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ORIGINAL ARTICLE

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In vitro activity of dimethylarsinic acid against human leukemia and multiple myeloma cell lines

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Abstract *Purpose*: Arsenic trioxide (As_2O_3), an inorganic arsenic compound, has recently been approved for the treatment of relapsed or refractory acute promyelocytic leukemia. However, systemic toxicity associated with As_2O_3 treatment remains a problem. Inorganic arsenic is detoxified in vivo by methylation reactions into organic arsenic compounds that are less toxic. *Methods and results*: We investigated the antiproliferative and cytotoxic activity of dimethylarsinic acid (DMAA), an organic arsenic derivative and major metabolic by-product of As_2O_3, against a panel of eight leukemia and multiple myeloma cell lines. As_2O_3 was tested in comparison. In clonogenic assay, the average concentration of DMAA that suppressed cell colony growth by 50% was 0.5–1 m*M*, while for As_2O_3 it was on average 1–2 μ*M*. At those concentrations DMAA and As_2O_3 had significantly less effect on colony growth of normal progenitor cells. Cytotoxic doses of DMAA and As_2O_3 in 3-day trypan blue dye exclusion assay experiments were similar to doses effective in clonogenic assay. Assessment of apoptosis by annexin V assay revealed a high rate of apoptosis in all cell lines treated with DMAA and As_2O_3, but significantly less effect on normal progenitor cells. DMAA, unlike As_2O_3, had no effect on the maturation of leukemic cells. *Conclusions*: DMAA exerts differential antiproliferative and cytotoxic activity against leukemia and multiple myeloma cells, with no significant effect on normal progenitor cells. However, concentrations of DMAA needed to achieve such efficacy are up to 1000 times those of As_2O_3. Evaluation of novel organic arsenic that would combine the high efficacy of As_2O_3 and the low toxicity of DMAA is warranted.

Keywords Dimethylarsinic acid · Arsenic trioxide · Leukemia · Multiple myeloma

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Introduction

The primary means of arsenic detoxification in vivo is methylation of inorganic arsenic to less-toxic, more rapidly excreted organic forms [1]. Dimethylarsinic acid (DMAA) is the main organic metabolite of arsenic trioxide (As_2O_3), an inorganic arsenic derivative. Due to its common use as a herbicide, DMAA has been extensively studied [2]. Animal and human studies have shown DMAA to have low environmental and occupational hazardous effects [3]. Excretion studies in men following ingestion of a single dose of inorganic arsenic or DMAA have found that inorganic arsenic is excreted mostly in methylated forms while about 80% of DMAA is excreted unchanged [4, 5, 6]. In addition, DMAA is excreted more rapidly than inorganic arsenic [6]. While the effects of DMAA on normal (non-malignant) cells have been extensively investigated [7, 8, 9, 10, 11, 12, 13], its possible anticancer activity has not been fully addressed. To our knowledge, the anticancer potential of DMAA

against human tumor cells has been evaluated in only two studies. Ochi et al. identified an apoptosis-inducing feature of DMAA against HL60 human acute myeloid leukemia cells and a role for glutathione in this process [14]. The system used was short-term (2-day) cell culture and MTT assay; DMAA was effective in the 2–10 m*M* range. On the other hand, Abdullaev et al. tested DMAA against HeLa human cervical carcinoma cells in a long-term (10-day) colony-forming assay and found DMAA to be effective in the 20–100 μ*M* range [15]. To properly address this issue, in the present study we determined the antiproliferative and cytotoxic activity of DMAA against a number of human malignant cell lines of hematologic origin, as well as against normal peripheral blood progenitor cells (PBPC), using both short- and long-term assays. The activity of DMAA as determined in these assays was compared with that of As_2O_3.

Material and methods

Cells and chemicals

The human cell lines used included acute myelocytic leukemia OCI/AML-3 cells, chronic myeloid leukemia blast-phase K562 cells, acute promyelocytic leukemia NB4 cells, acute myelomonocytic leukemia KBM3 cells, and chronic myeloid leukemia blast-phase KBM5 cells (kindly provided by Dr. Miloslav Beran, The University of Texas M.D. Anderson Cancer Center), and multiple myeloma RPMI 8226, ARK, and CAG cells (kindly provided by Dr. Joshua Epstein, Arkansas Cancer Research Center, Little Rock, AR). PBPC were isolated from blood samples from five healthy donors, obtained in the pheresis unit at M. D. Anderson Cancer Center after written consent had been obtained. The mononuclear layer was separated using Hypaque density gradient separation. All cells were maintained in alpha minimal essential medium with L-glutamine and ribo- and deoxyribonucleosides supplemented with 10% fetal bovine serum. DMAA (98% cacodylic acid, kindly supplied by Luxembourg Industries, Tel-Aviv, Israel) was dissolved in water to the appropriate concentrations. As_2O_3 (99% arsenous acid) was purchased from Sigma Chemical Company (St. Louis, Mo.). As_2O_3 was dissolved in NaOH, the pH was adjusted to 7.0 with HCl, and the volume was adjusted with water to give the appropriate concentration.

Colony-forming (clonogenic) assay

The growth-inhibitory effects of DMAA and As_2O_3 on the proliferation of various cell lines and PBPC were evaluated by clonogenic assay. Briefly, 5×10^4 cells/ml were cultured and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h with different concentrations of arsenic compounds. Cells were then washed twice in Hank's balanced salt solution and resuspended in semisolid "complete" methylcellulose medium containing recombinant cytokines (MethoCult GF H4434; StemCell Technologies, Vancouver, Canada). The cells were plated in quadruplicate at 2×10^2 cells/0.1 ml in 96-well microtiter plates (Linbro/Titertek; ICN Biomedicals, Aurora, Ohio). The volume of cell suspension added to the plating solution corresponded to the volume of liquid culture from which the cells had been taken. Cells were incubated at 37°C in an atmosphere containing 5% CO_2 for 5–7 days and then colonies containing more than 20 cells were counted using an inverted light microscope. Growth inhibition was defined as the percentage of cell growth/number of colonies in treated samples in relation to that in the control sample.

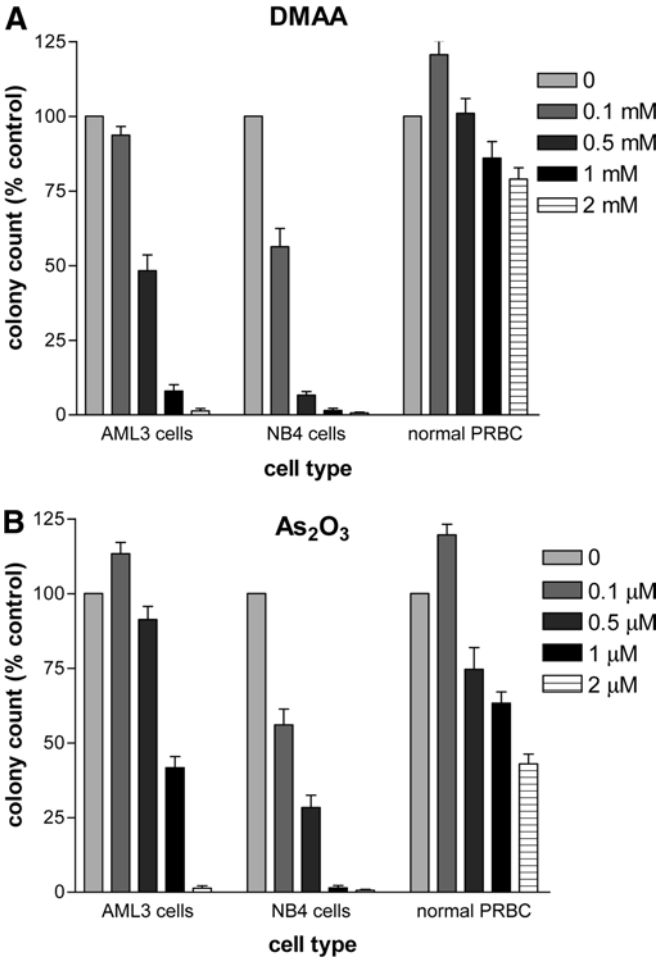


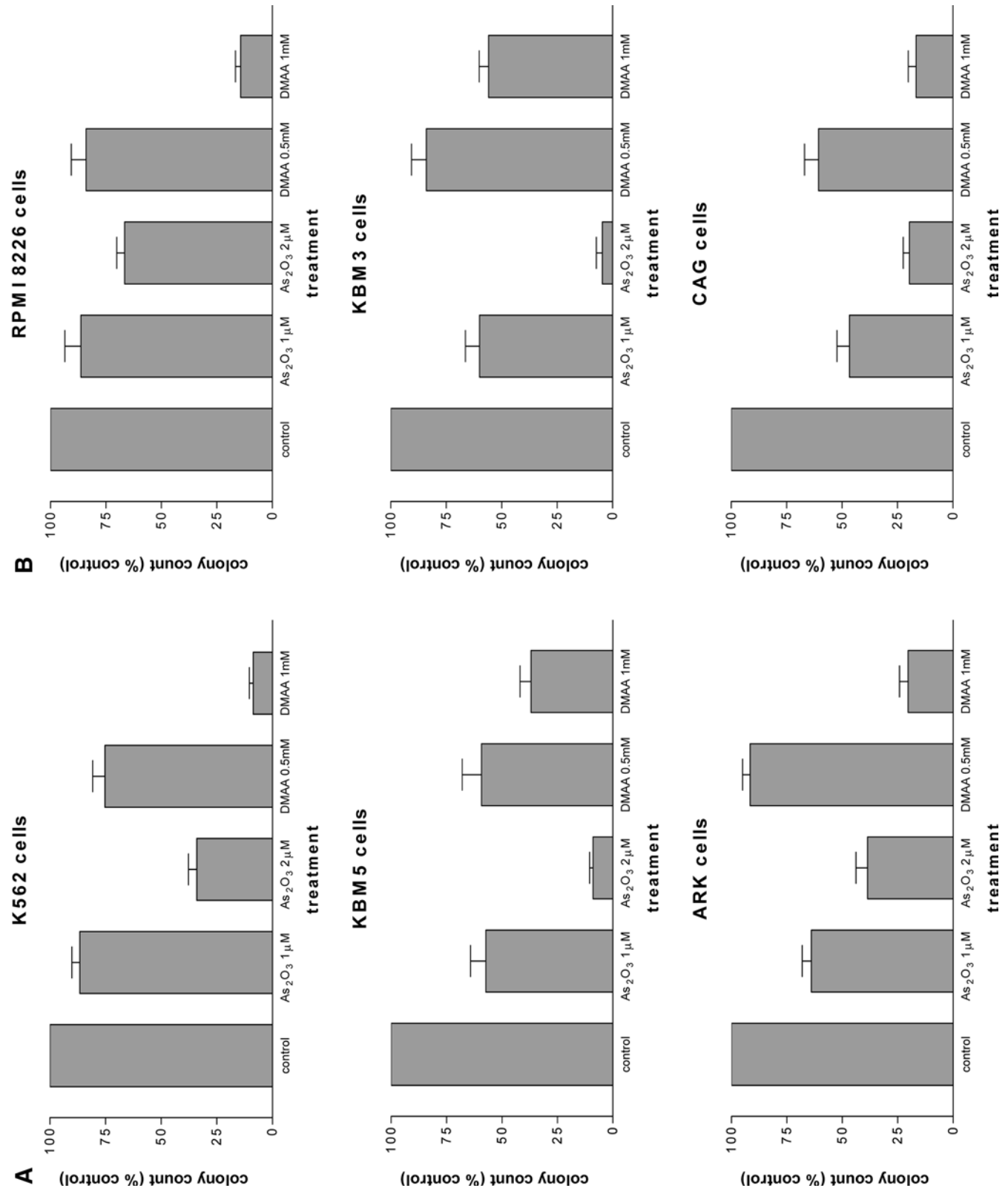
Fig. 1A, B Antiproliferative activity of DMAA and As_2O_3 against AML-3, NB4, and normal donor cells. AML-3, NB4, and normal donor cells were plated at a density of 5×10^4/ml and incubated for 72 h with the indicated concentrations of DMAA (**A**) and As_2O_3 (**B**). The cells were then washed twice and were plated in quadruplicate at a density of 2×10^3/ml. After 7 days, colonies containing more than 20 cells were counted. Data represent the percentage of colonies in relation to the number of colonies formed by untreated cells. The results shown are means ± SD and are representative of three independent experiments

Trypan-blue dye exclusion assay

To assess toxicity of arsenic compounds, cell lines at densities of 5×10^4 cells/ml and PBPC from healthy donors at densities of 1×10^6 cells/ml, were cultured for 72 h in 24-well plates (Linbro;

►

Fig. 2 Antiproliferative activity of DMAA and As_2O_3 against K562, KBM5, ARK, RPMI 8226, KBM3, and CAG cells. Cells at a density of 5×10^4/ml were incubated with the indicated concentrations of DMAA and As_2O_3 for 72 h. The cells were then washed twice and plated in quadruplicate at a density of 2×10^3/ml. After 7 days, colonies containing more than 20 cells were counted. Data represent the percentage of colonies in relation to the number of colonies formed by untreated cells. The results shown are means ± SD and are representative of an average of three independent experiments per cell line


A
K562 cells
B
RPMI 8226 cells
KBM5 cells
KBM3 cells
ARK cells
CAG cells
colony count (% control)
100
75
50
25
0
control
As2O3 1μM
As2O3 2μM
DMAA 0.5mM
DMAA 1mM
treatment

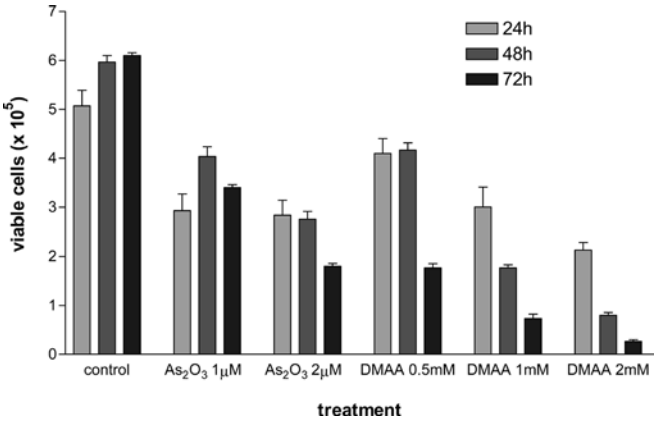


Fig. 3 Time- and dose-dependent cytotoxic activity of DMAA and As_2O_3 against RPMI 8226 cells. Cells were treated in triplicate with the indicated concentrations of DMAA or As_2O_3 for 24, 48, and 72 h. At the end of each incubation period, cells were assessed for viability using trypan blue staining. The results shown are means ± SD and are representative of two independent experiments

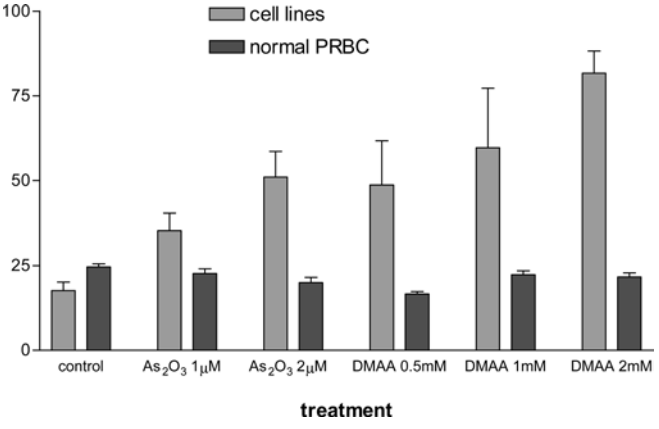


Fig. 4 Induction of apoptosis in leukemia and multiple myeloma cell lines by DMAA and As_2O_3. Cell lines at a density of 5×10^4 cells/ml and PBPC from healthy donors at a density of 1×10^6 cells/ml, were cultured with the indicated concentrations of DMAA or As_2O_3 for 72 h. Cells were then adjusted to a density of 1×10^6 cells/ml and stained with annexin V to assess apoptosis. Data were graphed as the mean percentage of positively stained cells relative to the total population (mean for eight cell lines vs mean for cells from five normal donors, ± SD)

ICN Biomedicals) with various concentrations of DMAA or As_2O_3. Cells were then washed in phosphate-buffered saline (PBS) and mixed with an equal volume of saline containing 0.4% trypan blue stain (GIBCO-BRL, Gaithersburg, Md.). Unstained cells (indicating viable cells) were counted.

Annexin V assay

To assess apoptotic effects, cell lines at densities of 5×10^4 cells/ml and PBPC from healthy donors at densities of 1×10^6 cells/ml, were cultured with various concentrations of DMAA or As_2O_3 for 72 h. Cells were then adjusted to a density of 1×10^6 cells/ml and stained with annexin V fluorescein isothiocyanate (Trevigen, Gaithersburg, Md.). The mixtures were incubated for 20 min on ice in the dark. Next, cells were washed once with PBS plus 0.1% sodium azide and rehydrated

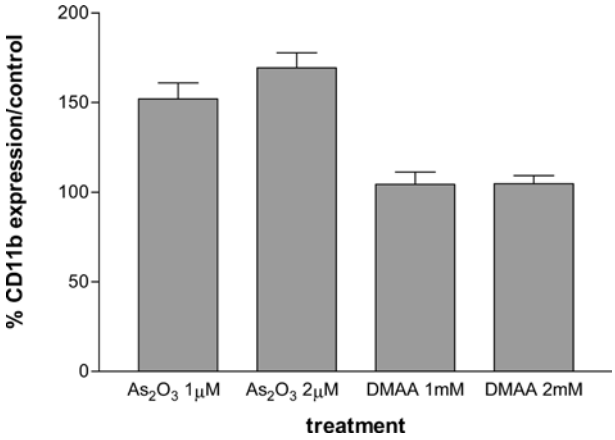


Fig. 5 Effect of As_2O_3 and DMAA on the maturation of promyelocytic leukemia NB4 cells. After 72 h of incubation with the indicated concentrations of DMAA or As_2O_3, cells were washed in PBS and incubated at a density of 1×10^6 cells/ml with phycoerythrin-conjugated anti-CD11b monoclonal antibody. To exclude nonspecific binding, an appropriate isotypic control was prepared in the same manner. Cells were then analyzed for the expression of CD11b using a flow cytometer. Data were graphed as the percentage expression of CD11b in treated sample in relation to the expression in control samples (means ± SD). The results shown are representative of two independent experiments

with the same buffer. Cells were collected using a fluorescence-activated cell sorter (Becton Dickinson) and analyzed using CellQuest Document Analysis (Becton Dickinson). For all staining procedures, nonspecific binding was controlled for with an isotypic control.

Maturation analysis

Human promyelocytic leukemia NB4 cells were used to test the maturation effect of DMAA and As_2O_3. Phycoerythrin-conjugated anti-CD11b monoclonal antibody (Becton-Dickinson) was used as a marker of mature myeloid cells. After 72 h of incubation with drugs, cells were washed in PBS. Cells at a density of 1×10^6 cells/ml were then incubated with monoclonal antibody at a dilution of 1:10 in the dark at room temperature for 15 min. Cells were then washed in PBS and the pellet was resuspended in 500 μl PBS. To exclude nonspecific binding an appropriate isotypic control was prepared in the same manner. Cells were sorted using a flow cytometer and analyzed using CellQuest Document Analysis.

Results

Effect of DMAA on cell proliferation

In the initial clonogenic assay experiment, using AML-3 and NB4 cells, the concentrations of DMAA that suppressed cell colony growth by 50% (IC_{50}) were determined to be 0.5 and 0.1 m*M*, respectively. In contrast, the IC_{50} values for As_2O_3 were found to be 1 μ*M* for AML-3 cells and 0.1 μ*M* for NB4 cells. At these concentrations DMAA and As_2O_3 had significantly less effect on colony growth of normal progenitor cells (Fig. 1). Following experiments confirmed similar activity of DMAA and As_2O_3 against K562, KBM3, KBM5, RPMI 8226, ARK, and CAG cells (Fig. 2). The IC_{50} values for DMAA were

on average in the range 0.5–1 mM, while for As_2O_3 they were on average in the range 1–2 μM.

Effect of DMAA on cell survival, apoptosis, and maturation

Cytotoxic doses of DMAA and As_2O_3 in trypan blue dye exclusion assay experiments were shown to be similar to doses effective in the clonogenic assay. Thus, an average IC_{50} for DMAA against the eight cell lines tested was about 500 μM, while for As_2O_3 it was about 1 μM; these doses had no significant effect on normal PBPC (data not shown). The cytotoxic effects of DMAA and As_2O_3 were shown to be time-dependent (as an example, results for RPMI 8226 cells are shown in Fig. 3). Assessment of apoptosis by the annexin V assay revealed a high rate of apoptosis in all eight cell lines treated with DMAA and As_2O_3 but no significant effect on PRBC from five normal donors (Fig. 4). DMAA, unlike As_2O_3, had no effect on the maturation of leukemic cells (Fig. 5) as assessed by the expression of CD11b on the leukemic cells after a 3-day incubation.

Discussion

As_2O_3, an inorganic arsenic compound, has recently been approved for the treatment of relapsed or refractory acute promyelocytic leukemia. However, systemic toxicity associated with As_2O_3 treatment is significant. Cardiotoxicity, in particular, has been a major problem [16, 17]. In ancient times, arsenic was used widely as a therapeutic drug to treat a variety of diseases, such as asthma, epilepsy, infections, and skin eruptions [18]. To enhance the therapeutic efficacy and reduce the toxicity associated with inorganic arsenic derivatives, organic arsenic compounds that retained the bactericidal and cytocidal properties of inorganic arsenic but showed reduced toxicity because of the presence of the organic portion of the molecule were synthesized. Atoxyl (sodium arsenilate), first used to treat sleeping sickness and syphilis, and Paul Erlich's magic bullets, salvarsan (arsphenamine), and neosalvarsan (neoarsphenamine), specifically directed against syphilis, were some of the first organic arsenic derivatives in chemotherapy [18]. Organic arsenic derivatives such as DMAA are used today as herbicides.

Our results reveal that DMAA exerts differential antiproliferative and cytotoxic activity against leukemia and multiple myeloma cells, with no significant effect on normal PBPC. We also showed that DMAA can induce apoptosis in malignant cells. However, concentrations of DMAA needed to achieve such efficacy against malignant cells are 500–1000 times those of As_2O_3. The antiproliferative and cytotoxic concentrations of As_2O_3 against eight cell lines tested were between 1 and 2 μM, results consistent with those of other investigators [19]. Although DMAA, when injected intraperitoneally into mice, has a median lethal dose of 500 mg/kg [20], significantly better than that of arsenic (10 mg/kg) [21], the fact that one needs to use DMAA in millimolar concentrations to achieve significant toxicity in vitro has lessened interest in this compound as a potential anticancer drug. Such concentrations are difficult to achieve in blood, and DMAA therefore would not be suitable for therapeutic studies. However, in light of the low systemic toxicity of organic arsenic compounds in general, research on other organic arsenicals is warranted. DMAA, in this regard, may serve as a backbone for new organic arsenic derivatives that would combine low toxicity with good anticancer activity. An example of such an effort is the work of Styblo et al. who found equivalent in vitro cytotoxicity among trivalent inorganic and methylated organic arsenicals [22]. Organic arsenic derivatives, in addition, may have different modes of action than As_2O_3; for example, glutathione plays a role in protecting cells from toxic effects of As_2O_3 while it enhances toxicity of DMAA [12, 23].

References

1. Vahter M, Concha G (2001) Role of metabolism in arsenic toxicity. Pharmacol Toxicol 89:1
2. Kenyon EM, Hughes MF (2001) A concise review of the toxicity and carcinogenicity of dimethylarsinic acid. Toxicology 160:227
3. US Environmental Protection Agency (1975) Initial scientific review of cacodylic acid (EPA 540/1-75-021). Criteria and Evaluation Division, Substitute Chemical Program
4. Crecelius EA (1977) Changes in the chemical speciation of arsenic following ingestion by man. Environ Health Perspect 19:147
5. Marafante E, Vahter M, Norin H, Envall J, Sandstrom M, Christakopoulos A, Ryhage R (1987) Biotransformation of dimethylarsinic acid in mouse, hamster and man. J Appl Toxicol 7:111
6. Buchet JP, Lauwerys R, Roels H (1981) Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethylarsonate, or dimethylarsinate in man. Int Arch Occup Environ Health 48:71
7. Vega L, Styblo M, Patterson R, Cullen W, Wang C, Germolec D (2001) Differential effects of trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in normal human epidermal keratinocytes. Toxicol Appl Pharmacol 172:225
8. Romach EH, Zhao CQ, Del Razo LM, Cebrian ME, Waalkes MP (2000) Studies on the mechanisms of arsenic-induced self tolerance developed in liver epithelial cells through continuous low-level arsenite exposure. Toxicol Sci 54:500
9. Sordo M, Herrera LA, Ostrosky-Wegman P, Rojas E (2001) Cytotoxic and genotoxic effects of As, MMA, and DMA on leukocytes and stimulated human lymphocytes. Teratog Carcinog Mutagen 21:249
10. Ochi T, Nakajima F, Fukumori N (1998) Different effects of inorganic and dimethylated arsenic compounds on cell morphology, cytoskeletal organization, and DNA synthesis in cultured Chinese hamster V79 cells. Arch Toxicol 72:566
11. Sakurai T, Qu W, Sakurai MH, Waalkes MP (2002) A major human arsenic metabolite, dimethylarsinic acid, requires reduced glutathione to induce apoptosis. Chem Res Toxicol 15:629
12. Sakurai T, Kaise T, Matsubara C (1998) Inorganic and methylated arsenic compounds induce cell death in murine macrophages via different mechanisms. Chem Res Toxicol 11:273

13. Ochi T (1997) Arsenic compound-induced increases in glutathione levels in cultured Chinese hamster V79 cells and mechanisms associated with changes in gamma-glutamylcysteine synthetase activity, cystine uptake and utilization of cysteine. Arch Toxicol 71:730
14. Ochi T, Nakajima F, Sakurai T, Kaise T, Oya-Ohta Y (1996) Dimethylarsinic acid causes apoptosis in HL-60 cells via interaction with glutathione. Arch Toxicol 70:815
15. Abdullaev FI, Rivera-Luna R, Garcia-Carranca A, Ayala-Fierro F, Espinosa-Aguirre JJ (2001) Cytotoxic effect of three arsenic compounds in HeLa human tumor and bacterial cells, Mutat Res 493:31
16. Li Y, Sun X, Wang L, Zhou Z, Kang YJ (2002) Myocardial toxicity of arsenic trioxide in a mouse model, Cardiovasc Toxicol 2:63
17. Chiang CE, Luk HN, Wang TM, Ding PY (2002) Prolongation of cardiac repolarization by arsenic trioxide. Blood 100:2249
18. Waxman S, Anderson KC (2001) History of the development of arsenic derivatives in cancer therapy. Oncologist 6 [Suppl 2]:3
19. Novick SC, Warrell RP Jr (2000) Arsenicals in hematologic cancers. Semin Oncol 27:495
20. Strem Chemicals (1998) Dimethylarsinic acid. Material safety data sheet. Strem Chemicals, Newburyport, MA
21. Lallemand-Breitenbach V, Guillemin MC, Janin A, Daniel MT, Degos L, Kogan SC, Bishop JM, de The H (1999) Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of acute promyelocytic leukemia. J Exp Med 189:1043
22. Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ (2000) Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Arch Toxicol 74:289
23. Ochi T, Kaise T, Oya-Ohta Y (1994) Glutathione plays different roles in the induction of the cytotoxic effects of inorganic and organic arsenic compounds in cultured BALB/c 3T3 cells. Experientia 50:115