Arsenic trioxide treatment decreases the oxygen consumption rate of tumor cells and radiosensitizes solid tumors

Caroline Diepart¹, Oussama Karroum¹, Julie Magat¹, Olivier Feron², Julien Verrax³, Pedro Bue Calderon³, Vincent Grégoire⁴, Philippe Leveque¹, Julie Stockis⁵, Nicolas Dauguet⁵, Bénédicte F. Jordan¹ and Bernard Gallez¹

1: Louvain Drug Research Institute, Biomedical Magnetic Resonance Group, Université catholique de Louvain, Avenue Mounier 73, B-1200 Brussels, Belgium
2: Laboratory of Pharmacotherapy, Université catholique de Louvain, Avenue Mounier 52, B-1200 Brussels, Belgium
3: Louvain Drug Research Institute, Laboratory of Pharmacokinetics, Metabolism, Nutrition and Toxicology, Université catholique de Louvain, Avenue Mounier 73, B-1200 Brussels, Belgium
4: Center for Molecular Imaging and Experimental Radiotherapy, Université catholique de Louvain, Avenue Hippocrate, 10, B-1200 Brussels, Belgium
5: de Duve Institute, Université catholique de Louvain, Avenue Hippocrate 75, B-1200 Brussels

Corresponding author:
Prof. Bernard Gallez, PhD
REMA
Avenue Mounier 73.40
B-1200 Brussels
Belgium
bernard.gallez@uclouvain.be
phone: 32 2 7647391
fax: 32 2 764.73.90

Manuscript submitted to Cancer Research.

Running Title: Arsenic trioxide radiosensitizes solid tumors
Abstract

Arsenic trioxide (As$_2$O$_3$) is an effective therapeutic against acute promyelocytic leukemia (APL) and certain solid tumors. Because As$_2$O$_3$ inhibits mitochondrial respiration in leukemia cells, we hypothesized that As$_2$O$_3$ might enhance the radiosensitivity of solid tumors by increasing tumor oxygenation (pO$_2$) via a decrease in oxygen consumption. Two murine models of radioresistant hypoxic cancer were used to study the effects of As$_2$O$_3$. We measured pO$_2$ and the oxygen consumption rate in vivo by electron paramagnetic resonance oximetry and $^{19}$F-MRI relaxometry. Tumor perfusion was assessed by Patent blue staining. In both models, As$_2$O$_3$ inhibited mitochondrial respiration, leading to a rapid increase in pO$_2$. The decrease in oxygen consumption could be explained by an observed decrease in glutathione in As$_2$O$_3$-treated cells, as this could increase intracellular reactive oxygen species (ROS) that can disrupt mitochondrial membrane potential. When tumors were irradiated during periods of As$_2$O$_3$-induced augmented oxygenation, radiosensitivity increased 2.2-fold compared to control mice. Notably, this effect was abolished when temporarily clamped tumors were irradiated. Together, our findings demonstrate that As$_2$O$_3$ acutely increases oxygen consumption and radiosensitizes tumors, providing a new rationale for clinical investigations of As$_2$O$_3$ in irradiation protocols to treat solid tumors.
Introduction

The partial pressure of oxygen (pO$_2$) is a crucial factor in the response of tumors to irradiation and other cytotoxic treatments. Several studies have shown superior outcomes for cancer patients whose tumors had lower hypoxic fractions (1). Tumor hypoxia results from an imbalance between oxygen delivery and oxygen consumption, either of which may potentially be targeted by therapeutic interventions in order to transiently alleviate tumor hypoxia and potentiate cytotoxic treatments. It has been suggested that modifying oxygen consumption is more efficient at alleviating hypoxia than modifying oxygen delivery (2). Several pharmacological drugs that inhibit cellular oxygen consumption have been characterized for their potential to increase tumor oxygenation and thereby enhance radiosensitivity. Meta-iodobenzylguanidine (3), insulin (4), anti-inflammatory drugs (5), corticoids (6), some antagonists of vascular endothelial (VEGF) receptor tyrosine kinase (SU5416 and ZD6474) (7, 8), and thyroid hormones (9), all play a major role in the metabolism of tumor cells by modifying the rate of oxygen consumption.

In the 1970s, arsenic trioxide (As$_2$O$_3$) was reported to induce complete remission in patients with acute promyelocytic leukemia (APL) in China (10). Additional studies confirmed that low doses of As$_2$O$_3$ could induce complete remission in 90% of relapsed APL patients (10). Importantly, it has become evident that the apoptotic effects of As$_2$O$_3$ are not restricted to APL cells but have also been observed in other malignant cells in pre-clinical studies, including myeloma cells, chronic myeloid leukemia cells, and various solid tumors cells, such as prostate, oesophageal and ovarian carcinomas (11-13).

Arsenic acts on cells through a variety of mechanisms, influencing numerous signal transduction pathways and resulting in a vast range of cellular effects that include apoptosis induction, growth inhibition, promotion or inhibition of differentiation and inhibition of angiogenesis (11, 14-16). As$_2$O$_3$ also seems to inhibit mitochondrial respiratory function in
human leukemia cells (17). We therefore hypothesized that As$_2$O$_3$ could be an important modulator of tumor oxygenation in solid tumors by affecting the oxygen consumption of tumor cells. The first step of the study was to monitor pO$_2$ in experimental tumors using EPR oximetry (18) after administration of As$_2$O$_3$ in order to determine the window of increased tumor oxygenation. These results were confirmed by $^{19}$F-MRI oxygen mapping, a technique that can probe the spatial heterogeneity of response (19, 20). Patent blue staining (blood flow) and in vitro oxygen consumption experiments were performed to investigate the origin of the observed increase in pO$_2$. The intracellular GSH content and the mitochondrial membrane potential were also evaluated to explain a possible mechanism by which As$_2$O$_3$ could decrease oxygen consumption. Finally, the window of increased oxygenation was exploited to enhance response of tumors to radiation therapy. Our study is the first report of the acute effect of As$_2$O$_3$ on oxygen consumption in solid tumors and provides a new rationale for combining As$_2$O$_3$ with radiation therapy.

**Materials and Methods**

**Tumor model**

Two tumor models were implanted by intramuscular injection in the rear leg of male mice: the transplantable mouse liver tumor (TLT) model in NMRI mice and the Lewis lung carcinoma (LLC) in C57Black6N mice. Measurements were performed when the tumor size reached 8.0 ± 1.0 mm. All animal experiments were conducted in accordance with national animal care regulations.

**Treatments**

As$_2$O$_3$ was purchased from Sigma-Aldrich (Bornem, Belgium). For the treated group, As$_2$O$_3$ was dissolved in PBS (Invitrogen) and given by intraperitoneal injection (5 mg/kg body weight, 100 μl injected). Control animals were treated with PBS only. Animals were
anesthetized by inhalation of isoflurane mixed with air in a continuous flow (3% induction, 1.8% maintain for a minimum of 15 minutes before any measurement).

**Tumor oxygenation**

**EPR oximetry**

EPR oximetry, using charcoal (CX 0670-1; EM Sciences, NJ) as the oxygen sensitive probe, was used to evaluate changes in tumor oxygenation after treatment with As$_2$O$_3$, using a protocol described previously (21). EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low frequency microwave bridge operating at 1.2 GHz and an extended loop resonator, or using a Bruker Elexsys system (Rheinstetten, Germany) with an L-band microwave bridge working at 1.1 GHz, and equipped with an E540R23 L-Band EPR coil resonator. A suspension of charcoal was slowly injected into the center of the tumor 1 day before measurement (100 mg/mL; 70 μL injected, particle size of 1-25 μm; needle diameter: 0.4 mm). The acute effect of As$_2$O$_3$ was measured by following the tumor pO$_2$ status before and for 2 hours after the single injection.

**$^{19}$F-MRI measurements**

MRI was performed with a 4.7T, 40 cm inner diameter bore system (Bruker Biospec, Ettlingen, Germany) and a tunable $^1$H/$^{19}$F surface coil. Parametric images of the spin-lattice relaxation time ($T_1$) were estimated using a snapshot inversion recovery (SNAP-IR) pulse sequence, using a protocol described previously (19). HFB was slowly injected into the tumor and deposited along three tracks (3×30 μl) encompassing central and peripheral regions. As$_2$O$_3$ was administered in 5 mice by a catheter and the tumor pO$_2$s were monitored for 2 h. Two measurements were acquired as baseline before injection. The mice used for this study were different from those used for EPR oximetry.

**Patent Blue staining**


Patent Blue (Sigma-Aldrich) was used to obtain a rough estimate of the TLT tumor perfusion fraction 1h30 after administration of As$_2$O$_3$ or PBS, using a protocol described previously (7,8). The assay was applied on a separate cohort. Briefly, this technique involved the injection of 200 μl of Patent Blue solution (1.25%) into the tail vein of the mice, sacrifice after 1 min, tumor excision and cutting into two size-matched halves. For each tumor, the percentage stained area of the whole cross section was determined using an in-house program running on MatLab, used as an indicator of tumor perfusion fraction.

**In-Vitro Evaluation of Oxygen Consumption Rate**

TLT cells were cultured in DMEM containing 10% FBS, 4.5 mg/L glucose and 1% penicillin-streptomycin. Confluent cells were suspended in medium without serum 2h before treatment with As$_2$O$_3$ (25μM) or PBS. EPR spectra were recorded on a Bruker EMX EPR spectrometer operating at 9 GHz. Tumor cells (2 X 10$^7$/ml) were suspended in 10% dextran in complete medium. A neutral nitroxide, $^{15}$N 4-oxo-2,2,6,6-tetramethylpiperidine-d$_{16}$-$^{15}$N-1-oxyl, at 0.2 mM (CDN Isotopes, Pointe-Claire, Canada), was added to 100 μl aliquots of tumor cells that were then drawn into glass capillary tubes. Oxygen consumption rates were obtained by measuring the pO$_2$ in the closed tube over time and finding the slope of the resulting linear plot, using a protocol described previously (9).

**Cell survival assay**

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH) both in the culture medium and in the cell pellet obtained after centrifugation (22). The results are expressed as the ratio of released activity to total activity.

**Tumor regrowth delay assay**

The TLT tumor-bearing leg was irradiated locally with 10Gy of 250 kV X-rays (RT 250; Philips Medical Systems; 1.2Gy/min). The tumor was centered in a 3 cm circular irradiation field. A single dose irradiation of 10 Gy was given 90 min after injection of As$_2$O$_3$ or PBS.
After radiotherapy, tumor growth was determined daily using a caliper until the diameter reached 16 mm, at which time the mice were sacrificed. A linear fit was performed between 8 and 16 mm, which allowed determination of the time to reach a particular size for each mouse.

**Measurement of intracellular GSH**

The glutathione content was determined using the Tietze enzyme recycling assay (23), with slight modifications (24). TLT cells were cultured in DMEM containing 10% FBS, 4.5 mg/L glucose and 1% penicillin-streptomycin. Tumor cells were treated with As$_2$O$_3$ (25 μM) or PBS during 90 minutes for TLT cells. Cells were then washed twice with ice-cold phosphate buffered saline and then lysed with a solution of 5-sulfosalicylic acid (5%). After two freeze-thaw cycles, samples were centrifuged at 10000 g for 10 minutes and the resulting supernatants were kept at -80°C. Ten microliters of the samples were then placed in a mixture containing 0.2 U/ml of glutathione reductase, 50 μg/ml 5,5'-Dithio-bis(2-nitrobenzoic acid) and 1 mM EDTA at pH 7. The reaction was initiated by the addition of 50 μM NADPH and changes in absorbance were recorded at 412 nm. Reduced (GSH) and oxidized (GSSG) glutathione were distinguished by the addition of methyl-2-vinylpyridine and their respective concentrations were determined from appropriate standard curves. Results were normalized to the protein content using the method of Lowry (25).

**Measurement of mitochondrial membrane potential**

TLT cells were treated with As$_2$O$_3$ (25μM) in 6 well plates. Mitochondrial membrane potential was monitored using a fluorescent cationic dye known as JC-1 (Sigma mitochondria staining kit). In healthy cells, JC-1 enters the negatively charged mitochondria where it aggregates and fluoresces red. In cells where the mitochondrial potential has collapsed, JC-1 exists as monomers throughout the cell. When dispersed in this manner JC-1 fluoresces green. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green
fluorescence intensity ratio (26). Data acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR). Valinomycin and carbonyl cyanide m-chlorophenylhydrazone (mCICCP) were used as positive controls (depolarizing agents).

**Statistical analysis**

Means ± SEs were compared using the unpaired Student’s *t* test or ANOVA (multiple comparisons post tests for 3 groups or more). *P* values < 0.05 were considered statistically significant.
Results

As$_2$O$_3$ rapidly increases tumor oxygenation

The administration of As$_2$O$_3$ at 5 mg/kg induced an acute increase in pO$_2$ in TLT (Fig.1A) and LLC (Fig.1B) tumors, an effect that was not observed for the control group (Fig. 1A-B) or for the group treated at 1 mg/kg of As$_2$O$_3$ (data not shown). After injection of As$_2$O$_3$, the pO$_2$ increased rapidly in TLT and LLC tumors, after which pO$_2$ decreased, enabling a window of the increased oxygenation to be determined (Fig.1). For TLT tumors, the mean tumor pO$_2$ (measured by EPR oximetry) after 90 min was 38.6 ± 7.2 mmHg for treated mice (n=6) and 4.3 ± 0.5 mmHg for controls (n=6) (P< 0.01, $t$-test). For LLC tumors, the mean pO$_2$ after 45 min was 10.5 ±1.3 mmHg for treated mice (n=5) and 4.3 ± 0.3 mmHg for control mice (n=5) (P<0.01, $t$-test). The effect of 5 mg/kg of As$_2$O$_3$ on pO$_2$ was further confirmed by $^{19}$F-MRI relaxometry in TLT tumors, which provides an estimation of the temporal and spatial heterogeneity of response. The mean tumor pO$_2$ was 15.9 ± 2.2 mmHg before the injection of the drug and 30.8 ± 5.5 mmHg 60 min after As$_2$O$_3$-injection (time of maximal pO$_2$, according to this oximetry technique) (P<0.05, $t$-test, n=5). The evolution of pO$_2$ over time, as measured by $^{19}$F-MRI relaxometry, is shown in Fig.2. $^{19}$F-MRI relaxometry confirmed the trend of an early increase in pO$_2$ after As$_2$O$_3$ administration, although the treated and control dynamic curves were not significantly different due to a large variation in the pO$_2$ readings. Using $^{19}$F-MRI relaxometry, it is possible to probe the spatial heterogeneity of response: color pO$_2$ maps and their corresponding histograms were generated, as shown in Fig.3A. The color maps show an increase in pO$_2$ after treatment with As$_2$O$_3$, marked by a global increase in the red color (corresponding to pO$_2$ higher than 10 mmHg). The histograms show a clear shift of the pO$_2$ values to the right after As$_2$O$_3$ treatment (n=5) (Fig.3B). For all further experiments (flow estimation, and therapeutic relevance), the experiments were conducted 90 min after injection of As$_2$O$_3$ in mice bearing the TLT tumor model.
The reoxygenation induced by As$_2$O$_3$ is mediated by an effect on oxygen consumption

To explain the increase in pO$_2$ induced by As$_2$O$_3$, blood perfusion in TLT tumors was investigated using the Patent blue staining assay 90 min after As$_2$O$_3$ injection (Fig.4A-B). This method has previously been validated by our group and compared with DCE-MRI data (6, 7). The colored area observed in tumors 1 min after injection of the dye (Patent blue) was decreased in As$_2$O$_3$-treated mice (n=8) compared to control mice (n=7) (38.8 ± 4.6% versus 61.2 ± 4.9%, P< 0.01, Fig.4A), indicating that blood perfusion fraction was decreased in treated mice. Typical images are shown in Fig.4B.

The oxygen consumption was investigated in vitro on TLT tumor cells exposed to As$_2$O$_3$ during 90 min. The incubation in the presence of As$_2$O$_3$ significantly decreased the rate of oxygen consumption (Fig.5), with mean slopes of -1.68 ± 0.07 μM/min (n=6) and -3.5 ± 0.1 μM/min (n=7) (P<0.001) for treated and control cells, respectively. As$_2$O$_3$-treated cells thus consumed oxygen 2 times slower than control cells. To exclude a possible direct cytotoxic effect of As$_2$O$_3$, we also measured the activity of LDH in TLT cells 90 min and 4 h after incubation in the presence of As$_2$O$_3$. Treatment induced LDH leakage of 23.4 ± 2.5% (90 min after treatment) and 27 ± 2.1% (4h after treatment) compared to 34.1 ± 2.1% and 34.2 ± 2.3% (similar timings) in control mice. These results indicate that this concentration of As$_2$O$_3$ (25μM) did not influence the viability of TLT cells early after the exposure (one-way ANOVA).

To determine whether the lowering of intracellular GSH content was a possible mechanism by which As$_2$O$_3$ could decrease the oxygen consumption of tumor cells, we measured the intracellular content of GSH. GSH contents were 18.3 nmol/mg protein and 42.6 nmol/mg protein for TLT treated and control cells, respectively (Fig.6A). Inhibiting GSH production may lead to oxidative stress by enhancing intracellular ROS, and accumulation of...
intracellular ROS leads to disruption of the mitochondrial membrane potential. This hypothesis is supported by the fact that the number of TLT cells with depolarized mitochondria was increased in the presence of As$_2$O$_3$ as shown in the JC-1 assay (Fig. 6B).

**As$_2$O$_3$ radiosensitizes tumors by an oxygen effect**

To evaluate the effect of As$_2$O$_3$ on tumor regrowth after irradiation, tumor-bearing mice were irradiated with a single dose of 10Gy of X-rays. Figure 7A shows the tumor growth of TLT tumors treated or not with As$_2$O$_3$, with or without irradiation. In the non-irradiated groups, there was no significant difference between tumors treated with PBS and those treated with As$_2$O$_3$ (P>0.05). All irradiated groups showed a significant regrowth delay compared to their respective control group (P<0.01). When irradiation was applied during the time window of increased oxygenation produced by As$_2$O$_3$ administration, the regrowth delay (33.2 ± 2 days to reach 12 mm) was significantly increased compared with irradiation alone (14.8 ± 2.4 days) (P<0.001, one-way ANOVA, Tukey’s multiple comparison test, Fig. 7B), resulting in a 2.2-fold increase in radiation response. Importantly, tumors were individually monitored with EPR oximetry after administration of As$_2$O$_3$ to make sure that the pO$_2$ was increased at the time of irradiation. To discriminate between an oxygen effect and a direct radiosensitizing effect, we also irradiated a group of As$_2$O$_3$-treated mice whose legs had been temporarily ligated to induce complete hypoxia at the time of irradiation. We checked the efficiency of the ligation by measuring pO$_2$ using EPR oximetry: in these conditions, the tumors were anoxic (pO$_2$ = 0.1±0.2 mm Hg (n=3) after leg ligation). The regrowth delays were similar for the control irradiated group and the As$_2$O$_3$+hypoxia-irradiated group (Fig. 7B). Finally, in an independent set of mice that were not treated by As$_2$O$_3$, we found no significant difference in regrowth delays between an irradiated group and a similar group that is deprived with oxygen at the time of irradiation (by leg ligation) (12+/− 0.6 days vs 11.1 +/- 0.9 days), indicating that
this tumor model presents a highly hypoxic pattern that is relevant to study hypoxia-induced radioresistance. \( \text{As}_2\text{O}_3 \) therefore induces an additional regrowth delay due to an oxygen effect. In Fig.7C, we used Kaplan-Meier curves to compare survival times (times at which mice were sacrificed, when the tumor diameter reached 16 mm) in the different groups. \( \text{As}_2\text{O}_3 \) administration combined with radiation extended the median survival of mice by more than 20 days compared to control.
Discussion

The major findings of the present study are: 1) As$_2$O$_3$ significantly reduced the tumor hypoxic fraction (pO$_2$ < 10mmHg) early after administration of a single dose; 2) the early increased oxygenation effect was linked to a decrease in tumor cell oxygen consumption rate; 3) As$_2$O$_3$ significantly increased the effectiveness of tumor radiotherapy when irradiation was given in the time window of increased oxygenation.

In this study, we report that As$_2$O$_3$ can induce an acute and transient increase in tumor oxygenation in experimental tumors. The basal pO$_2$ values and the time window of the increased oxygenation in TLT tumors measured using EPR and $^{19}$F-MRI relaxometry techniques were not exactly the same. The differences in observed pO$_2$ readings coming from these techniques can be partly explained by different factors that have been discussed in details in other methodological publications, including differences in sampling volumes (18-20, 27). The fact that we used two independent set of tumor-bearing mice may also explain some differences observed. Despite differences in the nature of the measurements, both techniques indicated that the increase in oxygenation was rapidly occurring after As$_2$O$_3$ administration, lasted for more than one hour, and that this effect was distributed in all areas of the tumors. A previous study reported an increase in pO$_2$, measured with an Eppendorf pO$_2$ histograph, after chronic administration of As$_2$O$_3$ in FSa II tumors; the maximal increased oxygenation was observed at day 3 (28). The authors interpreted the As$_2$O$_3$-induced increase in tumor oxygenation to be related to an increased supply of oxygen to the remaining viable regions of the tumor and a decrease in total oxygen demand of the tumor because of the significant amount of cell death involved in As$_2$O$_3$-induced necrosis (28). As the increased tumor oxygenation may result from an increase in oxygenation delivery and/or a decrease in oxygen consumption, we investigated both aspects to determine the origin of the observed effect responsible for the rapid change in tumor oxygenation. We showed a decrease in tumor
perfusion after administration of As$_2$O$_3$, which excludes this parameter as a possible cause of the early tumor reoxygenation. This is in accordance with other studies that demonstrated that a single dose of As$_2$O$_3$ induced a decrease in perfusion in tumors (28, 29, 30). Tumor cell oxygen consumption was reduced significantly after exposure to As$_2$O$_3$. We excluded a possible direct cytotoxic effect of As$_2$O$_3$ by measuring the activity of LDH in TLT cells after As$_2$O$_3$ exposure. LDH assay is a well recognized assay to assess plasma membrane breakdown as a sign of cytotoxicity. We excluded the MTT assay because it is dependent on the mitochondrial function, and a clonogenic assay because the effect of As$_2$O$_3$ was rapid and transient. Our results indicate that the concentration of As$_2$O$_3$ used in this assay (25μM) did not influence the viability of TLT cells early after the exposure. A mathematical model predicted that modification of oxygen consumption would be much more efficient at alleviating hypoxia than modification of oxygen delivery (2), a theoretical hypothesis that has been extensively demonstrated experimentally by our group (4-9). Thus, it is theoretically and experimentally possible for tumor oxygenation to improve even in the face of diminished perfusion (31). In comparison with previously described approaches to target oxygen consumption in TLT tumors (32), As$_2$O$_3$ induces a higher increase in oxygenation than all previously considered inhibitors. Also, the resulting factor of increase in tumor regrowth delay is above 2, which is superior to the effect observed with the majority of consumption inhibitors.

The effects of As$_2$O$_3$ on oxygen consumption have already been observed in vitro by others and the suggested mechanism was the inhibition of respiration upstream of complex IV in the mitochondrial respiratory chain (17). Another mechanism could involve the redox status of the tumor. The GSH (glutathione) redox system is known to modulate the effects of As$_2$O$_3$. Previous findings showed that sensitivity to As$_2$O$_3$-induced apoptosis was inversely related to the intracellular GSH content and that pharmacological modulation of intracellular
GSH content altered sensitivity to As$_2$O$_3$ (33). A study also showed that As$_2$O$_3$-induced adhesion molecule expression *in vitro* was abolished when the anti-oxidant N-acetyl-cysteine (NAC) was introduced prior to exposure, while the addition of NAC *in vivo* partially blocked As$_2$O$_3$-induced vascular shutdown (30). In our study, we observed a decrease in GSH content in As$_2$O$_3$-treated cells compared to control cells. This decrease in GSH levels could explain the inhibitory effect of As$_2$O$_3$ on oxygen consumption. Indeed, the GSH redox system represents one of the most important cellular defense systems against oxidative stress. Inhibiting GSH production may lead to oxidative stress by enhancing intracellular ROS, and accumulation of intracellular ROS leads to disruption of the mitochondrial membrane potential (34). This hypothesis is supported by the fact that the number of TLT cells with depolarized mitochondria was increased after exposure to As$_2$O$_3$. It is important to note that changes in oxygen consumption can occur a long time before changes in membrane potential become measurable (26). Overall, these observations indicate that As$_2$O$_3$ could act on the mitochondrial respiratory chain by enhancing intracellular ROS production mediated by decreased GSH levels. Although these experiments provide rational mechanisms that may explain the change in oxygen consumption by the tumor cells and increase in tumor oxygenation, it is important to note that it is difficult to extrapolate the kinetics of the effects observed in vivo from these in vitro experiments, as the result will be dependent on the dynamic evolution in concentration of As$_2$O$_3$ (perfusion and wash-out) inside the solid tumor.

We conducted radiosensitizing experiments to test the therapeutic value of the use of As$_2$O$_3$ in combination with radiation therapy. There was a significant increase in the response of tumors to radiotherapy (by a factor of 2.2) when X-ray irradiation was applied during the increased oxygenation window. As$_2$O$_3$ has previously been shown to induce tumor growth delay and to improve fractionated radiotherapy response in other studies that considered different mechanisms and timings of administration (28, 29, 35, 36). Lew et al. showed a
significant regrowth delay after a single dose or fractionated schedule of radiation when 
As$_2$O$_3$ was administered 60 min after radiation, explained by the increased production of 
TNF-α, known to enhance the antitumor effects of radiation (29). In other tumor cell lines, it 
appeared that As$_2$O$_3$ was also able to directly radiosensitize tumor cells, contrarily to our 
findings in TLT cells (36). One area related to As$_2$O$_3$ exposure that has been widely studied is 
the depletion of the glutathione level in cells, which may lead to oxidative stress and has been 
linked to increases in radiosensitivity (36, 37). Griffin et al. reported the greatest regrowth 
delay when combining treatment and radiation every 3 days, at the time of maximal tumor 
oxygenation in their model, suggesting that the oxygen level is a factor that is important in 
terms of radiosensitization by As$_2$O$_3$ (28). In these chronic experiments, the main factor was 
likely the decrease in oxygen demand due to the cell death. In the present study, the acute 
increased oxygenation after administration of a single dose of As$_2$O$_3$ was likely due to an 
effect on the mitochondrial respiration. Furthermore, tumors that were clamped during the 
irradiation were not radiosensitized, which identifies the ‘oxygen effect’ as the major factor 
responsible for the rapid radiosensitization of TLT tumors by As$_2$O$_3$, rather than an intrinsic 
direct radiosensitizing effect of the drug.

It is important to mention that there are large differences between the dose used in 
humans and animals. The usual dose used to treat APL is 0.15 mg/kg/day, and larger doses 
(up to 35 mg/kg/day) have been used in phase II clinical trials in patients with metastatic 
melanoma and renal cell carcinoma (38,39,40). In mice and rats, the usual doses ranged from 
2 to 8 mg/kg (28,30) and generally exceed the doses used in humans. This is related to the 
difference in route of administration (IV vs IP), the good tolerance of mice to arsenic trioxide 
(The LD50 in mice is 11-11.8 mg/kg i.p) which is linked to a difference in metabolism. The 
metabolism of arsenic in humans produces more toxic methylated arsenic compounds than in 
other animals (41). Even at high dose (6.5mg/Kg), the level of As$_2$O$_3$ in brain, kidney and
liver were low and the histological examination showed no pathological changes (42). No obvious sign of toxicity was observed in studies that investigated the possible adverse effects of combined treatment with arsenic trioxide and radiation (43,44). More information about arsenic toxicity and pharmacokinetics are available in the Official document linked to the Initial Marketing Authorization of Trisenox (45). Finally, it has also been shown that As$_2$O$_3$ selectively accumulated in tumors (28, 29, 41). As the difference in dose is approximately a 10-fold increased sensitivity to arsenic effects in humans, and as the extrapolation of the animal data to human is not straightforward, initial clinical studies that could benefit from our present observations should likely start with doses presently used in the clinic to treat APL or solid tumors.

In conclusion, we report for the first time that a single dose of As$_2$O$_3$ can decrease oxygen consumption by tumor cells in experimental tumors, resulting in a transient increased oxygenation of the tumors. This increased oxygenation window could be exploited to significantly enhance tumor radiation response, after individual monitoring of the tumor pO$_2$ before radiation therapy. The oxygen effect was identified as the major factor involved in the sensitization process induced by As$_2$O$_3$. Although additional fractionated radiation studies and TCD50 experiments should be conducted for further pre-clinical validation in a larger panel of tumor models, our study suggests that As$_2$O$_3$ could be used as a potential co-treatment for radiation therapy when this is applied at the time of maximum increased oxygenation induced by the drug.
Acknowledgments

This work is supported by grants from the Belgian National Fund for Scientific Research (FNRS), the Televie, the Fonds Joseph Maisin, the Saint-Luc Foundation, the "Actions de Recherches Concertées-Communauté Française de Belgique-ARC 09/14-020", and the « Pôle d’attraction Interuniversitaire PAI VI (P6/38) ». BJ is a research associate of the FNRS.
References


Figure legends

Fig. 1
Tumor pO₂ measured by EPR oximetry in TLT (A, n=6) and LLC (B, n=5) tumors as a function of time (mean ± SE). As₂O₃ dose of 5mg/Kg was injected at time 0 after establishing a baseline. **, P<0.01; ***, P<0.001.

Fig. 2
Tumor pO₂ measured by ¹⁹F MRI in TLT tumors. As₂O₃ dose of 5mg/Kg has been injected at time 0 after establishing a baseline.

Fig. 3
Typical oxygen maps of one TLT tumor before and after As₂O₃ treatment (A) and the corresponding histogram (B) (mean± SE, n=5). The white and grey pixels correspond to the anatomic image of the tumor and color pixels to the pO₂ measurements based on the distribution of the HFB in the tumor.

Fig. 4
Effect of As₂O₃ on blood perfusion measured by Patent blue staining. A: Percentage of coloration and perfusion decrease in treated mice (n= 8) compared to control mice (n=7). **, P<0.01. B: Typical image of tumor sections showing typical staining in control and treated tumor. The staining was lower in treated mice. Perfusion measurements were performed 90 min after As₂O₃ injection (5mg/Kg). The red lines are drawn to define regions of interest on non-perfused areas (see Material and Methods).

Fig. 5
Effect of As₂O₃ administration on tumor oxygen consumption rate in TLT tumor cells. Oxygen consumption measurements were performed 90 min after As₂O₃-treatment (25μM). As₂O₃-pretreated cells consumed oxygen significantly more slowly than control cells (n=6 for treated cells and n=7 for control cells).
Fig. 6

A. Intracellular GSH level in TLT tumor cells. GSH levels were measured 90 min after As$_2$O$_3$-treatment (25μM). The GSH levels decrease in As$_2$O$_3$-treated cells compared to control cells. GSH levels were expressed as nmol/mg protein. GSH values are the mean of triplicate measurements. **, P<0.01; ***, P<0.001.

B. % of TLT cells with depolarized mitochondria as measured by the JC-1 assay. Effect of incubation in the presence of As$_2$O$_3$ (25μM) compared to untreated cells. Valinomycin and mCICCP were used as positive controls.

Fig. 7

A: Effect of As$_2$O$_3$ on TLT tumor regrowth. Mice were treated with PBS (●, n=9), As$_2$O$_3$ (○, n=7), 10Gy of radiotherapy 1h30 after PBS (■, n=6), 10Gy of radiotherapy 1h30 after As$_2$O$_3$ (▲, n=9), or 10Gy of radiotherapy 1h30 after As$_2$O$_3$ plus tumor ligation at the time of irradiation (□, n=6). Each point represents the mean tumor size ± SE.

B: Time to reach 12mm (days). Results are mean ± SE, one-way ANOVA Tukey’s multiple comparison test.***, P < 0.001.

C: Kaplan-Meier analysis of survival. Survival curve (times when the tumor diameter reached 16 mm) for each group.
Fig. 3

A

\[ pO_2 \text{ (mmHg)} \]

CTRL \hspace{1cm} As_2O_3

B

Relative frequency

CTRL \hspace{1cm} As_2O_3

\[ pO_2 \text{ (mmHg)} \]

0.0 \hspace{1cm} 0.1 \hspace{1cm} 0.2 \hspace{1cm} 0.3 \hspace{1cm} 0.4 \hspace{1cm} 0.5 \hspace{1cm} 0.6 \hspace{1cm} 0.7

0-10 \hspace{1cm} 10-20 \hspace{1cm} 20-40 \hspace{1cm} 40-60 \hspace{1cm} 60-80
Fig. 6

A

GSH (nmol/mg protein)

CTRL

As$_2$O$_3$

**

B

% of cells with depolarized mitochondria

CTRL

Vincristine

mGDP7

As$_2$O$_3$-hsp

As$_2$O$_3$-Sh

As$_2$O$_3$-Sh

*** *** **
Fig. 7

A

Tumor size (mm)

Time (days)

B

Days to reach 12mm

CTRL As$_2$O$_3$ CTRL As$_2$O$_3$ +10Gy As$_2$O$_3$ +10Gy +hypoxia

C

Percent survival

Time (days)
Arsenic trioxide treatment decreases the oxygen consumption rate of tumor cells and radiosensitizes solid tumors

Caroline Diepart, Oussama Karroum, Julie Magat, et al.

Cancer Res  Published OnlineFirst December 2, 2011.