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Increasing sensitivity to arsenic trioxide-induced apoptosis by altered telomere state

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Abstract

In this work, we investigated the synergic effects between low-dose arsenic trioxide and diethyloxadicarbocyanine (DODC), a telomerase inhibitor, on cell apoptosis. Results revealed that low-dose arsenic could block cell cycle arrest at the G2/M phase and induce apoptosis, whereas DODC could block cell cycle arrest at the G0/G1 phase but not induce apoptosis. However, cells pretreated with DODC showed greater sensitivity to arsenic than untreated cells. The percentage of apoptosis produced by combination treatment with the two agents increased and that was similar to the effect of high-dose arsenic treatment alone. Further studies showed that DODC alone could induce hairpin G-quadruplex formation and inhibit telomerase activity in a dose-dependent manner. Compared with HT1080 cells, 293 cells were more sensitive to cell growth inhibition and apoptosis and were less sensitivity to telomerase activity. These results indicate that DODC can synergistically enhance the apoptosis induced by arsenic, suggesting the increased cell senescence in response to arsenic is induced by an altered telomere state rather than by a loss of telomerase. Thus clinical application of combination treatment with arsenic and telomerase inhibitor may have potential in cancer therapy.
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Keywords: Apoptosis; Arsenic trioxide; DODC (diethyloxadicarbocyanine); Cell cycle; Sensitivity; Telomere

1. Introduction

Arsenic trioxide (As_2O_3), a compound present in numerous traditional Chinese medicines has been used to treat several human diseases, such as anemia, dyspepsia and some tumors. In the last decade, the efficiency of arsenic trioxide in both newly diagnosed and relapsed patients with acute promyelocytic leukemia has been established (Zhang et al., 1996; Shen et al., 1997; Soignet et al., 2001). Currently, numerous clinical trials are under way with hematopoietic malignancies and solid tumors, and great success with cultured cell lines has been achieved. Such studies demonstrated that the compound works by inducing cells to go into apoptosis (Chen et al., 1996a; Zhang et al., 1999; Chou et al., 2001). Recent evidence also suggests that the formation of reactive oxygen species is involved in apoptosis induced by arsenite (Liu et al., 2001; Chen et al., 2002), and arsenic exerts its function by some protein kinase, caspases, and intracellular glutathione system (Akao et al., 2000; Zhang and Cao, 2002). These findings put the traditional Chinese remedy squarely in the mainstream of modern chemotherapy research (Mervis, 1996).

However, one critical factor that limits the utility of As_2O_3 may be its cytotoxicity to normal tissue because it is a well-known poison. We should still take its toxicity into account when emphasizing its anticancer effect. This means that seeking a way to reduce its toxicity is both important and necessary. In general, decreasing its toxicity and improving its anticancer effect might depend on two approaches. First, it is necessary to find out how sensitive tumor cells are to arsenic in order to reduce its dose. Second, the way a drug is given can reduce its systemic effect. Recently, some studies demonstrated that the potential exists for synergism with other agents to provide enhanced therapeutic benefits. The combination of all-transretinoic acid and arsenic trioxide has been reported to accelerate tumor regression by enhancing both differentiation and apoptosis in some models (Jing et al., 2001). Synergy between arsenic trioxide and vitamin C has been shown in vitro and in vivo by several groups (Grad et al., 2001; Bachleitner-Hofmann et al., 2001). The profound increase in arsenic sensitivity in

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vitro is associated with glutathione depletion by buthionine sulfoximine (Gartenhaus et al., 2002). Combined arsenic trioxide and cAMP during maturation of acute promyelocytic leukemia cells submits novel signal cross-talk (Zhu et al., 2002). These data have renewed interest in arsenic as anticancer agents.

Telomeres are essential for chromosome stability and regulate the replicative life-span of somatic cells (Klapper et al., 2001). Telomerase may serve as an important target for anticancer drugs. Telomere dysfunction triggers developmentally regulated cell apoptosis (Hemann et al., 2001). The 3′ end of each telomere consists of a single stranded G-rich overhang that has been proposed to stabilize a loop structure at the end of chromosomes (Griffith et al., 1999; Chen et al., 2001). Telomere length can be maintained by telomerase (Greider, 1996). Telomerase activity has been found in 85% of human cancers, including stomach, breast, colon, and in immortalized cell lines, but not in normal tissue (Kim et al., 1994; Hiyama et al., 1995, 1996; Chadeneau et al., 1995). Recent experiments also suggest that replicative senescence in primary cells is induced by an altered telomere state and not by telomere loss (Kariseder et al., 2002), and apoptosis is induced by an altered telomere 3′ overhang-specific DNA structure (Eller et al., 2002). However, how these events play a role in low-level arsenic-mediated cell apoptosis is not known.

It has been reported that 3, 3′-diethyloxadicarbocyanine (DODC), a telomerase inhibitor, can induce hairpin G-quadruplex formation in telomere DNA (Chen et al., 1996b). There is a different telomeric DNA length in 293 cells (transformed embryonic kidney cells) and HT1080 cells (fibrosarcoma). To investigate the effects of telomeric structure on cell apoptosis induced by low-level arsenic and to open new strategies for improving chemotherapy, we used the above two types of solid malignancy cell lines as a model to address the synergetic effects of arsenic and DODC on cell growth and apoptotic death. Results showed that cells pretreated with DODC showed a greater sensitivity to arsenic than did untreated cells. The percentage of apoptosis produced by the two agents increased, suggesting that the increased cell senescence in response to arsenic is induced by an altered telomere state rather than by a loss of telomerase. DODC decreased the dose of arsenic needed for treatment and may have potential in cancer therapy.

2. Materials and methods

2.1. Reagents

Arsenic trioxide, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and 3, 3′-diethyloxadicarbocyanine (DODC), and propidium iodide were purchased from Sigma (St. Louis, MO, USA). All the oligodeoxynucleotides were obtained from SBS Genetech. Purification and desalination were performed by reverse high performance liquid chromatography (HPLC) (C-18). Purity was determined from the UV spectrum. Oligodeoxynucleotides were dissolved in a 1 mM Tris–HCl (pH = 7.5) buffer containing a certain concentration of NaCl.

2.2. Cell culture and treatment

The 293 (human transformed embryonic kidney cells, ATCC No. CRL-1573) cells and HT1080 (human fibrosarcoma, ATCC No. CRL-121) cells were maintained in monolayers in tissue culture flasks in PRMI1640 supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were used for experiments. Immediately prior to experimental treatment, medium was replaced and arsenic in concentrations from 1 to 100 μM was added to the medium. Telomerase inhibitor was added 1 h beforehand and remained in the medium during the rest of the experiment.

2.3. MTT cytotoxicity assay

The viability of cells plated on culture dishes was measured by adding MTT reagent, which was dissolved in phosphate-buffered saline (PBS), to a final concentration of 0.5 mg/ml (Mossman, 1983). Briefly, about 5000 cells/pore were seeded in 96-well plates and for preincubated 24 h. The drug was added to a final concentration ranging from 1 to 100 μM for 24 or 48 h. Following culture, the cells were incubated with MTT reagent. Four hours later, 200 μl dimethyl sulfoxide (DMSO) was added to dissolve the formazan product. The plate was then incubated at 37 °C for another 30 min. The absorbance at 570 nm was measured. Percentage of cell growth inhibition is expressed as: $(A - B)/A \times 100\%$, where A is the absorbance value from control groups and B is the absorbance value from experimental groups.

2.4. Fluorescence microscopy

The cells were seeded at a density of 3×10^4 cells/cm^2 and preincubated for 24 h. Following experimental treatments, cells were collected and washed twice with cold PBS, fixed 20 min, and then stained with the fluorescent DNA binding dye Hoechst 33342. Hoechst-stained cells were visualized and photographed under fluorescent microscopy. Apoptotic cells were determined by evaluating the nuclear morphology, using a fluorescence microscope (NIKON Inverted Microscope DIAPHOT 300).

2.5. Flow cytometry

Flow cytometry measurements were carried out according to the methods described by Ishibashi and Lippard

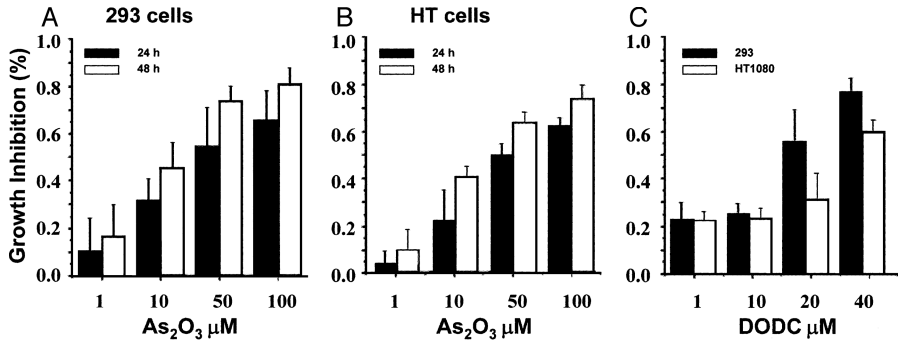

Fig. 1. (A) and (B) show dose- and time-dependent effects of As_2O_3 on the growth of 293 and HT1080 cell lines. (C) presents inhibitory effects of DODC alone. Data indicate means ± S.D.

(1998). About 1×10^6 cells from control and treated dishes were collected, washed twice with cold PBS, and fixed in 70% ethanol overnight. Then the cells were incubated in 1 ml PBS containing 50 μg/ml propidium iodide and 250 μg/ml RNase at 37 °C for 30 min to stain the DNA and to eliminate RNA. The fluorescence intensity was measured using a Coulter EPICS XL Flow cytometer. Percentage of cells in sub-G1 phase was regarded as the percentage of apoptotic cells. The cell cycle distribution of cells was also determined from the fluorescence of individual cells measured by flow cytometry (FACSSVantage, BD, USA).

2.6. DNA laddering

Low molecular size DNA was isolated according to the methods of Zhang et al. (1999). Briefly, about 10^6 cells were collected and washed three times with PBS. The pellets were resuspended in 400 μl of lysis buffer containing 150 mM NaCl, 10 mM Tris–HCl at pH 8.0, 20 mM EDTA at pH 8.0, and 0.5% sodium dodecyl sulfate. After mixing, proteinase K (final concentration 20 ng/ml) was added and incubated at 50 °C for 4 h. DNA was precipitated with two volumes of ethanol at −20 °C overnight, and collected by centrifugation (12,000 × *g*) DNA samples were dissolved in 20–30 μl TE buffer (10 mM Tris, 1 mM EDTA), and treated with Rnase (20 mg/ml) at 37 °C for 1 h. Total DNA was analyzed using 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide, and the DNA fragments were visualized using ultraviolet light and photographed.

2.7. Telomerase activity assay

Telomerase activity was assayed using the polymerase chain reaction (PCR)-based telemetric repeat amplification protocol (TRAP) assay as previously described (Broccoli et al., 1995). The cells were collected as pellets after centrifugation at 1000 × *g* for 5 min at 4 °C. The pellets then were washed and lysed in 1, 3-((3-cholamidopropy) dimethylammonio)-1-propanesulfonic acid (CHAPS, Sigma) buffer, incubated on ice for 30 min, and the lysates were centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatants were collected and the protein concentration was measured. Each of the TRAP reactions contained 5 μg of total protein, 10 pmol forward TS primer, 10 mM dNTPs, 2.5U Taq DNA polymerase, 10× buffer, and 20 mM $MgCl_2$ to a final volume of 50 μl; the reaction mixture was incubated 30 min at 37 °C. Then 10 pmol reverse CX primer was added before PCR. PCR was performed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s. Samples were separated by 10% polyacrylamide gel electrophoresis and stained with $AgNO_3$.

2.8. Spectroscopic analysis of hairpin quadruplexes (Chen et al., 1996b)

Oligodeoxynucleotides were dialyzed against 1 mM Tris–HCl (pH 7.5) and stored at −20 °C in the same buffer. A stock solution of DODC was prepared in methanol. Stock solutions of Tris–HCl buffer, dye, salt (NaCl), and oligodeoxynucleotide were mixed to give the desired dye concentrations. Final buffer concentrations were 10 mM Tris–

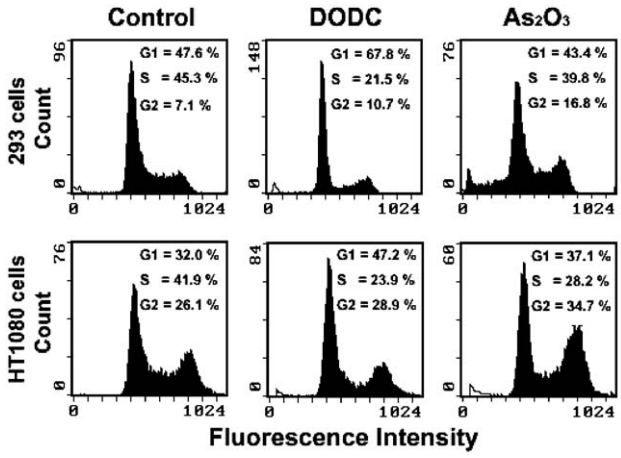

Fig. 2. Flow cytometric analysis of cellular DNA content in control and arsenic- or DODC-incubated 293, or HT1080 cell lines, stained with propidium iodide.

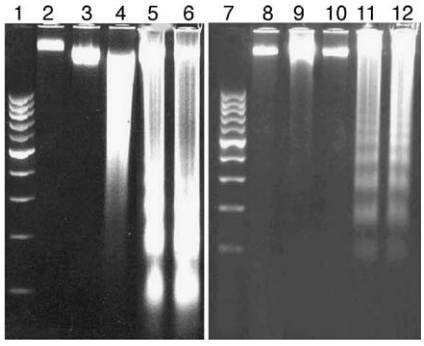

Fig. 3. DNA ladder formation. Cells (293 for lanes 1–6, HT1080 for lanes 7–12) were incubated with As_2O_3 alone (10 or 100 μM), DODC alone (20 μM) or with a combination of both As_2O_3 (10 μM) and DODC (20 μM) for 24 h. Lanes 1 and 7: marker; lanes 2, 8: control; lanes 3, 9: 20 μM DODC alone; lanes 4, 10: 10 μM As_2O_3 alone; lanes 5, 11: 1-h preincubation of DODC combined with 10 μM As_2O_3; lanes 6 and 12: 100 μM As_2O_3 alone.

HCl and 150 mM NaCl (pH 7.5). The dye concentration was less than 6 μM, while the concentration of DNA samples ranged from 10 to 36 μM strand. The UV experiments were carried out on a Hitach U-3200 spectrophotometer.

3. Results

3.1. Effects of As_2O_3 on cell growth and cell cycle distribution

As shown in Fig. 1A and B, the cell viability decreased with increasing of As_2O_3 dose and time course. For example, after exposure to 10 μM As_2O_3, 31% of cells showed growth inhibition at 24 h, increasing to 46% at 48 h in the 293 cell line, and 23% and 41% in the HT1080 cell line, respectively. Both cell lines showed similar results in terms of cell viability. The 293 cell line seemed to be more sensitive to inhibition of cell growth than the HT1080 cell line, although there was not a significant difference between the cell lines. Fig. 1C presents the inhibitory effects of DODC alone. Cell viability decreased with increasing DODC dose, and the 293 cell line was more sensitive to inhibition of cell growth than the HT1080 cell line at the higher dose.

Table 1
Comparison of absorbance spectrum between free DODC and DNA complexed with DODC

	Peak 1 (Abs/λ nm)	Peak 2 (Abs/λ nm)	A_{Peak1}/A_{Peak2}
Free DODC	0.1260/576.0	0.0401/536.0	3.14
T_2AG_3	0.1244/576.6	0.0395/536.0	3.14
$(T_2AG_3)_2$	0.0822/579.8	0.0277/536.0	2.96
$(T_2AG_3)_4$	0.0676/584.0	0.0236/536.0	2.86
$(G_3T_2AG_3)_2$	0.0737/576.6	0.0214/535.4	3.44

Measurement in 10 mM Tris/150 mM NaCl, pH 7.5 at 25 °C. DODC: 1.0 μM; oligodeoxynucleotide (ODN): 10 μM.

Fig. 2 shows the cellular DNA content and cell cycle changes, determined by flow cytometric analysis. Low-dose arsenic inhibited cell growth in the G2/M phase, although some cells were in a sub-G1 phases, representing apoptosis (Fig. 4C and E). Similar results were obtained with the two cell lines, but the 293 cell line was more sensitive. Flow cytometry results also revealed that in both 293 and HT1080 cells, the percentage of cells in S phase after treatment with DODC decreased compared with control cells. Cells treated with DODC alone arrested in the G0/G1 phase and few were present in the sub-G1 phase (Fig. 2).

3.2. Induction of cell apoptosis

Further studies showed that the inhibition of cell growth induced by As_2O_3 was caused by apoptosis, as indicated by the DNA fragmentation assay using 1.5% agarose gel electrophoresis. Fig. 3 shows the effects of DODC, arsenic alone and combined treatment on DNA ladder formation in 293 cells and HT1080 cells. As_2O_3 treatment alone produced a typical DNA ladder, corresponding to internucleosomal cleavage and showing the biochemical characteristics

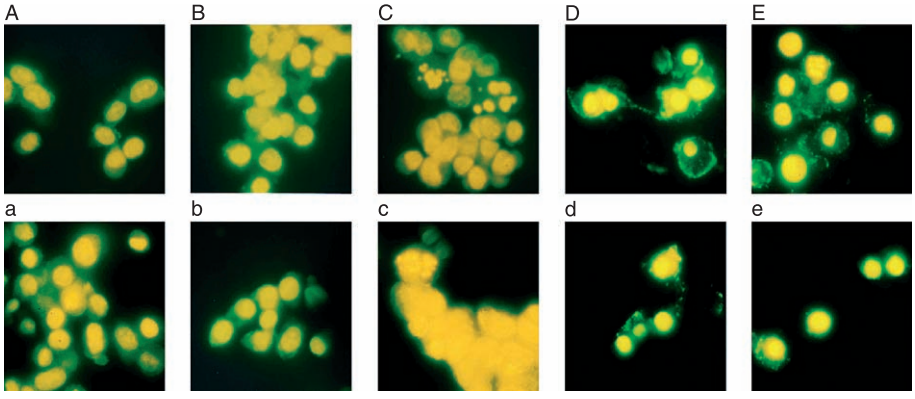

Fig. 4. Morphological changes in 293 and HT1080 cell lines. Cells were incubated as described in the legend of Fig. 3. (A) and (a): control; (B) and (b): 20 μM DODC alone; (C) and (c): 10 μM As_2O_3 alone; (D) and (d): combination of DODC and 10 μM As_2O_3; (E) and (e): 100 μM As_2O_3 alone. The groups treated with two agents or high-dose As_2O_3 alone showed characteristic nuclear condensation and fragment (D, d, E, e).

of apoptosis (Fig. 3, lanes 6 and 12); there was a weaker ladder in lane 4, whereas there was no ladder after DODC treatment alone (lanes 3 and 9). The cells incubated with DODC and arsenic showed a typical DNA ladder on agarose gel electrophoresis, and the density was almost equal to that of cells incubated with high-dose arsenic (100 μM). The only difference between the two cell lines was the weaker ladder in the293 cells after incubation with arsenic 10 μM alone.

These results are consistent with the fluorescence microscopy findings shown in Fig. 4. After incubation with 10 μM As_2O_3 the cell nucleus showed minor apoptotic morphology, while that of cells incubated with 100 μM As_2O_3 showed prominent apoptotic morphology, such as a shrunken cell membrane, nuclear condensation and fragment, and formation of a nuclear body (Fig. 4E and e). There were no obvious morphological changes after DODC treatment (Fig. 4B and b). The above results indicate that at the same concentrations of As_2O_3, the growth of cells of both cell lines was inhibited by apoptosis, but the 293 cell line seemed to be more susceptible to apoptosis. Fluorescence images also showed that combined DODC and As_2O_3 incubation as well as high-dose arsenic incubation resulted in clear apoptotic morphological changes compared with those of cells incubated with low-dose arsenic (Fig. 4D, E and d, e).

3.3. Telomere DNA structure alteration and inhibition of telomerase activity by DODC

To show the effects of DODC on telomere DNA structure state, TTAGGG, $(TTAGGG)_2$, $(TTAGGG)_4$ and $(GGGTTAGGG)_2$ were synthesized. In aqueous solution, the visible absorbance spectrum of DODC at a low concentration has a maximum at 576 nm with a small shoulder at 530−550 nm and a relative intensity ratio of A576/A536 ≈ 3.1. Upon addition of TTAGGG, $(TTAGGG)_2$, $(TTAGGG)_4$ and $(GGGTTAGGG)_2$, there was a decrease in intensity and a small red shift of the 576 nm peak, and a decrease in the relative intensity ratio of A_{Peak1}/A_{Peak2}. Results are summarized in Table 1. The red shift of the 576 nm peak and relative intensity ratio of A_{Peak1}/A_{Peak2} decreased with $(TTAGGG)_2$, and $(TTAGGG)_4$ which indicate the formation of bimolecular hairpin quadruplex structures under these conditions (Chen et al., 1996b).

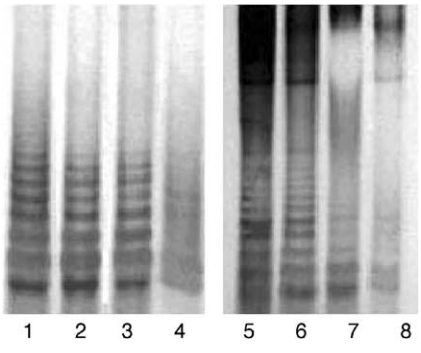

Fig. 5. Direct assay of telomerase activity. Lanes 1 and 5 indicate normal 293, HT1080 cell lines, respectively. Lanes 2, 6: 1 μM DODC; lanes 3, 7: 20 μM DODC; lanes 4, 8: 40 μM DODC.

As shown in Fig. 5, DODC could inhibit telomerase activity in a dose-dependent manner after incubation for 24 h over a concentration range from 1 to 40 μM. Telomerase activity was inhibited almost completely by 40 μM DODC, and partly inhibited by 20 μM in both cells. The change in telomerase activity in the 293 cell lines was weaker than that in the HT1080 cells.

4. Discussion

Since application of As_2O_3 was found to be effective in patients with resistant and refractory acute promyelocytic leukemia, numerous studies have focused on the mechanism of arsenic, and clinical trials are also under way for hematopoietic malignancies and solid tumors. Our group studies previously showed that human gastric cancer MGC-803 cells exhibited the highest susceptibility to As_2O_3 with an IC_{50} of 1 μM, while other cell lines of cervical and hepatocarcinoma cell lines were slightly less sensitive (Zhang et al., 1999). Recently, our group and other groups have correlated the sensitivity of cancer cell lines to As_2O_3 with the intracellular glutathione (GSH) content and the generation of reactive oxygen species (Chen et al., 2002; Gartenhaus et al., 2002; Choi et al., 2002). One critical factor that limits the application of As_2O_3 may be its cytotoxicity in normal tissue because it is a well-known poison. We should take its toxicity into account when emphasizing its anticancer effect. It is important to increase sensitivity in less susceptible cell lines and to seek a way to reduce its toxicity.

To decrease the dose of arsenic and to enhance therapeutic benefits, we investigated the synergetic effects of arsenic and DODC in the induction of apoptosis. Results showed that As_2O_3 can inhibit the growth of both 293 and HT1080 cells by inducing apoptosis. In both cell lines, As_2O_3 had similar effects on cell viability, apoptosis and cell cycle. Results from a collaborating group showed that the change in Ca^{2+} and mitochondrial membrane potential in the both cell lines treated with As_2O_3 paralleled the change in the ratio of apoptotic cells (unpublished). But compared with HT1080 cell line, the 293 cell line was more sensitive to the same dose of As_2O_3.

DODC is used as a telomerase inhibitor and could induce the formation of hairpin G-quadruplex in vitro (Chen et al., 1996b). In this work, DODC resulted in a marked and progressive decrease in cell number compared to control and inhibited cell growth in a dose-dependent manner (Fig. 1C). Flow cytometry results revealed that the cell cycle was arrested at G0/G1 phase in both 293 and HT1080 cells treated DODC compared with control cells. One possible explanation is that the inhibition of telomerase by DODC

blocked DNA replication and cells could not enter S phase. According to research with cultured cells in vitro, telomerase activity is different in different phases of cell cycle. It begins to increase from G1/S and is greatest strongest in S phase; it is almost undetectable in G2/M. Thus telomere maintenance by telomerase is active in a replicative phase but not in a stable phase. Telomerase activity was inhibited by DODC in a dose-dependent manner over the concentration range 1–40 μM, but flow cytometry results showed that 20 μM DODC alone did not cause apoptosis; a characteristic sub-G1 peak and a ladder were not observed in the DODC alone treatment group. This suggests that cell apoptosis might not be associated with a decrease in telomerase activity at a certain time. Both HT1080 cells and 293 cells incubated with DODC and As_2O_3 had a typical DNA ladder, indicative of apoptosis. The effect was almost equal to that of high-dose arsenic (100 μM). Our experiments provide evidence that (1) DODC alone might induce the formation of hairpin quadruplex structures in telomeric DNA and inhibit telomerase, and does not cause apoptosis within 24 h; (2) low-dose arsenic (10 μM) alone dose not lead to apoptosis if not combined with DODC. Other studies from our group also showed that 10 μM arsenic inhibited telomerase activity. Based on the above data, we suggest the following scenario. It is possible that DODC increases the sensitivity of cells to As_2O_3 by altering the structure of DNA, but not by decreasing telomerase activity (Kariseder et al., 2002). It has reported that p53 protein can bind telomeric single-strand overhangs and t-loop junctions, suggesting that telomeres signal p53 directly (Stansel et al., 2002). It is also possible that DODC altered the telomere DNA structure, leading to a change in binding with other protein, for example p53. Normally, p53 translocates from the cytoplasm to the nucleus at the G1/S transition and shuttles back to the cytoplasm shortly thereafter. Cell arrest or apoptosis can then be triggered. Perhaps these factors may be essential for the synergetic effects of arsenic and DODC in the induction of apoptosis.

The 293 cells were clearly more sensitive than HT1080 cells when both cells were treated with the same dose of arsenic or DODC alone. What is the cause for the different sensitivity of the two cell lines to As_2O_3? One possible explanation is that this phenomenon may relate to telomere length or other unknown factors. It has been reported that 293 cells maintain a stable terminal restriction fragment of average length ≈ 3 kb and two stable bands of 12 and 23 kb (Counter et al., 1992), whereas in the human fibrosarcoma cell line HT1080, the terminal restriction fragment has an average length ≈ 1.5 kb (Van Steensel and De Lange, 1997). The telomere length in 293 cells is longer than in HT1080 cells, and this may be related to the formation of more hairpin G-quadruplex in vivo.

These results suggest the existence of synergism between the two agents. This may provide a potential method to decrease the dose of arsenic in order to avoid the cytotoxicity of As_2O_3. DODC application increased the effects of low-dose arsenic on the cells. That is, the synergy made the less sensitive cell become more susceptible to As_2O_3. This may provide useful information for clinical application. More work was needed to find new treatment protocols for cancer therapy.

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