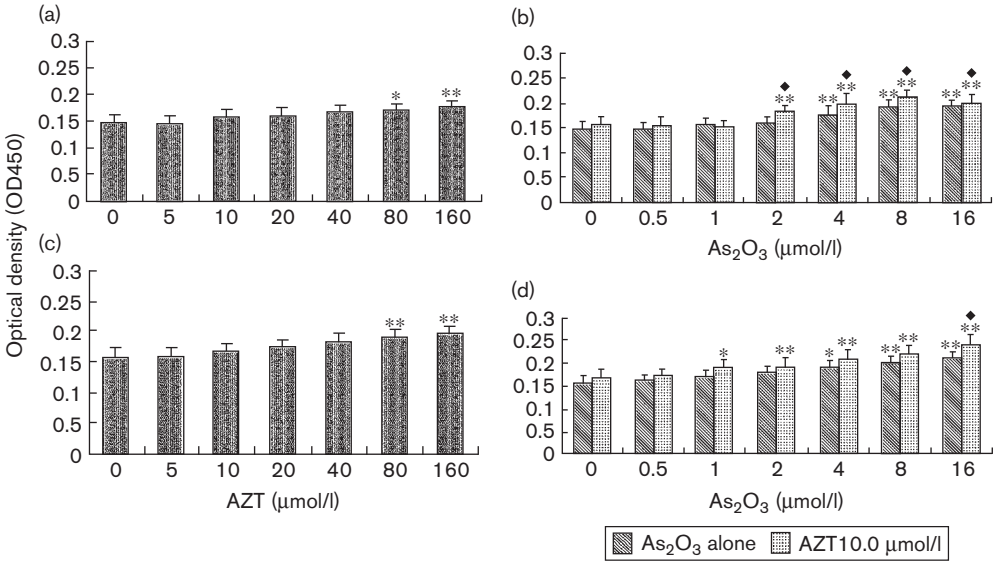
As_2O_3, arsenic trioxide; AZT, 3′-azido-3′-deoxythymidine; SD, standard deviation. Compared with control, $*P<0.05$, $**P<0.01$; compared with As_2O_3 alone, $***P<0.01$; compared with AZT (10 μmol/l), $\dagger P<0.01$; compared with AZT (15 μmol/l), $\ddagger P<0.01$.

of telomerase increases the susceptibility of tumor cells to apoptosis induced by anticancer agents. Our results showed that As_2O_3 + 10.0 μmol/l of AZT significantly increased the inhibition of telomerase activity in HepG2 and SMMC-7721 cells compared with 10.0 μmol/l of AZT alone. These results also showed that the combination of As_2O_3 and AZT dramatically and significantly increased the number of apoptotic cells in HepG2 and SMMC-7721. All these findings indicated that combining As_2O_3 with AZT offers the dual advantages of reducing toxic side effects while at the same time improving therapeutic efficacy.

The therapeutic effects of arsenic are mainly dependent on its ability to induce cell cycle arrest and induction of apoptosis [41–46]. Some of the apoptotic effects of arsenic are attributed to its ability to upregulate caspases [47–49]. Caspase 3 is a member of the caspase family of aspartate-specific cysteine proteases that play a central role in the execution of programmed cell death [50–53]. Our results indicated that AZT enhanced the positive effect of As_2O_3 on caspase-3 activity in HepG2 and SMMC-7721 cells. However, it is surprising that 10 or 15 μmol/l of AZT alone, and 2 μmol/l of As_2O_3 alone, caused the two types of cell apoptosis to some extent, but not caspase-3 activation. This contradiction suggested that AZT and As_2O_3 may also promote apoptosis of HepG2 and SMMC-7721 cells through a caspase-3-independent pathway. The reason needs further investigation.

Earlier studies have shown that effective dosages of As_2O_3 used to treat solid tumors in mice range from 2 to 6.5 mg/kg [54–57]. Such dosages are considerably higher (approximately 12–40-fold) than the standard dosage of 0.16 mg/kg used to treat human patients with acute promyelocytic leukemia over a period of 6 weeks [28,58].

Fig. 4



Effect on caspase-3 activity in hepatocellular carcinoma HepG2 and human liver cancer SMMC-7721 cell lines: HepG2 (a and b), SMMC-7721 (c and d). Compared with control, $*P<0.05$, $**P<0.01$; compared with the same dose of arsenic trioxide (As_2O_3) alone. $\blacklozenge P<0.01$. AZT, 3′-azido-3′-deoxythymidine.

Table 5 The effect of As_2O_3 or/and AZT on growth of H22 cells in tumor-bearing mice (Mean ± SD)

		Body weight (g)			
Group	Dose (mg/kg)	Pretreated	Additional weight	Tumor weight (mg)	Inhibition rate (%)
NS	0	20.25 ± 1.48	9.78 ± 1.12	3.63 ± 0.53	
As_2O_3	1	19.87 ± 1.17	6.25 ± 0.51*	2.42 ± 0.43*	39.23
AZT	50	19.21 ± 1.22	7.32 ± 0.58*	2.83 ± 0.49	21.27
As_2O_3 + AZT	–	20.39 ± 1.27	6.10 ± 0.82*	1.58 ± 0.31**,***,†	56.35

As_2O_3, arsenic trioxide; AZT, 3′-azido-3′-deoxythymidine; NS, normal saline; SD, standard deviation.
Compared with control, $*P<0.05$, $**P<0.01$; compared with As_2O_3 alone, $***P<0.05$; compared with AZT alone, $^{\dagger}P<0.01$.

Dosages ranging from 1 to 2 mg/kg are appropriate for treating HCC tumors in mice, as tumor growth is suppressed and the side effects are endurable [59].

Our studies *in vivo* showed that the combination of As_2O_3 and AZT was statistically superior to either As_2O_3 or AZT alone in the treatment of tumor-bearing mice. As_2O_3 (1 mg/kg) containing AZT (50 mg/kg) inhibits proliferation of implanted H22 by 56.35%. However, it is not known whether the results are translatable to the effects of As_2O_3 in patients with cancer, but the fact that lower doses of As_2O_3 could be effectively used if As_2O_3 was to be combined with AZT, warrants follow-up studies in humans.

In summary, our studies show a synergiic interaction between As_2O_3 and AZT in the treatment of hepatoma. This has the advantages of reducing toxic side effects while improving therapeutic efficacy. Further research is required to show the molecular mechanism through which As_2O_3 and AZT inhibit telomerase and activate caspase 3.

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