Arsenic trioxide synergizes with B7H3-mediated immunotherapy to eradicate hepatocellular carcinomas

Liqiong Luo1, Haiquan Qiao1, Fanqiang Meng1, Xuesong Dong1, Baoguo Zhou1, Hongchi Jiang1, Jagat R Kanwar2, Geoffrey W Krissansen1 and Xueying Sun1,2*

1The Hepatosplenic Surgery Center of Heilongjiang Province/Department of General Surgery, The First Clinical College of Harbin Medical University, Harbin, China
2Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

Key words: arsenic trioxide; B7H3; hepatocellular carcinoma; angiogenesis; immunotherapy; apoptosis

Arsenic trioxide (As2O3), a valuable anticancer drug for the treatment of acute promyelocytic leukemia, may also have therapeutic potential for the treatment of solid tumors. However, its therapeutic efficacy against solid tumors is lacking even at high dosages. Other therapeutic strategies are required to enhance the efficacy of As2O3 against solid tumors such as hepatocellular carcinoma (HCC), which is refractory to chemotherapy. B7H3, a new member of the B7 family, has been shown to induce antitumor immunity. Intratumoral injection of B7H3 plasmids eradicates small EL-4 lymphomas, but monotherapy is ineffective against large tumors. Here we investigated whether As2O3 would synergize with B7H3 immunotherapy to combat HCC. Large subcutaneous H22 HCCs (0.5–0.8 cm in diameter) established in BALB/c mice were rapidly and completely eradicated when intratumoral administration of As2O3 was preceded by in situ gene transfer of B7H3. In contrast, neither As2O3 nor B7H3 monotherapy was effective. The antitumor activity of As2O3 was attributed to increased tumor-cell apoptosis, perhaps as a result of direct cytotoxicity as well as decreased tumor angiogenesis. Combination therapy generated potent systemic antitumor immunity mediated by CD8+ and NK cells that was active in combating a systemic challenge of 1 × 107 parental H22 cells. It led to the simultaneous and complete regression of multiple distant tumor nodules, concomitant with increased levels of serum IFN-γ and cytotoxic T lymphocyte (CTL) activity. In conclusion, combining B7H3-mediated immunotherapy with As2O3 warrants investigation as a therapeutic strategy to combat HCC, and other malignancies.

© 2005 Wiley-Liss, Inc.

Material and methods

Mice, cell lines, As2O3 and antibodies

Male BALB/c mice (H-2b), 6–8 weeks old, were obtained from the Animal Research Center, Harbin Medical University, China. The syngeneic H22 hepatocellular carcinoma cells (CCTCC No. GDC091 from Dianzang Cell Bank of Wuhan University, Wuhan, China) were kind gifts from the Animal Research Center.

Abbreviations: APL, acute promyelocytic leukemia; As2O3, arsenic trioxide; CAM, cell adhesion molecule; CTL, cytotoxic T lymphocyte; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; HCC, hepatocellular carcinoma; ROS, reactive oxygen species.

Grant sponsor: National Natural Scientific Foundation of China; Grant numbers: 30471681 and 30170916; Grant sponsor: Collaborative Fund for Overseas Scholars from the Scientific and Technological Bureau of Heilongjiang Province, China; Grant number: WH05C02; Grant sponsor: Wellcome Trust (UK).

*Correspondence to: The Hepatosplenic Surgery Center of Heilongjiang Province/Department of General Surgery, The First Clinical College of Harbin Medical University, Harbin 150001, China. Fax: +86-451-53643628. E-mail: k.sun@auckland.ac.nz

Received 8 April 2005; Accepted after revision 16 August 2005 DOI 10.1002/jc.21557
Published online 10 October 2005 in Wiley InterScience (www.interscience.wiley.com).
of Heilongjiang Provincial Tumor Hospital. The H22 cells were maintained by successive transplantation into the abdominal cavity of BALB/c mice. The cancerous ascites were collected, washed with PBS, stained with Trypan blue and counted by microscopy. An As$_2$O$_3$ solution was purchased by Yida Pharmaceutical Co. Ltd, Harbin Medical University, China. The rabbit anti-FLAG and anti-CD4 (clone Gk1.5) antibodies were purchased from Sigma (MO) and Chemicon International, Inc., respectively. The anti-CD8 (clone 53-6.7), anti-NK (clone PK136) and anti-CD31 (MEC13.3) antibodies were purchased from Pharmingen, CA.

**Plasmid/PVP formulation**

The $B7H3$-pcDNA3.1 plasmid containing a 951 bp cDNA encoding full-length mouse $B7H3$, and Flag-$B7H3$-pcDNA3.1 containing cDNA encoding a FLAG tag (DYKDDDDK) fused to the N-terminal sequence of mouse $B7H3$ which was used to detect expression in situ, have been described previously. PVP (plasdone C-30, M, 50,000) was kindly supplied by Alchemy Chemicals Ltd., Auckland, New Zealand. Purified plasmids were formulated with 5% PVP to generate a plasmid/PVP formulation with a concentration of 1 mg DNA/ml, as described previously.

**Tumor implantation and treatment**

All surgical procedures and care administered to the animals were in accordance with institutional guidelines. All experiments included 6 mice per treatment group, and each experiment was repeated at least once.

**Single tumor model.** Tumors were established by subcutaneous injection of $1 \times 10^6$ H22 HCC cells into a site in the left flank of BALB/c mice. The growth of tumors was determined by measuring 2 perpendicular diameters. Animals were randomly assigned to treatment when tumors reached around 0.7–0.8 cm in diameter after ~14 days. The $B7H3$ plasmid/PVP formulation (100 µl) containing 100 µg of $B7H3$ plasmid was intratumorally injected at multiple sites. As$_2$O$_3$ (5 µg) in 100 µl of PBS$^{16}$ was injected into the tumor at multiple sites every 2 days, and mice were euthanized when tumors reached 2 cm in diameter. For combination treatment, the $B7H3$ plasmid/PVP formulation solution was intratumorally injected, and 48 hr later, As$_2$O$_3$ was injected into the tumor as above, every 2 days until the tumors disappeared. Empty vector (pcDNA3.1)/PVP formulation solution served as a control for the $B7H3$ plasmid/PVP formulation, whereas PBS served as a control for As$_2$O$_3$. The tumors in the $B7H3$ monotherapy group were injected with $B7H3$ plasmid followed by an equivalent volume of PBS 48 hr later; tumors in the As$_2$O$_3$ monotherapy group were injected with empty vector/PVP formulation solution followed by As$_2$O$_3$, and tumors in the control group received empty vector/PVP complex followed by PBS. Mice whose tumors completely regressed were rechallenged 4 weeks after the disappearance of tumors by injecting $1 \times 10^6$ H22 cells subcutaneously into the opposing flank (right flank). Mice that resisted this challenge were given a second challenge of $1 \times 10^6$ H22 cells 2 weeks later.

**Multiple tumor model.** A primary tumor was established by subcutaneous injection of $1 \times 10^6$ H22 tumor cells into a site in the left flank of BALB/c mice, and the same number of H22 cells were injected at 4 different sites in the right flank, 5 days later, to represent secondary tumors. Primary tumors reached around 0.5 cm in diameter after about 12 days, at which stage the 4 tumors in the right flank were around 0.15–0.2 cm in diameter. $B7H3$ plasmid/PVP formulation (100 µl) was injected into the primary tumor in the left flank, and 48 hr later, As$_2$O$_3$ was given every 2 days as above, until tumors disappeared. The growth of all the tumors was monitored and tumor size determined by measuring 2 perpendicular diameters.

**Immunohistological analysis**

Tumor cryosections (10 µm) prepared 2 days after intratumoral injection of plasmids were treated with acetone, rinsed with PBS, blocked with 2% BSA for 2 hr and incubated overnight with the primary antibody. They were subsequently incubated for 30 min with appropriate secondary antibodies using the SABC kit (Boster Biological Technology Ltd., Wuhan, China), and developed with Sigma FAST DAB (3,3′-diaminobenzidine tetrahydrochloride) and CoCl$_2$ enhancer tablets (Sigma). Sections were counterstained with Mayer’s hematoxylin, mounted and examined by microscopy.

**Western blotting**

The method for detecting the expression of proteins in tumors has been previously described. Briefly, tissues were excised, minced and homogenized in protein lysate buffer. Debris was removed by centrifugation at 10,000 g for 10 min at 4°C. Protein samples (50 µg) were resolved on 10% polyacrylamide SDS gels, and electrophoretically transferred to nitrocellulose Hybond C extra membranes. The membranes were incubated with primary antibodies, and subsequently with horseradish peroxidase-conjugated secondary antibodies. They were developed by enhanced chemiluminescence (Amersham International, Buckingham, England), and exposure to x-ray film.

**Assessment of tumor vascularity**

The methodology to determine tumor vascularity has been described previously. Briefly, 10 µm frozen tissue sections prepared from tumors 3 weeks after treatment were immunostained with the anti-CD31 antibody, as described above. Stained blood vessels were counted in 5 blindly chosen random fields (0.155 mm²) at 40× magnification, and the mean of the highest 3 counts was calculated.

**In situ detection of apoptotic cells**

This method has been described previously. Briefly, serial sections of 6 µm thickness were prepared from tumors 3 weeks following treatment, stained with the TUNEL agent (Boehringer Mannheim, Germany) and examined by fluorescence microscopy. Adjacent sections were counterstained with haematoxylin and eosin. The total number of apoptotic cells in 10 randomly selected fields was counted. The apoptosis index (AI) was calculated as the percentage of positive staining cells, namely apoptosis index (AI) = number of apoptotic cells × 100/total number of nucleated cells.

**Detection of IFN-γ by ELISA**

Serum samples and splenocytes were harvested from mice, 4 days after treatment. The splenocytes were cultured in RPMI1640 supplemented with 10% FCS at 37°C and 5% CO$_2$ for 48 hr, and the culture supernatant was collected. The levels of IFN-γ in both serum and supernatant were measured with an ELISA kit (Boster Biological Technology Ltd., Wuhan, China).

**Cytotoxicity assays**

Splenocytes were harvested from mice, 10 days after treatment, and incubated at 37°C with H22 parental target cells in graded E:T ratios in 96-well round-bottom plates, supplemented with recombinant interleukin-2 at a final concentration of 1,000 U/ml. After a 4 hr incubation, 50 µl of supernatant was collected, and lysis was measured using a Cyto Tox 96 Assay kit (Promega, Madison, WI). Background controls for nonspecific target and effector cell lysis were included. After background subtraction, the percentage of cell lysis was calculated using the formula: 100 × (experimental—spontaneous effector—spontaneous target)/maximum target—spontaneous target. For antibody-mediated depletion of leukocyte subsets, splenocytes were incubated with specific antibody at a concentration of 2 µg of antibody per 10$^6$ cells for 60 min before they were mixed with H22 parental target cells in 100:1 ratios, and splenocyte cytotoxicity was measured as above.
As$_2$O$_3$ SYNERGIZES WITH IMMUNOTHERAPY TO COMBAT HCC

**FIGURE 1** - Analysis of mouse B7H3 transgene expression. (a) Engineered expression of Flag-B7H3 in the H22 HCC. H22 tumors of 0.7–0.8 cm in diameter were injected with empty vector (pcDNA3.1), and a Flag-B7H3 expression vector (Flag-B7H3), as indicated. Illustrated are representative tumor sections prepared 2 days following plasmid injection, and stained brown with a mAb against the Flag tag (×100 magnification). (b) Western blot analysis of Flag-B7H3 in HCCs. H22 tumors of 0.7–0.8 cm in diameter were injected 2 days earlier with a Flag-B7H3 plasmid (right lane), or empty vector (left lane) and homogenized. The homogenates were resolved by SDS-PAGE and Western blotted with an anti-Flag mAb. Blots were stained with an anti-tubulin antibody to confirm that each lane contained similar amounts of tumor homogenate. The positions of the 45 kDa Flag-B7H3 protein and tubulin are indicated.

**Results**

*Intratumoral gene transfer of a plasmid encoding mouse B7H3 results in intense in situ transgene expression*

A Flag tag was fused to the N-terminus of mouse B7H3 so as to detect expression of mouse B7H3 plasmids injected directly into H22 tumors in situ. H22 tumors that had been established 14 days earlier by subcutaneous injection of H22 cells were injected with 100 µg of Flag-B7H3 (pcDNA3.1) expression plasmid and sectioned 2 days following gene transfer. Representative photographs reveal expression of Flag-tagged B7H3 throughout tumors, whereas control sections from vector-only-treated tumors were not stained with the anti-Flag antibody (Fig. 1a). Western blot analysis of homogenates of tumors prepared 2 days after gene transfer confirmed that the 45 kDa Flag-tagged B7H3 protein was expressed in situ (Fig. 1b, right lane). As expected, control homogenates of vector-only-treated tumors were not stained with the anti-Flag tag antibody (Fig. 1b, left lane).

*Arsenic trioxide synergizes with B7H3 immunotherapy to eradicate hepatoma*

It was possible that As$_2$O$_3$ might impair B7H3-mediated antitumor immunity, as tumor cells dying in response to As$_2$O$_3$ would not be able to adequately express the transgene. Hence established HCCs were first injected with B7H3 plasmid to stimulate antitumor immunity, and then, As$_2$O$_3$ was intratumorally injected 48 hr later. Remarkably, tumors rapidly diminished in response to the combination of B7H3 and As$_2$O$_3$, accompanied by massive necrosis, such that by the third week of treatment, tumors had completely disappeared leaving perfectly healed skin. In contrast, monotherapy with either B7H3 plasmid or As$_2$O$_3$ failed to eradicate tumors, but nevertheless each agent inhibited the growth of tumors for 1–3 weeks. As$_2$O$_3$ monotherapy was more effective than B7H3 monotherapy in inhibiting tumor growth. The tumors of control mice treated with either empty vector or PBS grew rapidly, reaching 2 cm in diameter within 2 weeks (Fig. 2).

**Statistical analysis**

Results were expressed as mean values ± standard deviation (SD), and a Student’s t-test was used for evaluating statistical significance. A value of less than 0.05 ($p < 0.05$) was used for statistical significance.

**Combinational therapy generates potent antitumor immunity**

Animals cured of their tumors by combination therapy completely rejected the challenge of 1 × 10⁶ parental H22 tumor cells, and a subsequent more substantial burden of 1 × 10⁷ H22 cells (Fig. 2). The level of IFN-γ detected in the sera of mice 4 days after treatment of tumors with the As$_2$O$_3$ and B7H3 combination was significantly increased compared with the levels in the sera of mice treated by As$_2$O$_3$ monotherapy, or with the control agents PBS and empty pcDNA3.1 vector. There was no significant difference in the levels of IFN-γ in the sera of mice treated with combinational therapy vs. those treated by B7H3 monotherapy, indicating that the production of IFN-γ can be attributed to B7H3 gene transfer (Fig. 3a). The level of IFN-γ detected in supernatants from cultured splenocytes harvested from mice 4 days after treatment showed a similar pattern to that of sera (Fig. 3b). Moreover, the antitumor cytotoxic activity of splenocytes obtained from mice 10 days after treatment with the combination of As$_2$O$_3$ and B7H3 was significantly augmented, compared with animals treated with either As$_2$O$_3$ monotherapy or PBS or empty pcDNA3.1 vector. Once again, there was no significant difference in the antitumor cytotoxic activity of splenocytes generated by mice treated with combinational therapy vs. those with B7H3 monotherapy, indicating that B7H3 gene transfer was responsible for the generation of cytotoxicity (Fig. 3c). These results suggested that As$_2$O$_3$ did not participate in the generation of antitumor immunity, but neither did it impair antitumor immunity. CD4⁺ and CD8⁺ lymphocytes and NK cells were individually depleted by specific antibodies in the assay, which measured the cytotoxic activity of splenocytes in order to define the effector-cell types responsible for antitumor activity. As shown in Figure 3d, the antitumor cytotoxic activity of splenocytes was significantly reduced by 43% ($p < 0.01$) upon depletion of CD8⁺ T cells, and by 37% ($p < 0.01$) upon depletion of NK cells, compared to controls in respect of splenocytes derived from mice treated by B7H3 monotherapy and the combi-
nation of B7H3 plus As$_2$O$_3$. In contrast, depletion of CD4$^+$ cells only slightly affected the cytotoxic activity of splenocytes ($p > 0.05$) (Fig. 3d). The results are consistent with our previous report that B7H3 mediated antitumor immunity largely depends on CD8$^+$ T cells and NK cells.16

Combination therapy targeted to a single tumor generates antitumor immunity capable of simultaneously eradicating multiple distant tumor nodules

The ultimate objective of cancer immunotherapy is to generate potent systemic antitumor immunity capable of simultaneously eradicating multiple tumor foci wherever they are located in the body and not to simply cause the destruction of a single treated tumor nodule. As reported above, mice cured by combination therapy completely rejected a substantial rechallenge of parental H22 cells, indicating that potent systemic antitumor immunity had been induced. A multiple tumor model was developed, in which a large tumor was established in 1 flank, and 4 small tumors in the opposing flank so as to provide a clinically relevant model for testing the therapeutic efficacy of As$_2$O$_3$ and B7H3 combination therapy. The B7H3 expression plasmid was injected into the large tumor, followed 48 hr later and every 2 days by injection of As$_2$O$_3$ until mice were cured of the primary tumor. The 4 smaller tumors were left untreated. Surprisingly, all 5 tumors completely regressed. The large tumor completely disappeared by the fourteenth day and the 4 smaller tumors were completely eradicated 1 week later (Fig. 4a). In contrast, all 5 tumors grew largely unchecked in mice treated by either B7H3, or As$_2$O$_3$ monotherapy, and in control mice.

The distant tumor nodules were immunostained with antibodies against specific leukocyte subsets so as to determine whether they had been subjected to an immune attack. The multiple distant tumor nodules from the control group contained few CD8$^+$ and CD4$^+$ T cells, and sparse numbers of NK cells (Fig. 4b). In contrast, highly elevated numbers of CD8$^+$ T cells and NK cells, and conversely small numbers of CD4$^+$ T cells infiltrated the tumors of mice treated with the combination of B7H3 and As$_2$O$_3$ (Fig. 4b). The results indicate that B7H3-mediated antitumor immunity, which relies largely on CD8$^+$ T cells and NK cells,16 is systemic since it is able to eradicate tumors wherever they are located in the body.

As$_2$O$_3$ suppresses tumor growth by inhibiting tumor angiogenesis and inducing tumor apoptosis, but does not induce antitumor immunity

Nodular tumors established in the left flank of mice were removed 3 weeks after intratumoral injection of As$_2$O$_3$, or PBS, sectioned and stained with an anti-CD31 antibody (Fig. 5a vs. 5b). As$_2$O$_3$ therapy resulted in a statistically significant ($p < 0.01$) 40%
reduction in tumor vessel density compared with mock treatment with PBS (Fig. 5). 

As$_2$O$_3$ has previously been demonstrated to induce cell apoptosis in many different types of tumors. Therefore, we examined whether HCCs underwent programmed cell death in response to As$_2$O$_3$ as measured by *in situ* labeling of fragmented DNA using the TUNEL method. Small numbers of apoptotic cells were de-

**Figure 4** – Combination therapy targeting a single tumor leads to the immune-mediated eradication of multiple distant tumor nodules. (a) Subcutaneous H22 HCCs (~0.5 cm in diameter) were established in the left flank (Tumor 1) and 4 small tumors (Tumors 2–5; 0.15–0.2 cm in diameter) in the right flank. B7H3 plasmid (100 μg) was injected into Tumor 1, and As$_2$O$_3$ was administered 48 hr later and thereafter every 2 days. Eradication of Tumor 1 is denoted by a vertical arrow, and eradication of Tumors 2–5 is denoted by asterisks. (b) Sections prepared from the distant tumor nodules of mice treated with B7H3 and As$_2$O$_3$ combinational therapy (Photos 2, 4 and 6), and those of control mice (Photos 1, 3 and 5) were immunostained with leukocyte-type specific antibodies against CD8$^+$ T cells (Photos 1 and 2), NK cells (Photos 3 and 4) and CD4$^+$ T cells (Photos 5 and 6).

**Figure 5** – Intratumoral injection of As$_2$O$_3$ inhibits tumor angiogenesis and increases tumor-cell apoptosis. Illustrated are representative tumor sections prepared 3 weeks following intratumoral injection of either PBS (a and c) or As$_2$O$_3$ (b and d). Tumor microvessels were stained with an anti-CD31 mAb (a and b, ×40 magnification), and by TUNEL analysis of apoptotic cells (c and d; green fluorescent cells showing condensed fragmented nuclei). Tumor blood vessels stained with the anti-CD31 mAb were counted in blindly chosen random fields to record mean vessel density per high power field (0.155 mm$^2$). n, number of tumors assessed. A significant difference in mean vessel counts between tumors treated with As$_2$O$_3$ vs. PBS (p < 0.01) is denoted by an asterisk (e). TUNEL-positive cells were counted to record the AI, which was significantly (p < 0.01, denoted by asterisk) different between tumors treated with As$_2$O$_3$, and PBS. n, numbers of tumors assessed (f).
ected in tumors treated with PBS (Fig. 5c), whereas tumor cell apoptosis was tripled following As2O3 treatment (Fig. 5d). Adjacent sections were stained with haematoxylin/eosin, and the AI was calculated. The AI for As2O3-treated tumors was significantly (p < 0.01) higher than that for PBS-treated tumors (Fig. 5f).

Sections prepared from As2O3-treated tumors were immunostained with specific antibodies against CD4+ and CD8+ lymphocytes and NK cells in order to determine whether repetitive injection of As2O3 might have an effect on the recruitment and penetration of immune cells within the tumor. The results demonstrated that there was no increase in the infiltration of immune cells into tumors treated by intratumoral injection of As2O3, compared with control tumors (data not shown).

**Discussion**

This present study has demonstrated for the first time that the activity of As2O3 against solid tumors can be dramatically enhanced by combining it with B7H3 immunotherapy. Intratumoral injection of As2O3 inhibited tumor angiogenesis and led to increased tumor-cell apoptosis. The combination of As2O3 and B7H3 immunotherapy completely eradicated large subcutaneous HCCs, and generated potent and memorized antitumor immunity as evidenced by protection against subsequent challenge of cured mice with a heavy burden of parental tumor cells. The systemic antitumor immunity generated by treating a single primary tumor was able to simultaneously eradicate multiple distant tumor nodules that were left untreated. The antitumor immunity directed against distant tumor nodules was largely dependent on the presence of CD8+ T and NK cells. The outcome was more successful than that achieved by treating HCC with As2O3 in combination with the chemotherapeutic agents cisplatin and doxorubicin.30

Following the discovery of As2O3 as a new and promising treatment for various types of leukemia, particularly APL, a large number of studies have investigated the use of As2O3 in the treatment of solid cancers, including neuroblastoma,27 head and neck,28 gastrointestinal,13 and renal cell carcinoma,29 esophageal,10 prostate,11 colorectal12 and hepatocellular13,26 cancers. However, the results achieved were not comparable with the previous success obtained with APL. As2O3 was found to induce the apoptosis of various cancer cell lines, including human hepatocarcinoma cell lines,32 and inhibited their growth in vitro, but the concentrations of As2O3 required were higher than those used against hematologic malignancies and not clinically achievable without the risk of As2O3-mediated side effects. Consequently, a search for agents suited to increase the efficacy of As2O3 against less sensitive solid tumors was initiated,30–32 based on the results achieved with APL.33 Strategies to reduce its toxicity, including intratumorally injected As2O3 exhibits antiangiogenic effects. The vascular status of the distant tumor nodules that had not directly received As2O3 was not examined. Theoretically, intratumorally injected As2O3 should leak into the circulation at a very low concentration. Roboz et al. reported that As2O3 causes dose-dependent apoptosis of the endothelium,46 indicating that As2O3 needs to reach a certain level to exert an antiangiogenic effect on tumors not directly injected with As2O3.

B7H3 immunotherapy stimulates the generation of antitumor cytotoxic T cells, and selectively enhances IFN-γ expression. IFN-γ plays a critical role in the immune-mediated destruction of tumors when expressed within the tumor bed.58 Its antitumor activity stems from its ability to control antigen processing and presentation, leukocyte trafficking and indirect tumor cytotoxicity.44 B7H3 immunogene therapy activates both acquired and innate immunity, as it leads to NK cell and CD8+ T-cell dependent killing of tumor cells.59 Moreover, NK cells are crucial to eradicating tumors that express low levels of MHC Class I. B7H3 was shown to co-stimulate a tumor-specific CD8+ CTL response in a recent report.60 It may also serve as an NK-cell receptor like other B7 family members, enabling NK-cell dependent killing.22 It also induces modest amounts of TNF-α, which could be cytotoxic to tumor cells. As2O3 may facilitate a B7H3-stimulated immune attack on large primary tumors by disrupting the integrity of the tumor so that it is more accessible to immune cells. Further, by preventing tumor cells from growing, it will inhibit the generation of immune escape variants. The B7H3-mediated immune response appears sufficient to eradicate small distant tumor nodules that, unlike large primary tumors, have not developed sophisticated immune defense mechanisms to ward of an immune attack, and are more readily accessible to immune effector cells.

In conclusion, these results confirm that cancer therapy employing either B7H3 or As2O3 alone is of limited therapeutic use against large HCCs. However, the efficacy of B7H3 immunotherapy and As2O3 therapy can be harnessed by combining the two therapies thereby obtaining a synergistic result that renders large tumors susceptible to immune attack. This approach warrants consideration for the treatment HCC.
As$_2$O$_3$ SYNERGIZES WITH IMMUNOTHERAPY TO COMBAT HCC

References

1. Antman KH. Introduction: the history of arsenic trioxide in cancer therapy. Oncologist 2001;3:1–2.
2. Sun BD, Ma L, Hu XC, Zong J. Al-Li treatment cured 32 cases of acute promyelocytic leukemia. Chin J Integrated Traditional Chin West Med 1992;12:170–1 [in Chinese].
3. Soignet SL. Clinical experience of arsenic trioxide in relapsed acute promyelocytic leukemia. Oncologist 2001;6:11–6.
4. Shen Y, Shen ZX, Van H, Chen J, Zeng XY, Li JM, Li XS, Wu W, Xiong SM, Zhao WL, Tang W, Wu F, et al. Studies on the clinical efficacy and pharmacokinetics of low-dose arsenic trioxide in the treatment of relapsed acute promyelocytic leukemia: a comparison with conventional dosage. Leukemia 2001;15:735–41.
5. Dai J, Weinberg SR, Waxman S, Jing Y. Malignant cells can be sensi-
tised to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood 1999;93:268–77.
6. Chen YC, Lin-shiau SY, Lin JK. Involvement of reactive oxygen species and caspase 3 activation in arsenic-induced apoptosis. J Cell Physiol 1998;177:324–33.
7. Park WH, Seol JG, Kim ES, Hyun JM, Jung CW, Lee CC, Kim BK, Lee YY. Arsenic trioxide-mediated growth inhibition in MC/CAr myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. Cancer Res 2000;60:3065–71.
8. Zhu XH, Shen YL, Jing YK, Cai X, Jia PM, Huang Y, Tang W, Shi KY, Sun YP, Dai J, Wang ZY, Chen SJ, et al. Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. J Natl Cancer Inst 1999;91:772–8.
9. Usho R, Sanli UA, Sezgin C, Karabulut B, Terzioglu E, Omary SB, Goker E. Arsenic Trioxide-mediated cytotoxicity and apoptosis in prostate and ovarian cell lines. Clin Cancer Res 2000;6:4957–64.
10. Shen ZY, Zhang Y, Chen YJ, Chen MH, Shen J, Luo WH, Zeng Y. Intratumoral injection of arsenic to enhance tumor efficacy in human esophageal cancer cell xenografts. Oncol Rep 2004;11:155–9.
11. Maeda H, Hori S, Nishitoh H, Ichijio H, Ogawa O, Kakehi Y, Kakinouchi O, et al. Arsenic trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis. Biochem Biophys Commun 2003;300:230–5.
12. Bauernfeind-Hofmann T, Gitteringer B, Grumbeck E, Gisslinger H. Arsenic trioxide and ascorbic acid: synergy with potential implications for the treatment of acute myeloid leukemia? Br J Haematol 2001;112:73–8.
13. Seol JG, Park WH, Kim ES, Jung CW, Hyun JM, Kim BK, Lee YY. Effect of arsenic trioxide of cell cycle arrest in head and neck cancer cell line PCH-1. Biochem Biophys Commun 1999;265:400–4.
14. Hyun PW, Hoc CY, Won IC, Oh PJ, Kim K, Hyuck JY, Lee MH, Ki KW, Park K. Arsenic trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis. Biochem Biophys Commun 2003;300:230–5.
15. Goker E. Arsenic Trioxide-mediated cytotoxicity and apoptosis in human neuroblastoma cells and function in a PML and PML-RARa translocated cell line. J Neurooncol 2004;69:497–504.
16. Hyun PW, Hee CY, Won IC, Lee SJ, Park MJ, Rhee CH, Hong SI, Chung HY. Caspase-independent signals apoptosis-inducing factor release from mitochondria. Cancer Res 2004;64:9603–7.
17. Vinh D-T, Zhang T, Wang XJ, Hong L, Qi HQ. Effect of arsenic trioxide on rat hepatocarcinoma and its renal cytotoxicity. World J Gastroenterol 2003;9:930–5.
18. Ishiwa K, Nakajo S, Aiuchi T, Nakaya K. Apoptosis induction by arsenic trioxide in leukemia U937 cells is independent of activation of p38, inactivation of ERK and the Ca2+–dependent production of superoxide. Int J Cancer 2001;92:518–26.
19. Shen ZY, Shen WY, Shen MH, Jai CJ, Wu ZY, Zeng Y. Morphological and functional changes of mitochondria in apoptotic esophageal carcinoma cells induced by arsenic trioxide. World J Gastroenterol 2002;8:31–5.
20. Shen ZY, Shen WY, Shen MH, Jai CJ, Wu ZY, Zeng Y. Nitric oxide and calcium ions in apoptotic esophageal carcinoma cells induced by arsenic trioxide. World J Gastroenterol 2002;8:40–3.
21. Woo SH, Park IC, Park MJ, Lee HC, Lee SJ, Chun YJ, Lee SH, Hong SI, Rhee CH. Arsenic trioxide induces apoptosis through a reactive oxygen species-dependent pathway and loss of mitochondrial membrane potential in HeLa cells. Int J Oncol 2002;21:57–63.
22. Ohnishi K, Yoshida H, Shigeno K, Nakamura S, Fujisawa S, Naito K, et al. Nitric oxide and ascorbic acid: synergy with potential implications for the treatment of acute myeloid leukemia? Br J Haematol 2001;112:73–8.
23. Takayama T, Sekine T, Makuuchi M, Yamashita K, Kusuda Y, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy for postoperative recurrence rates of hepatocellular carcinoma in randomised trial. Lancet 2000;356:802–7.
24. Sun X, Qiao H, Jiang H, Zhu X, Liu F, Wang J, Liu M, Xu R, Kanwar JR, Krissansen GW. Intramuscular delivery of anti-angiogenesis genes supplements secondary metastasis after removal of primary tumors. Cancer Gene Ther 2005;12:35–45.
25. Mendiratta SK, Quezada A, Matar M, Wang J, Hebel HL, Long S, Nordstrom JL, Pericle F. Intratumoral delivery of IL-12 gene by poly- vinyl poly(ethylene glycol) vector system results in potent antitumor immunity. Gene Ther 1999;6:833–9.
26. Wang W, Qing S-K, Chen B-A, Chen H-Y. Experimental study on antitumor effect of arsenic trioxide in combination with cisplatin or doxorubicin on hepatocellular carcinoma. World J Gastroenterol 2001;7:702–5.
27. Akae Y, Nakagawa Y, Akiyama K. Arsenic trioxide induces apoptosis in neuroblastoma cell lines through the activation of caspase3 in vitro. FEBS Lett 1999;455:61–62.
28. Seol JG, Park WH, Kim ES, Jung CW, Hyun JM, Kim BK, Lee YY. Effect of arsenic trioxide of cell cycle arrest in head and neck cancer cell line PCH-1. Biochem Biophys Commun 1999;265:400–4.
29. Hyun PW, Hoc CY, Won IC, Oh PJ, Kim K, Hyuck JY, Lee MH, Ki KW, Park K. Arsenic trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis. Biochem Biophys Commun 2003;300:230–5.
30. Bachleitner-Hofmann T. Arsenic trioxide-mediated apoptosis using docosa-
heaxenoic acid in arsenic trioxide-resistant solid tumor cells. Int J Cancer 2004;112:707–12.
31. Shen ZY, Shi ZZ, Fang J, Gu BW, Li JM, Zhu YM, Shi JY, Zheng FY, Han HY, Liu YF, Chen Y, Shen Y, et al. All-trans retinoic acid/As$_2$O$_3$ combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc Nat Acad Sci USA 2004;101:5328–35.
32. Baumgartner M, Sturlan S, Roth E, Wessner B, Bachleitner-Hofmann T. Enhancement of arsenic trioxide-mediated apoptosis using docosa-
heaxenoic acid in arsenic trioxide-resistant solid tumor cells. Int J Cancer 2004;112:707–12.
33. Takayama T, Sekine T, Makuuchi M, Yamashita K, Kusuda Y, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy for postoperative recurrence rates of hepato-cellular carcinoma in randomised trial. Lancet 2000;356:802–7.
44. Gochi A, Orita K, Fuchimoto S, Tanaka N, Ogawa N. The prognosis advantage of preoperative intratumoral injection of OK-432 for gastric cancer patients. Br J Cancer 2001;84:443–51.
45. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nature Rev Cancer 2004;4:423–36.
46. Roboz GJ, Dias S, Lam G, Lane WJ, Soignet SL, Warrell RP, Jr, Rafii S. Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. Blood 2000;96:1525–30.
47. Yeh JY, Cheng LC, Liang YC, Ou BR. Modulation of the arsenic effects on cytotoxicity, viability, and cell cycle in porcine endothelial cells by selenium. Endothelium 2003;10:127–39.
48. Tannenbaum CS, Hamilton TA. Immune-inflammatory mechanisms in IFN-γ-mediated anti-tumor activity. Sem Cancer Biol 2000;10:113–23.
49. Luo L, Chapoval AI, Flies DB, Zhu G, Hirano F, Wang S, Lau JS, Dong H, Tamada K, Flies AS, Liu Y, Chen L. B7-H3 enhances tumor immunity in vivo by costimulating rapid clonal expansion of antigen-specific CD8+ cytolytic T cells. J Immunol 2004;173:5445–50.
50. Wilson JL, Charo J, Martín-Fontecha A, Dellabona P, Gasorati G, Chambers BJ, Kiessling R, Bejarano MT, Ljunggren HG. NK cell triggering by the human costimulatory molecules CD80 and CD86. J Immunol 1999;163:4207–12.