TROLOX ENHANCES ANTI-LEUKEMIC EFFECTS OF ARSENIC: THE ROLE OF OXIDATIVE STRESS

by

Zuanel Diaz Heredia

Department of Medicine
Division of Experimental Medicine
McGill University, Montréal, Québec, Canada
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ABSTRACT

Arsenic trioxide (As_2O_3) has considerable efficacy in the treatment of acute promyelocytic leukemia (APL), inducing partial differentiation and promoting apoptosis of malignant promyelocytes. Although initial studies focused on the role of the characteristic APL fusion protein, PML-RARα, in mediating the response to As_2O_3, recent investigations indicate that its cytotoxic activities are mediated by mechanisms independent of this fusion protein. As_2O_3 affects numerous intracellular targets mainly through the accumulation of free radicals and consequent induction of oxidative stress and causes a wide range of alterations leading to apoptosis.

The intracellular oxidative status has been shown to be important for As_2O_3 sensitivity. Hematologic cancers other than APL and, solid tumors are less responsive to As_2O_3 monotherapy in part because their increased redox buffering capacity. Thus, the use of As_2O_3 in other malignancies is limited by the toxicity of concentrations required to induce apoptosis. The primary goal of the work presented in this thesis was a search for agents that could enhance As_2O_3 efficacy in malignant cells, but not in normal cells.

We demonstrated that trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid), a widely known antioxidant, enhances As_2O_3-mediated apoptosis in APL, P388 murine lymphoma, myeloma and breast cancer cells through the potentiation of As_2O_3-induced oxidative stress. We performed *in vivo* experiments in P388 tumor-bearing mice, and show that As_2O_3 treatment prolonged survival, and the addition of trolox provided a significant further increase in life span and decreased the number of animal with visible macrometastasis. Importantly, trolox protected normal blood mononuclear cells and non-malignant hepatocytes from As_2O_3-mediated cytotoxicity *in vitro* and protected non-tumors and tumors-bearing animals from arsenic-induced hepatotoxicity. We next investigated the mechanisms responsible for the opposite effects of the As_2O_3 and trolox combination in malignant *vs* normal cells, i.e., a cooperative action of As_2O_3 and trolox against lymphoma growth and metastases with the concomitant protection by trolox of normal cells. We analyzed, under isogenic conditions, the effects of oncogenes on trolox and As_2O_3 synergism and demonstrated that oncogenic transformation of mouse embryonic fibroblasts causes elevated ROS

generation and decreased total antioxidant capacity, which renders these cells highly sensitive to As_2O_3 and trolox.

Taken together, the work presented in this thesis suggests that trolox might prevent some of the clinical manifestations of As_2O_3-related toxicity while increasing its pro-apoptotic capacity and therapeutic potential.

SOMMAIRE

Le trioxyde d'arsenic (As_2O_3) induit l'apoptose ainsi qu'une différentiation partielle des promyélocytes malins et de cette façon, il est considérablement efficace dans le traitement de la leucémie promyelocytic aigüe (APL). Les premières études sur le trioxyde d'arsenic ont mis en lumière l'importance de la protéine de fusion PML-RARα. Cependant, plusieurs études récentes démontrent que l'acitvité cytotoxiques de l'As_2O_3 dépend de mécanismes indépendants de PML-RARα. Entres autres, la présence d'As_2O_3 dans la cellule engendre une accumulation de radicaux libre qui affecte de nombreuses cibles intracellulaires et provoque une variété de changement qui vont mener à l'apoptose.

L'équilibre oxydatif intracellulaire à démontré être important pour la sensibilité des cellules cancéreuse face à l'As_2O_3. Les cancers hématologiques autres que l'APL ainsi que les tumeurs solides semblent moins sensibles à l'As_2O_3 puisqu'elles possèdent une grande capacité d'oxydoréduction. L'utilisation de l'As_2O_3 contre plusieurs cancers est par conséquent limitée; les doses effectives pour induire l'apoptose étant toxique pour les cellules saines. L'objectif de la recherche présenté dans cette thèse est d'augmenter l'efficacité de l'As_2O_3 par le biais de thérapies combinatoires.

Un antioxydant largement reconnu, le trolox (acide 6-hydroxy-2,5,7,8-tétraméthylchroman-2-carboxilique) augmente l'effet de l'As_2O_3 sur l'apoptose dans des cellules APL, de myélome, de cancer du sein en potentialisant le stress oxydatif. Par des expériences *in vivo,* nous observons que l'As_2O_3 prolonge la survie de souris possédant les tumeurs P388. De plus, la combinatoire avec le trolox augmente significativement la durée de vie de ces souris et diminue la quantité de candidats présentant des macrométastases. D'une autre part, des études *in vitro* montre que le trolox protège les cellules mononucléaires normales et les hépatocytes non-malins de la toxicité par l'As_2O_3. De la même façon, les expériences *in vivo* montre que le trolox protège les souris de la toxicité hépatique par l'As_2O_3 et ce, autant chez les individus sains que chez ceux porteur d'une tumeur. Par la suite, les mécanismes responsables des effets opposés de l'As_2O_3 et du trolox dans les cellules normales et malignes sont étudiés. Nous avons établi une raison pour laquelle on observe un effet coopératif dans l'inhibition de la croissance des lymphomes et métastase en même temps qu'on constate une protection

contre la toxicité de l'As_2O_3 dans les cellules saines. Des analyses démontrent que la transformation oncogénique de fibroblastes embryonnaires murins occasionnent une élévation des radicaux libres intracellulaires et une diminution de la capacité antioxydante ce qui a pour conséquence de rendre ces cellules beaucoup plus sensible à l'As_2O_3 et au trolox.

En conclusion, les résultats de recherche présentés dans cette thèse, suggèrent que le trolox pourrait augmenter l'efficacité de l'As_2O_3 dans le traitement du cancer tout en prévenant sa toxicité sur des cellules saines.

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PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis preparation from the Faculty of Graduate Studies and Research of McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. For this, the guidelines state: "…Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. …..The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. ….The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. ….In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. …..In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. ….."

As chapters of this thesis, I have included the texts and figures of two original research manuscripts that have been published (Chapter 2 and 3) and a manuscript of novel, yet unpublished material (Chapter 4). Each of these chapters contains its own summary, introduction, materials and methods, results, discussion, and references sections. In addition, a preface is included at the beginning of each chapter in order to introduce and bridge the papers with connecting texts. A general introduction and literature review is presented in Chapter 1, and a final discussion is included in Chapter 5. The references for chapters 1 and 5 are included at the end of the thesis.

The manuscripts included in this thesis are as follows:

Chapter 2 **Diaz Z**, Colombo M, Mann KK, Su H, Smith KN, Bohle DS, Schipper HM and Miller WH Jr. Trolox selectively enhances arsenic-mediated oxidative stress and apoptosis in APL and other malignant cell lines. Blood. 2005 Feb 1;105(3):1237-45.

Chapter 3 **Diaz Z**, Laurenzana A, Mann KK, Bismar TA, Schipper HM, Miller WH Jr.Trolox enhances the anti-lymphoma effects of arsenic trioxide, while protecting against liver toxicity. Leukemia. 2007 Oct;21(10):2117-27.

Chapter 4. **Diaz Z**, Mann KK, Schipper HM, Miller WH Jr. Selectively killing of oncogenically transformed cells through a ROS-mediated mechanism by As_2O_3 and trolox.

CONTRIBUTION OF AUTHORS

The candidate performed the majority of the research described in this thesis and with the support of Dr Wilson H. Miller and Hyman M. Schipper. The contribution of other authors to this work is as follows:

In Chapter 2, M.Sc. Myrian Colombo provided assistance with the PARP western blots in Figure 2.2D and the kinase assays in Figure 2.5. Haixiang Su performed protein carbonyl and lipid peroxidation quantification in Figure 2.3B, and C. Kamilah Smith and Dr. Scott Bohle provided help with the electronic paramagnetic resonance experiments shown in Figure 2.6. In Chapter 3, Anna Laurenzana, Kathy Ann Forner, Veronique Michaud, and Scott Hartigan provided technical help with the injections and animal manipulations. Dr. Bismar and Dr. Xiujie Lee provided expertise and helped with the identification of lymphocyte foci and binucleated cells in H/E stained liver samples (Figure 3.3). Koren Mann provided analysis of the research plan in Chapter 2, 3 and 4 and developed the flow cytometric assays to determine superoxide levels in Chapter 4. Sophie Marcoux performed cell death assays in Figure 5.2D and atomic absorption experiments depicted in Figure 5.3. Maria Kourelis performed the staining with MitoSoxRed in Figure 5.2C. Drs. Hyman M. Schipper and Wilson H. Miller supervised all the studies and provided critical analysis of the results.

CONTRIBUTIONS NOT INCLUDED IN THIS THESIS

In addition to the manuscripts included in this thesis, the candidate contributed to the following studies, which have been published or submitted:

Diaz Z, Marcoux S, Mann KK, Kourelis M, Colombo M, Miller WH Jr. A novel arsenical has anti-tumor activity toward As_2O_3-resistant and MRP1/ABCC1-overexpressing cell lines. Blood 2008 (submitted)

Assaraf MI, **Diaz Z**, Liberman A, Miller WH Jr, Arvanitakis Z, Li Y, Bennett DA, Schipper HM. Brain erythropoietin receptor expression in Alzheimer disease and mild cognitive impairment. J Neuropathol Exp Neurol. 2007 May;66(5):389-98.

Mann KK, Davison K, Colombo M, Colosimo AL, **Diaz Z**, Padovani AM, Guo Q, Scrivens PJ, Gao W, Mader S, Miller WH Jr. Antimony trioxide-induced apoptosis is dependent on SEK1/JNK signaling. Toxicol Lett. 2006 Jan 5;160(2):158-70.

Diaz Z, Assaraf M, Miller WH Jr, Schipper HM. Astroglial cytoprotection by erythropoietin pre-conditioning: implications for ischemic and degenerative CNS disorders. J Neurochem. 2005 Apr;93(2):392-402.

Mann KK, Rephaeli A, Colosimo AL, **Diaz Z**, Nudelman A, Levovich I, Jing Y, Waxman S, Miller WH Jr. A retinoid/butyric acid prodrug overcomes retinoic acid resistance in leukemias by induction of apoptosis. Mol Cancer Res. 2003 Oct;1(12):903-12.

LIST OF ABBREVIATIONS

8-iso-PGF2α	8-iso- prostaglandin F2α
AA	Ascorbic acid
ADP	Adenosine diphosphate
AIF	Apoptosis inducing fator
AKT	v-akt murine thymoma viral oncogene /serine/threonine protein kinase Akt
AML	Acute Myeloblastic leukemia
AMP	Adenosine monophosphate
AP-1	Activator protein 1
APL	Acute Promyelocytic Leukemia
As_2O_3	Arsenic trioxide
Asc^-	Ascorbate
$AscH^-$	Reduced ascorbate
GSSG	Reduced glutathione
ASK1	Apoptosis signal-regulating kinase 1
ATLL	T-cell leukemia/lymphoma
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
Bak	BCL2-antagonist/killer
Bax	Bcl2-associated X protein
Bcl2	B cell protein 2
Bcl-XL	B cell protein XL
Bcr-Abl	Fusion protein Bcr (breakpoint cluster region) and Abl (Abelson murine leukemia viral oncogene)
BH4	5,6,7,8-tetrahydrobiopterin
Bid	BH3 interacting domain death agonist
Bim	Bcl2 interacting mediator
Bmf	Bcl2 modifying factor
BSO	L-Buthionine Sulfoximine

CAT	Catalase
Cdc42	Cell division cycle 42 protein
CR	Complete remission
CT scan	Computational tomography scan
DHA	Dehydroascorbic acid
DMA	Dimethylarsinic acid
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FKHD	Forkhead
Gab1	Growth factor receptor bound protein 2-associated protein
$gp91^{PHOX}$	Glycoprotein 91 from the phagocyte oxidase complex
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione (γ-glutamylcysteinylglycine)
GSTπ	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HL	Hodgkin's lymphoma
HNE	4-hydroxy-2-nonenal
HO-1	Heme oxygenase-1
HTLV-1	Human T-cell Lymphotropic Virus
IFNα	Interferon α
jBid	JNK-cleaved Bid
JIP	JNK Interacting Protein (scaffold protein)
JNK	c-jun N-terminal kinase
kD	kiloDalton
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPK kinase kinase
Mcl-1	Myeloid cell leukemia sequence 1 (Bcl-2-related protein)
MDA	Malondialdehyde
MEF	Mouse embryonic fibroblast
MM	Multiple myeloma
MMA	Monomethylarsenous acid
mRNA	messenger RNA
Mrp-1	Multidrug resistance protein 1
mtDNA	mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nuclear bodies
NF-κB	Nuclear factor kappa B
NHL	Non-Hodgkin's lymphoma
$NO^{\bullet}$	Nitric oxide
NPM	Nucleophosmin
NQO1	NADPH:quinone oxidoreductase 1
NuMA	Nuclear mitotic apparatus
$O_2^{\bullet-}$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
$p47^{PHOX}$	47 kD Protein 1 from the phagocyte oxidase complex
PARP	poly (ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
PLZF	Promyelocytic leukemia zinc finger
PML	Promyelocytic leukemia gene
Prx	Peroxiredoxin
PTPC	Permeability transition pore
PUFA	Polyunsaturated fatty acid
Raf-1	v-raf-1 Murine Leukemia Viral Oncogene Homolog 1
RARα	Retinoic acid receptor α
Ras	Rat sarcoma virus oncogene

Rho	Ras homologous protein
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical
ROOH	Organic peroxides
ROS	Reactive oxygen species
RS	Reactive species
SEK	Stress-enhanced kinase
siRNA	Small interfering RNA
SOD	Superoxide dismutase
Src	Rous sarcoma oncogene
STAT	Signal transducer and activator of transcription
STI-571	Signal transduction inhibitor 571
tBid	truncated Bid
TGFβ	Tumor growth factor Beta
TMA	Trimethylarsonic acid
T-PLL	T-cell prolymphocytic leukemia
TRAF2	Tumor necrosis factor receptor-associated factor 2
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UV	Ultraviolet
XO	Xanthine oxidase

CHAPTER 1

Introduction and Literature Review

1.1 General Introduction

Arsenic trioxide (As_2O_3) is an effective therapy in acute promyelocytic leukemia (APL). However, its use in other malignancies is limited by the toxicity of concentrations required to induce apoptosis in non-APL tumor cells. The objective of this thesis is to identify new compounds that increase As_2O_3–mediated toxicity *in vitro* and *in vivo* and to understand the mechanisms of action of this combination in malignant and non-malignant cells. The first topic will describe the clinical use of arsenic compounds from a historical point of view, As_2O_3–associated toxicity and the hematological malignancies where it has been proved to be an effective treatment. Topic 2 will describe the molecular mechanism of cellular response to As_2O_3, which includes the generation of ROS, and the activation of signaling and cell death pathways. Topic 3 will focus on different compounds that have been demonstrated to increase As_2O_3-mediated toxicity in a variety of cancer cell lines. Topic 4 will cover the role of free radicals in cellular biology discussing the consequences of redox imbalance in the cellular environment. Topic 5 will address the complex relationship between cancer and oxidative stress and will propose strategies to distinguish the response of malignant and non-malignant cells to reactive oxygen species. Lastly, a general discussion will be presented in Topic 6 to highlight the importance of the findings presented in this thesis.

1.2 History of the clinical use of arsenic compounds

Arsenic is an element that occurs naturally in the earth's surface at 1.5 to 2 ppb[1]. Apart from the organic arsenicals, there are three main inorganic arsenic forms: red arsenic (As_2S_2, also known as "realgar"), yellow arsenic (As_2S_3 or "orpiment") and white arsenic (As_2O_3; arsenolite) (Figure 1.1). Strictly speaking, the name arsenic applies to this latter compound, not to the metal. In German, the metal is called Arsen, the oxide Arsenik, but in English "arsenic" is used for both. In the trioxide form As_2O_3, arsenic has valence +3, while in the pentoxide As_2O_5 has valence +5.

The use of arsenic for medicinal purposes arose in parallel in China and Greece more than 2400 years ago, but among the general public the word "arsenic" became almost synonymous of the word "poison" thanks to the Savellis, the Borgias, and Agatha Christie (Figure 1.1).

In 1785, the English physician William Withering, who extracted a powerful cardiotonic compound from the flower Digitalis, stated that "poisons in small doses are the best medicines and the best medicines in too large doses are poisonous", and based on this thought, different arsenic-based treatments were prepared. In 1786, Thomas Fowler formulated the "Liquor Potassii Arsenitis", a 1% solution of potassium arsenite and lavender extract that was originally conceived to treat periodic fever. This solution was later used to treat conditions such as paralytic afflictions, rheumatism, hypochondriasis, epilepsy, hysteria, melancholia, dropsy, rachitis, heart palpitations, convulsions, syphilis, ulcers, cancer, and dyspepsia. As such, Fowler's liquor, nicknamed 'the mule' for both its strength and the unpredictable toxicity, became the cornerstone of nineteenth-century *materia medica*[2].

The first uses of arsenic as an anticancer drug dates from 1878, when it was reported by Cutler *et al.* at Boston City Hospital that Fowler's solution reduced the number of white cells in two normal people and one patient with "leucocythemia"[3]. The popularity of Fowler's solution resurged in 1931 when Forkner and Scott administered this solution to patients with chronic myeloid leukemia (CML). Nine of 10 patients responded to arsenic treatment[4]. These results were subsequently confirmed by other reports[5] and arsenic was considered the most effective treatment of chronic myelogenous leukemia (CML) next to irradiation.

The development of modern chemotherapy decreased the medicinal use of arsenic compounds substantially. However, in the early 1970's, it was known that for more than 1000 years the Chinese medications *pi shuang* and *xiong huang*, which are now known to contain arsenic trioxide (As_2O_3) and arsenic disulfide (As_2S_2) respectively, were used in remote regions of Northeastern China for the treatment of cancer and other conditions[6]. Initial studies at Harbin Medical University, followed by a careful clinical trial at the Shangai Second Medical University, documented remarkable clinical efficacy in patients with newly diagnosed and relapsed acute promyelocytic leukemia (APL) (see section

1.4.1). Extensions of this pivotal Chinese study by investigators in Japan, Europe and USA have shown that As_2O_3 is a very effective and relatively safe drug in relapsed APL. Currently, As_2O_3 is used worldwide to treat patients with this disease who have undergone relapse from their primary treatment.

1.3 Arsenic toxicity

The cytotoxic effects of arsenic compounds have been well documented, and reveal a greater toxicity for inorganic and trivalent arsenicals than organic and pentavalent ones.

Together, food and drinking water usually account for 99% of the total human intake of arsenic (Ontario Ministry of the Environment, 2006). Arsenic is quickly absorbed from the gastrointestinal tract and rapidly cleared from the blood with a half-life of 1 to 2 hours[7], where the liver is the primary site of arsenic metabolism in mammals. In the hepatocytes, arsenate can be reduced to arsenite, followed by conjugative methylation reaction to form monomethylarsonous acid (MMA), then dimethylarsinic acid (DMA), and finally trimethylarsonic acid (TMA), with these methylated species found in urine[8] (Figure 1.1). Arsenic is eliminated from the body primarily through the kidneys and other less important routes of elimination include feces, sweat, skin desquamation, and incorporation into hair and nails.

The first symptoms of chronic long-term exposure to low levels of arsenic (arsenicosis) are dermatologic pathologies including hyperkeratosis, hyperpigmentations, and cutaneous malignancies. In addition, chronic indigestion and stomach cramps have been observed. Longer-term effects include diabetes, hypertension, peripheral vascular disease, polyneuropathy as well as gangrene-like sores. The symptoms of chronic exposure differ among individuals, population groups, and geographic areas[9].

Epidemiologic studies have demonstrated that chronic arsenic exposure causes tumors of the skin, urinary bladder, lung, liver, prostate, kidney, and possibly other sites[10,11] although the internal cancers in affected patients may not appear for 20 or 30 years after exposure[12-14]. Until recently, arsenic was considered a “paradoxical” human carcinogen because of strong human evidence but limited evidence for animal carcinogenesis[10,15,16]. Recently, important advances have been made in the development

of rodent models of inorganic arsenic carcinogenesis. These include skin cancer models in which inorganic arsenic acts as a co-promoter with 12-O-tetradecanoyl phorbol-13-acetate in Tg.AC mice[17], or as a co-carcinogen with ultraviolet irradiation in hairless mice[18] and a transplacental model in which short-term exposure to inorganic arsenic in utero produces a variety of internal tumors in the offspring when mice reach adulthood[16,19].

Thus, despite its long history of medicinal use, arsenic has earned a reputation as a toxic compound and poison. While the risk of disease associated with chronic arsenic exposure is not negligible, it is important to note that these are graded, long-term effects that may not be manifested after careful, clinical administration of As_2O_3 for the treatment of hematological malignancies.

A.

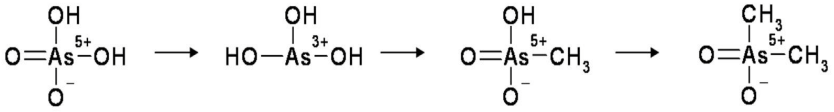
Orpiment (As_2S_3)

Realgar (As_2S_2)

Arsenolite (As_2O_3)

($O{=}As{-}O{-}As{=}O$)

B.



Arsenate (As^{5+}) Arsenite (As^{3+}) MMAs DMAs

C.

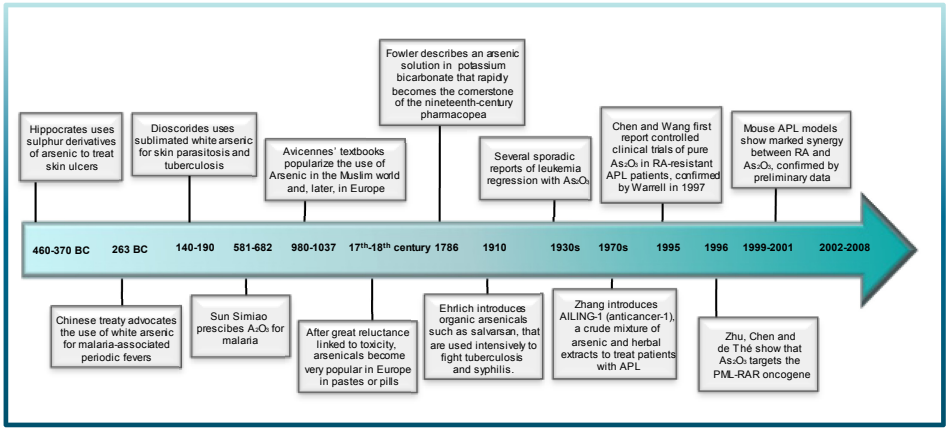


Figure 1.1: Arsenic compounds.

A.Mineral arsenicals. B.Common inorganic arsenicals and their organic arsenical metabolites. C.Milestones of arsenic development as a drug (Adapted from Zhu et al. How acute promyelocytic leukaemia revived arsenic. Nature Reviews Cancer 2, 705-714, 2002)

1.4 Clinical use of As_2O_3 in hematological malignancies

Arsenic trioxide has been used as an anticancer drug in different hematological malignancies. Recent investigations have suggested that As_2O_3 may also have clinical activity in certain tumors.

1.4.1 Acute Promyelocytic Leukemia

Acute Promyelocytic Leukemia is a rare subtype of acute myeloblastic leukemia (AML) that comprises roughly 15% of all myeloid leukemias and has been classified as M3 disease in the French-American-British classification system.

APL is associated with a reciprocal and balanced translocation which involves the retinoic receptor α gene on chromosome 17, which translocates to the PML gene (promyelocytic leukemia gene) on chromosome 15[20-22]. In an few variant cases, RARα fuses to the promyelocytic leukemia zinc finger (PLZF) gene, nuclophosmin (NPM) gene, nuclear mitotic apparatus (NuMA) gene, and signal transducer and activator of transcription 5b (STAT5b)[23-26]. The expression of this fusion protein blocks the differentiation process at the promyelocyte stage. Therefore, APL is characterized by abnormal, heavily-granulated promyelocytes that accumulate in the bone marrow and peripheral blood of patients diagnosed with APL.

Several research groups have made APL the most curable leukemia in adults and a model disease for both differentiation therapy and oncogene-targeted treatment. The combination of all-trans retinoic acid (ATRA) plus chemotherapy has shown remarkable efficacy in the treatment of APL with relatively few side effects[27,28]. However, approximately 20%-30% of patients treated with this combination will relapse and die, unless rescued by bone marrow transplantation[29,30]. Therefore, alternative treatment strategies have been administered to patients with advanced APL including As_2O_3, gemtuzumab, ozogamicin, and several other novel agents.

As_2O_3 has emerged as the single most active agent in advanced APL. Researchers from Harbin Medical University in China introduced a crude As_2O_3 preparation (Ailing-1) in advanced APL patients. The clinical complete remission rate (10 mg As_2O_3/day, intravenous infusion for 28 to 60 days) was reportedly from 65.6% to 84%, respectively. Moreover, an impressive 28.2% survival rate after 10 years was achieved and most

patients showed neither bone marrow depression nor other severe clinical side effects during the treatment[31]. A clinical trial with pure As_2O_3 was then conducted in Shanghai Second Medical University that confirmed its efficacy in patients with APL who had undergone relapse after ATRA plus chemotherapy[32]. These results have been reproduced in Western populations[33,34], where complete remission rate ranged from approximately 80% to 90%[30,35]. A follow-up of these results suggests that As_2O_3 is not only able to produce high levels of complete remission, but also extends its effectiveness to relapse-free survival and overall survival[36]. In addition, it was proven that single agent As_2O_3 is effective in patients in first, second, or third relapse after ATRA and anthracycline therapy[33].

Although As_2O_3 monotherapy is now used worldwide to treat patients with APL who have undergone relapse from their primary therapy, combining As_2O_3 with other agents in this setting may also be useful, particularly As_2O_3 with anthracycline-based chemotherapy. A report from China suggested that patients receiving combination therapy of As_2O_3 and chemotherapy may have a superior outcome compared to patients treated with As_2O_3 alone[32]. However, the combination of ATRA and As_2O_3 does not appear to be beneficial in the setting of relapsed disease[37].

1.4.2 Myeloma

Multiple myeloma (MM) is an incurable cancer that occurs in 3 to 4 individuals per 100,000 people in the United States[38], and comprises more than 10% of all hematological malignancies. Therefore, MM is one of the most common primary cancers of the bone marrow. The tumor arises from somatically mutated plasma cells that accumulate in the bone marrow which leads to bone destruction and bone marrow failure.

Standard of care for MM currently includes salvage single-agent or combination chemotherapy; thalidomide-, lenalidomide-, or bortezomib-based regimens; or melphalan-based autologous stem cell transplantation[39]. These treatment approaches typically achieve response rates of only 10% to 30%, which generally last only several months[40] and the disease eventually relapse and become refractory in all patients.

Initial successful animal experiments from Russelot *et al.*[41] prompted investigations of the effectiveness of As_2O_3 in patients with this multiple myeloma. In a

phase II trial at the University of Arkansas[42], treatment of relapsed MM patients refractory to conventional salvage therapy with As_2O_3 (0.15 mg/kg daily for 60 days) resulted in significant responses. A multicenter trial in the United States was then initiated using a higher dose of As_2O_3 and a shorter schedule[43]. Patients received 0.25 mg/kg As_2O_3 intravenously, 5 days per week for 2 weeks followed by no therapy for 2 weeks, in repeated 4-week cycles. Eight relapsed patients and 16 refractory patients were treated. Of the treated patients, 43% had an objective response as measured by a >25% decrease in serum M-protein concentrations. In addition, several Phase I/II studies have analyzed the efficacy and safety of combining As_2O_3 with bortezomib, mephalan, and/or ascorbic acid with promising results[44,45].

1.4.3 Lymphoma

Lymphomas are subdivided into Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). NHL are more specifically classified into two subtypes, B-cell neoplasms and T-cell/natural killer-cell neoplasms[46]. HLs involve the lymph nodes predominantly and only approximately 5% arise in extranodal sites, whereas 30% of NHLs present in extranodal sites. Malignant lymphomas are the fifth most frequently occurring type of cancer in the United States. In 2008, an estimated 8,220 new cases of HL and 66,120 new cases of NHL are expected to be diagnosed and deaths from these diseases will exceed 20,330[47].

Lymphoma represents a diverse group of neoplasms that include some of the most treatment-responsive cancers currently known. More than 80% of patients with HL can be cured with current chemotherapy, radiation therapy or combined modality therapy regimens[48] and many patients with aggressive types of NHL can now be cured with appropriate intensive therapy[49]. Recent advances have significantly improved the results of treatment for patients with lymphoma and include; the use of monoclonal antibody-based therapies for B cell lymphomas[50], the more widespread use of early high dose chemotherapy and haematopoietic stem-cell transplantation for patients who have failed initial therapy[51]. Despite these therapeutic advances in HL and aggressive NHL that have increased cure rates, some subtypes such as adult T-cell leukemia/lymphoma and T-cell prolymphocytic leukemia are resistant to conventional chemotherapies.

Recently, As_2O_3 has demonstrated activity in lymphoma[52,53]. A preliminary report from China indicated activity of arsenic compounds in NHL. In this study, twenty-seven patients with malignant lymphoma were treated by Ailing-1 injections (As_2O_3) plus Chinese herbal medicine. The total remission rate was 70.37%[54]. However, such activity has not been confirmed in a prospective study in the West. Another group has observed activity of As_2O_3, in combination with ascorbic acid and steroids, in a patient with refractory Burkitt's-like lymphoma, as indicated by transient stabilization of radiological findings and decrease in serum lactate dehydrogenase[55]. In a study recently published[56], it was shown that IFNα and As_2O_3 have dramatic synergistic effects resulting in induction of cell cycle arrest and apoptosis in cells infected with HTLV-1, a virus that can cause of T-cell leukemia/lymphoma (ATLL). This effect was substantiated by *ex vivo* experiments using peripheral blood mononuclear cells from a patient with ATLL. Based in these results, a phase II trial using dual IFNα/As_2O_3 treatments was developed and seven patients were included in the study. Four of them exhibited a clear initial response (one complete remission and three partial remissions). Yet, the treatment was discontinued after a median of 22 days because of toxicity (three patients) or subsequent progression (four patients). Six patients eventually died from progressive disease (five patients) or infection (one patient), but the remaining patient was still alive and disease free at 32 months[57].

1.4.4 Other malignancies

Based on *in vitro* data of As_2O_3 activity against non-APL cell lines and good tolerability with less toxicity than intensive chemotherapy, the use of As_2O_3 has been evaluated in patients with non-APL hematological malignancies[58-60] as well as advanced metastatic melanoma[61], hepatocellular carcinoma[62], and advanced head and neck cancer[63]. Ongoing clinical trials have included patients with advanced primary carcinoma of the liver, metastatic liver cancer, esophageal cancer, advanced or metastatic non-small cell lung cancer, and metastatic kidney cancer (Table 1.1).

Although some promising results have been seen in other hematological malignancies, the clinical effects of As_2O_3 have not been close to the APL success. In addition, these initial investigations suggest that As_2O_3 may have limited clinical activity

as a single agent in certain tumors. Therefore, it has been proposed that a combination therapy with drugs targeting specific pro-survival molecules or the capability to enhance pro-apoptotic pathways may lead to an improvement in the anticancer efficacy of As_2O_3 (this aspect will be reviewed in section 1.7). Ongoing clinical trials using arsenic alone or in combination with other drugs have included patients with multiple myeloma, malignant glioma, neuroblastoma, pheochromocytoma or paraganglioma, and colorectal cancer (Table 1.1).

Title	Condition(s)	Phase
Evaluation of Disulfiram Plus Arsenic Trioxide In Patients With Metastatic Melanoma and at Least One Prior Systemic Therapy	Metastatic Melanoma	Phase I
Study of Low-Dose Cytarabine in Combination With Arsenic Trioxide, Compared With Low-Dose Cytarabine Alone, for the Treatment of Elderly Patients With Acute Myeloid Leukemia	Acute Myeloid Leukemia	Phase III
Single Agent Arsenic Trioxide in the Treatment of Newly Diagnosed Acute Promyelocytic Leukemia	Acute Promyelocytic Leukemia	Phase II/III
Trial of Arsenic Trioxide With Ascorbic Acid in the Treatment of Adult Non-APL Acute Myelogenous Leukemia	Acute Myelogenous Leukemia	Phase II
Arsenic Trioxide and Ascorbic Acid Combined With Bortezomib, Thalidomide, and Dexamethasone in Treating Patients With Relapsed or Refractory Multiple Myeloma or Plasma Cell Leukemia	Multiple Myeloma and Plasma Cell Neoplasm	Phase I
Arsenic Trioxide, Temozolomide, and Radiation Therapy in Treating Patients With Malignant Glioma That Has Been Removed By Surgery	Brain and Central Nervous System Tumors	Phase I/II
Tretinoin and Arsenic Trioxide With or Without Idarubicin in Treating Patients With Acute Promyelocytic Leukemia	Leukemia	Phase II
Arsenic Trioxide, Cytarabine, and Idarubicin in Treating Patients With Acute Myeloid Leukemia	Leukemia	Phase I
Cephalon Decitabine, Arsenic Trioxide and Ascorbic Acid for Myelodysplastic Syndrome	Myelodysplastic Syndrome	Phase II
A Phase II Protocol of Arsenic Trioxide (Trisenox) in Subjects With Advanced Primary Carcinoma of the Liver	Carcinoma, Hepatocellular	Phase II
Treatment Protocol for Relapsed Acute Promyelocytic Leukemia (APL) With Arsenic	Relapsed Acute Promyelocytic Leukemia; Refractory Acute Promyelocytic Leukemia	Phase IV
All-Trans Retinoic Acid, and Arsenic +/- Gemtuzumab and Theophylline	Acute Promyelocytic Leukemia	Phase I
Treatment of Relapsed Promyelocytic Leukemia With Arsenic Trioxide (ATO)	Acute Promyelocytic Leukemia	Phase IV
Arsenic Trioxide and Pamidronate in Treating Patients With Advanced Solid Tumors or Multiple Myeloma	Multiple Myeloma and Plasma Cell Neoplasm; Unspecified Adult Solid Tumor, Protocol Specific	Phase I
Arsenic Trioxide and Radiation Therapy in Treating Young Patients With Newly Diagnosed Gliomas	Brain and Central Nervous System Tumors	Phase I
Arsenic Trioxide, Thalidomide, Dexamethasone, and Ascorbic Acid in Treating Patients With Relapsed or Refractory Multiple Myeloma	Multiple Myeloma and Plasma Cell Neoplasm	Phase II
Study of IV Decitabine, Arsenic Trioxide and Vitamin C in Patients With MDS	Myelodysplastic Syndrome	Phase I
Arsenic Trioxide and Cholecalciferol (Vitamin D) in Treating Patients With Myelodysplastic Syndromes	Leukemia; Myelodysplastic Syndromes; Myelodysplastic/Myeloproliferative Diseases	Phase I
Arsenic Trioxide Plus Radiation Therapy in Treating Patients With Newly Diagnosed Malignant Glioma	Brain and Central Nervous System Tumors	Phase I
Arsenic Trioxide in Relapsed/Refractory Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma	Chronic Lymphocytic Leukemia; Small Lymphocytic Lymphoma	Phase II
Iodine I 131 Metaiodobenzylguanidine and Arsenic Trioxide in Treating Patients With Recurrent, Progressive, or Refractory Neuroblastoma or Malignant Pheochromocytoma or Paraganglioma	Neuroblastoma; Pheochromocytoma	Phase II
Arsenic Trioxide in Treating Patients With Recurrent Bladder or Urinary Tract Cancer	Bladder Cancer; Transitional Cell Cancer of the Renal Pelvis and Ureter; Urethral Cancer	Phase II
Arsenic Trioxide in Treating Women With Locally Advanced or Metastatic Breast Cancer	Breast Cancer	Phase II
Arsenic Trioxide and Thalidomide in the Treatment of Refractory Multiple Myeloma	Multiple Myeloma	Phase II
Arsenic Trioxide in Treating Patients With Metastatic Liver Cancer That Cannot Be Removed by Surgery	Liver Cancer	Phase II
Arsenic Trioxide in Treating Patients With Locally Advanced or Metastatic Non-Small Cell Lung Cancer	Lung Cancer	Phase II
Arsenic Trioxide and Gemtuzumab Ozogamicin in Treating Patients With Advanced Myelodysplastic Syndromes	Leukemia; Myelodysplastic Syndromes	Phase II
Azacitidine and Arsenic Trioxide in Treating Patients With Myelodysplastic Syndromes	Leukemia; Myelodysplastic Syndromes	Phase I/II
Tretinoin, Cytarabine, and Daunorubicin With or Without Arsenic Trioxide Followed by Tretinoin With or Without Mercaptopurine and Methotrexate in Treating Patients With Acute Promyelocytic Leukemia	Leukemia	Phase III
Arsenic Trioxide in Treating Patients With Myelodysplastic Syndromes	Leukemia; Myelodysplastic Syndromes; Myelodysplastic/Myeloproliferative Diseases	Phase II
Arsenic Trioxide in Treating Patients With Multiple Myeloma	Multiple Myeloma and Plasma Cell Neoplasm	Phase II
Arsenic Trioxide With or Without Tretinoin in Treating Patients With Hematologic Cancer That Has Not Responded to Previous Therapy	Leukemia; Lymphoma; Multiple Myeloma and Plasma Cell Neoplasm; Myelodysplastic Syndromes	Phase I/II
Arsenic Trioxide in Treating Patients With Refractory or Recurrent Acute Promyelocytic Leukemia	Leukemia	Phase I/II
Phase I Trial of Arsenic Trioxide and Stereotactic Radiotherapy for Recurrent Malignant Glioma	Brain Cancer	Phase I
Arsenic Trioxide in Treating Patients With Relapsed or Refractory Hodgkin's Disease	Lymphoma	Phase II
Arsenic Trioxide and Dexamethasone in Treating Patients With Recurrent or Refractory Stage II or Stage III Multiple Myeloma	Multiple Myeloma and Plasma Cell Neoplasm	Phase II
Arsenic Trioxide in Treating Patients With Recurrent or Refractory Acute Leukemia, Chronic Myeloide Leukemia, Myelodysplasia, Lymphoma, or Myeloma	Leukemia; Lymphoma; Multiple Myeloma and Plasma Cell Neoplasm; Myelodysplastic Syndromes	Phase II
New Retinoid Agent Combined With Arsenic Trioxide for Untreated Acute Promyelocytic Leukemia	Acute Promyelocytic Leukemia	Phase II
Arsenic Trioxide and Imatinib Mesylate in Treating Patients With Chronic Myelogenous Leukemia	Leukemia	Phase I/II
Velcade, Trisenox, Vitamin C and Melphalan for Myeloma Patients	Myeloma	Phase I/II
Acute Promyelocytic Leukemia 2006 (APL)	Leukemia, Promyelocytic, Acute	Phase III
Trisenox, Ascorbic Acid and Bortezomib in Patients With Relapsed/Refractory Multiple Myeloma	Multiple Myeloma	Phase I/II
Safety, Efficacy, & Pharmacokinetic Study of Tamibarotene to Treat Patients With Relapsed or Refractory APL	Acute Promyelocytic Leukemia; APL	Phase II
Effects of Arsenic on Keratinocytes in a Skin Equivalent Model	Bowen Disease	Phase I
Gemtuzumab and Combination Chemothearpy in Treating Patients With Previously Untreated Acute Promyelocytic Leukemia	Leukemia	Phase II
Combination Chemotherapy in Treating Patients With Newly Diagnosed Acute Promyelocytic Leukemia	Leukemia	Phase III

Table1.1: Ongoing clinical trials using As_2O_3 (Trisenox) as single agent or in combination therapy.

(Listed at the U.S. National Institute of Health (http://clinicaltrials.gov) in May 2007)

1.5 The role of free radicals in cellular biology

1.5.1 Brief history of free radical research

Remarkable progress has been achieved in the past 100 years in the field of free radical chemistry, biology and medicine. The causes of oxygen toxicity were unknown until the publication of Gershman's free radical theory of oxygen poisoning properties in 1954, which states that the toxicity of oxygen is due to the formation of oxidizing free radicals[64]. With the adaptation of electronic paramagnetic resonance absorption techniques to living materials, Commoner *et al*. reported the same year that free radicals occur in a variety of lyophilized biological samples and provided evidence relating free radicals to metabolic activity[65]. The world of free radicals in biological systems was, thereafter, explored by Denham Harman who, in 1956, proposed and provided experimental evidence that free radicals could be involved in the aging process[66]. This work gradually triggered intense research into the field of free radicals in biological systems. In 1969, McCord and Fridovich provided convincing evidence about the importance of free radicals in living systems; without antioxidant mechanisms, aerobic life could not exist[66].

A third era in free radical research began with the first reports describing advantageous biological effects of reactive species and demonstrating their involvement in cell signaling. Mittal and Murard[67] provided evidence that the superoxide anion ($O_2^{\bullet -}$) through its derivative, the hydroxyl radical ($OH^{\bullet}$), stimulates the activation of guanylate cyclase and formation of the "second messenger" cGMP. Furthermore, Ignarro and Kadowitz[68] and Moncada and colleagues[69] discovered independently the role of nitric oxide ($NO^{\bullet}$) as a regulatory molecule in the control of smooth muscle relaxation and in the inhibition of platelet adhesion. Roth and Dröge[70] found that, in activated T cells, the superoxide anion or low micromolar concentrations of hydrogen peroxide (H_2O_2) increase the production of the T-cell growth factor interleukin-2, an immunologically important T-cell protein. Studies from Keyse and Tyrrell[71] showed that H_2O_2 induces the expression of the heme oxygenase-1 (HO-1) gene. Storz and colleagues[72] reported the induction of various genes in bacteria by H_2O_2, and Schreck and Baeuerle[73] reported that

H_2O_2 mediated the activation of the transcription factor nuclear factor κB (NF-κB) in mammalian cells.

The field of redox regulation is now receiving attention from clinical researchers in view of the role that oxidative stress has been found to play in numerous disease conditions. These pathological conditions demonstrate the biological relevance of redox regulation.

1.5.2 Free Radicals and related "reactive species"

Free radicals can be defined as any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons[74], and these unpaired electron(s) make the free radical highly reactive. Radicals can be formed by losing a single electron from a non-radical ($X - e^- \rightarrow X^{\bullet+}$) or by gaining one ($Y + e^- \rightarrow Y^{\bullet-}$). Radicals can also be formed when a covalent bond is broken if one electron from the bonding pair remains on each atom (homolytic fission). Reactive oxygen species (ROS) is a collective term that includes not only the oxygen radicals but also some non-radical derivatives of O_2 (Table 1.2). On the other hand, the reactive nitrogen species (RNS) term refers to various nitrogenous products from nitric oxide synthases. Although ROS represent the most important class of reactive species (RS) generated in living systems[75], there is significant cross talk between ROS and RNS via either chemical reaction or functional interplay. For instance, ONOO is formed via $NO^{\bullet}$ and $O_2^{\bullet-}$, which can exert both oxidant and nitrosant activity[76].

The most important source of $O_2^{\bullet-}$ in most cells under physiological conditions is the mitochondrial electron transport chain, where an estimated 1-2% of the total O_2 consumed by the cell is released in a partially reduced state rather than being converted to water[77] (Figure 1.2). ROS can arise from complexes I and III of the respiratory chain, where electrons can be directly transferred to O_2 to generate $O_2^{\bullet-}$. Another important source of cellular ROS generation is the action of non-mitochondrial electron transport chains in several different organelles. The nuclear envelope also contains an electron transport chain functionally different from the mitochondria as it possesses altered substrate specificities and regulation mechanisms. Although poorly characterized, the

Free Radicals	Non-Radicals
<u>Reactive oxygen species (ROS)</u> Superoxide, $O_2^{\bullet-}$ Hydroxyl, OH• Hydroperoxyl, $HO_2^{\bullet}$ Carbonate, $CO_3^{\bullet-}$ Peroxyl, $RO_2^{\bullet}$ Alkoxyl, $RO^{\bullet}$ Carbon dioxide, $CO_2^{\bullet-}$ Singlet $O_2^1 \sum g^+$ <u>Reactive chlorine species (RCS)</u> Atomic chlorine, $Cl^{\bullet}$ <u>Reactive bromide species (RBS)</u> Atomic bromine, $Br^{\bullet}$ <u>Reactive nitrogen species (RNS)</u> Nitric oxide, $NO^{\bullet}$ Nitrogen dioxide, $NO_2^{\bullet}$ Nitrate, $NO_3^{\bullet}$	<u>Reactive oxygen species (ROS)</u> Hydrogen peroxide, H_2O_2 Hypobromous acid, HOBr Hypochlorous, HOCl Ozone, O_3 Organic peroxides, ROOH Peroxynitrite, $ONOO^-$ Peroxynitrate, O_2NOO Peroxynitrous acid, ONOOH Nitrosoperoxycarbonate, $ONOOCO_2^-$ Peroxomonocarbonate, $HOOCO_2^-$ <u>Reactive chlorine species (RCS)</u> Hypochlorous acid, HOCl Nitryl chlorine, NO_2Cl Chloramines Chlorine gas, Cl_2 Bromide chlorine, BrCl Chlorine dioxide, ClO2 <u>Reactive bromine species (RBS)</u> Hypobromous acid, HOBr Bromine gas, Br_2 Bromine chlorine, BrCl <u>Reactive nitrogen species (RNS)</u> Nitrous acid, HNO_2 Nitrosyl cation, NO^+ Nitroxyl anion, NO^- Dinitrogen tetroxide, N_2O_4 Dinitrogen trioxide, N_2O_3 Peroxynitrite, ONOO Peroxynitrate, O_2NOO^- Peroxynitrous acid, $ONOOH^-$ Nitronium (nityl) cation, NO_2^+ Alkyl peroxynitrites, ROONO Alkyl peroxinitrates, RO_2ONO Nitryl chlorine, NO_2Cl Peroxylacetyl nitrate, $CH_3C(O)OONO_2$

Table1.2: Nomenclature of reactive species.

(Adapted from Free Radicals in Biology and Medicine, B. Halliwell and J.M.C Gutteridge. Fourth Edition, 2007)

nuclear electron transport chain has been suggested to play a role in ATP generation for RNA synthesis, however it may also function as an ROS generating system, liberating both $O_2^{\bullet-}$ and H_2O_2[78].

A third electron transport chain is contained in the endoplasmic reticulum (ER)[79]. The ER electron transport chain consists of multiple isoenzymes of cytochrome P450 oxidase, cytochrome b5 and an NADPH cytochrome P450 reductase. Due to the broad substrate range of the P450 oxidases, the ER is the central location for xenobiotic detoxification, however substantial amounts of superoxide can also be released[80,81]. Additionally to the electron transport chains, cellular oxidoreductases can contribute to ROS formation at multiple locations. At the plasma membrane, NADPH oxidase functions to transport electrons across the membrane, oxidizing NADPH on the cytosolic side and generating extracellular $O_2^{\bullet-}$. This enzyme was originally characterized in phagocytes, which utilize a multicomponent complex to generate a respiratory burst of $O_2^{\bullet-}$ for defense against pathogens. The complex consists on the catalytic $gp91^{PHOX}$ and its cytosolic regulators $p47^{PHOX}$, $p67^{PHOX}$ and Rac1. A homologue (Mox1/Nox1) to the $gp91^{PHOX}$ has recently been identified in non-phagocytic cells, where its generation of $O_2^{\bullet-}$ is implicated in mitogenic signaling[82,83]. This enzyme is functionally discrete from the phagocytic oxidase as its activity is unaffected by the addition of $p47^{PHOX}$, $p67^{PHOX}$ and Rac1, the regulatory components of the phagocyte respiratory burst oxidase. It has been hypothesized that this oxidase may be activated in response to receptor ligand interactions, increasing extracellular $O_2^{\bullet-}$ formation. ROS can then diffuse back inside the cell to interact with components of signaling pathways in close proximity to the membrane.

The cell also possesses several cytosolic ROS generating mechanisms. The molybdoenzyme xanthine oxidase (XO) is the rate limiting enzyme in the purine metabolic cycle. XO catalyzes the oxidation of hypoxanthine to xanthine, which further converts to uric acid, generating both $O_2^{\bullet-}$ and H_2O_2. The enzyme's subcellular location remains controversial, and while it is certainly present in the cytosol, XO may also become associated with cellular membranes and organelles[84]. Additionally, many NAD(P)H utilizing redox enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1)

are located in the cytosol and will also reduce oxygen in addition to their natural substrates[85].

The superoxide anion in itself is not highly reactive; therefore, its potential effects are restricted to a single intracellular compartment. However, $O_2^{\bullet-}$ can further interact with other molecules to generate other reactive radicals such as hydroxyl radical ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$). In addition, through the action of superoxide dismutase (SOD), $O_2^{\bullet-}$ is rapidly converted to hydrogen peroxide (H_2O_2), a non-radical reactive species that can readily penetrate membranes. Although H_2O_2 is a relatively weak oxidant compared to other ROS such as $OH^{\bullet}$, it has emerged as a particularly important signaling molecule based on its unique biochemical properties. H_2O_2 is ubiquitously present in biological systems with a relatively long half-life, and more importantly, H_2O_2 is soluble in both lipid and aqueous environments and is thereby capable of reaching its cellular targets when applied extracellularly[86,87] (Figure 1.3). Frequently, different reactive species coexist in the cellular environment and make it difficult to identify unequivocally which agent is responsible for a given biological effect.

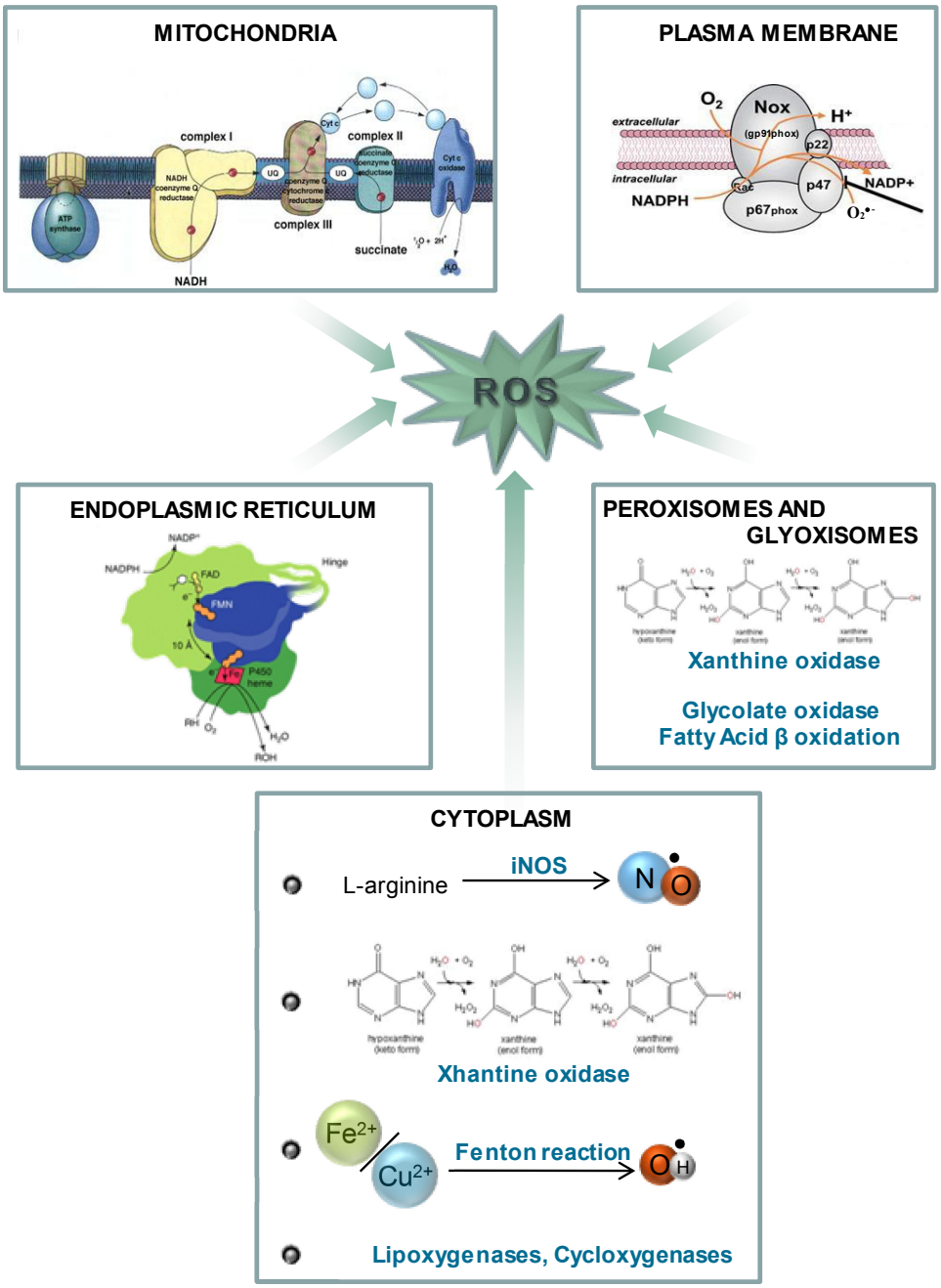


Figure 1.2: Cellular compartments where ROS are generated.

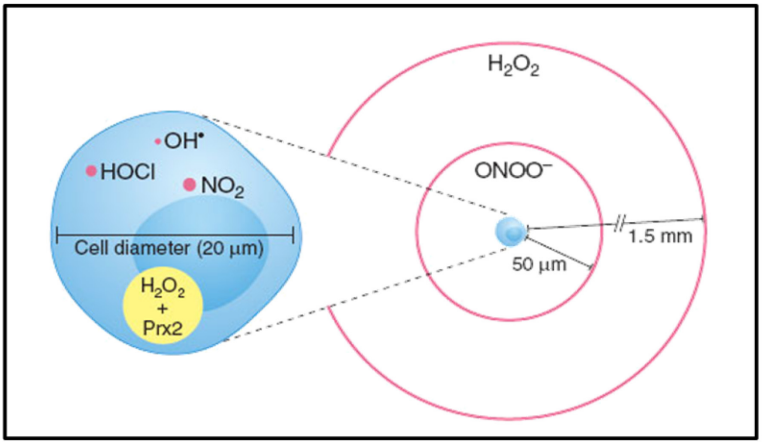


Figure 1.3: Estimated diffusion distances of selected oxidants.

Diffusion distances are shown for oxidants in the presence of a nominal cellular GSH concentration of 2 mM (pink circles) relative to the diameter of a generic cell (20 µm; blue). Also shown (yellow circle) is the diffusion distance for H_2O_2 in the presence of peroxiredoxin 2 (Prx2) at an estimated cellular concentration of 20 µM. The cell is scaled down ten-fold from left to right to illustrate that distances for $ONOO^-$ and H_2O_2 are greater than its diameter. Diffusion distances are calculated from the expression $\ln(C/Co) = l\sqrt{\Sigma k[S]/D}$, where l is the distance over which the oxidant drops from an initial concentration of Co to C, D is its diffusion coefficient, k is the reaction constant rate and S is the substrate concentration.

(Adapted from Winterbourn CC, Nat Chem Biol. 2008 May;4(5):278-86.

1.5.3 Oxidative damage to DNA, lipids and proteins.

ROS can be important mediators of damage to nucleic acids[88]. Physiologically relevant levels of $O_2^{\bullet-}$, $NO^{\bullet}$, H_2O_2 or ROOH do not react at significant rates with any of the DNA bases or with the deoxyribose sugar. However, $O_2^{\bullet-}$ and $NO^{\bullet}$ could react with radicals formed after DNA is attacked by the more aggressive radical $OH^{\bullet}$. Both the purine and pyrimidine bases and also the deoxyribose backbone are fragmented by $OH^{\bullet}$, yielding multiple products[74]. The most extensively studied DNA lesion is the formation of 8-deoxy-guanosine. Permanent modification of DNA resulting from oxidative damage might result in an increase in the mutagenic rate. A mutation in an exon might lead to an altered protein. Mutations in introns are not necessarily innocuous because they can affect mRNA splicing. Mutations in promoter regions could affect transcription factor binding and alter gene expression. RNA bases and riboses can also be targets of radical species. Since most RNA turns over rapidly, the significance of its oxidation in normal cells is unclear. Different studies suggested that oxidative base damage is higher in mitochondrial than in nuclear DNA possibly due to the proximity of mitochondrial DNA to ROS generated during the electron transport chain and that mitochondrial DNA is not protected by histones. However, the significant disparity between published measurements of oxidative damage makes it impossible to conclude that mitochondrial DNA suffers greater oxidation than nuclear DNA[89].

Reactive species can also react with polyunsaturated fatty acids, lipoproteins and membranes to create peroxyl radicals, the primary free radical intermediate of lipid peroxidation. Initiation of lipid peroxidation can be caused by addition of an RS or by hydrogen atom abstraction from a methylene ($-CH_2-$) group by an RS, and in both cases, a carbon radical results. $OH^{\bullet}$ readily initiates lipid peroxidation as do $HO_2^{\bullet}$, $RO^{\bullet}$ and $ROO^{\bullet}$. By contrast, neither $NO^{\bullet}$ nor $O_2^{\bullet-}$ are sufficiently reactive to abstract H from lipids; in any case, the charge of $O_2^{\bullet-}$ tends to preclude it from entering the lipid phase of membranes. Although carbon radicals are often stabilized by molecular dienes, the most likely fate of carbon radicals under aerobic conditions is to combine with oxygen resulting in the formation of a peroxyl radical ($ROO^{\bullet}$). Peroxyl radicals can extract $H^{\bullet}$ from an adjacent fatty-acid side-chain. This is the propagation state of lipid peroxidation; it forms new carbon radicals, and so the chain reaction of lipid peroxidation continues.

The $ROO^{\bullet}$ combines with $H^{\bullet}$ that it extracts to yield lipid peroxides. Thus, a single initiation event has the potential to generate multiple peroxide molecules by a chain reaction. Once formed, peroxyl radicals can be re-arranged and form numerous intermediates: hydroxyperoxides or monocyclic peroxides. Some hydroxyperoxides are unstable and can decompose to aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). The cyclic peroxides can form bicyclic structures that can give rise to isoprostanes. Isoprostane measurement, especially 8-*iso*-PGF2α, is recognized as a reliable marker of lipid peroxidation and is currently used as a sensitive index of oxidative stress *in vivo*. Although the quantification of MDA and HNE has been used as an indication of lipid peroxidation, data should be interpreted with caution. These aldehydes can arise from peroxide decomposition rather than by peroxide formation.

Lipid peroxidation result in a chain of events that decrease membrane fluidity making it easier for phospholipids to exchange between the two halves of the bilayer. The alteration in the membrane array increases the flux of substances that do not normally cross it other than through specific channels (e.g. Ca^{2+}). Alterations in lipid structures can also cause cross-linking of membrane proteins which decreases their lateral and rotational mobility resulting in the inactivation of enzymes and ion channels. Continued oxidation of the fatty acid side-chains and their fragmentation ultimately lead to loss of the membrane integrity compromising the cellular function. Lipid peroxidation also occurs in other membranes, such as the membranes of mitochondria, the ER, nucleus and microsomes.

Proteins are also susceptible to reactions with free radicals. Damage to proteins can occur by direct attack of RS, or by "secondary damage" involving reaction with end-products of lipid peroxidation. The chemistry of oxidative protein damage is even more complex than for DNA damage; instead of four bases and one sugar there are 20 amino acids residues, each capable of forming several oxidation products. Peptide bonds can also be attacked by RS. Some protein damage is reversible, such as peroxiredoxin inactivation, methionine sulphoxide formation, S-nitrosylation, destruction of Fe-S clusters by $O_2^{\bullet-}$, glutathionylation, and possibly nitration. Other damage, for example oxidation of side-chain residues to carbonyl residues (protein carbonylation), appears irreversible and the protein is destroyed and replaced. Several RS (including $OH^{\bullet}$, $RO_2^{\bullet}$,

O_3, HOCl, ONOO- and singlet O_2) oxidize amino acids residues in proteins generating a multiplicity of end-products with carbonyls groups, which can be measured after the reaction with 2,4-dinitrophenylhydrazine (DNPH). Indeed, the carbonyl assay is a reliable general assay widely used for measuring oxidative protein damage. When cells are exposed to oxidative stress, proteomic analysis reveals that often only a small number of proteins are damaged, although the mechanism for this selectivity remains undetermined[90]. Oxidative damage to proteins is important *in vivo* because it can impair the functioning of receptors, antibodies, signal transduction, transport proteins and enzymes. In addition, protein damage can lead to secondary damage to other biomolecules, for example, by raising Ca^{2+} levels and activating nucleases. Damage to DNA repair raises oxidative DNA damage levels and increases mutation frequency, whereas damage to polymerases may decrease their fidelity in replicating DNA.

Different antioxidants are needed to protect against several of these events and when the disturbance in the prooxidant (RS) - antioxidant balance is in favor of the former, the cell is in a state defined as oxidative stress. Oxidative stress can produce injury by multiple pathways that overlap and interact in complex ways. For example, DNA can suffer direct oxidative damage, by $OH^{\bullet}$, indirectly by damage in binding to end-products of lipid peroxidation such as HNE and MDA, failure to repair it because of oxidative damage to polymerases and repair enzymes, inaccurate replication by damaged polymerases, and cleavage by nucleases activated by raises in intracellular free Ca^{2+}.

1.5.4 Antioxidants

To defend against excess ROS production, the cell contains high concentrations of antioxidant molecules (Figure 1.4). In the cytosol, high micromolar levels of the antioxidants vitamin C, β-keto acids, such as pyruvate, and purine species, such as urate, can act as ROS scavengers. Similarly, the membrane-bound antioxidants, vitamin E and lipoic acid, can serve a protective function in a more hydrophobic environment. Of particular importance is the cellular tripeptide glutathione (γ-glutamylcysteinylglycine, GSH). GSH is present in the cell in millimolar concentrations and functions as a redox buffer to maintain the overall cellular redox state. Upon oxidation, it forms glutathione

disulfide (GSSG), which can be reduced by glutathione reductase (GR) using NADPH as an electron source.

While ROS can effectively interact with these antioxidant molecules *in vitro,* the rates of these reactions are generally slow on a physiological timescale and the cell uses several antioxidant enzymes as a supplemental means for ROS detoxification. Superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to H_2O_2 and O_2 whereas catalase (CAT) dismutates H_2O_2 to O_2 and H_2O[91]. In addition, there are two families of antioxidant enzymes that require GSH as an electron donor. Glutathione peroxidase (GPx) and the 1- and 2- cysteine peroxiredoxins (Prx) both utilize a similar catalytic cycle to detoxify peroxides[92,93]. Although both serve analogous functions, GPx is much more efficient (rate = 10^7 $M^{-1}s^{-1}$) whereas the Prxs are slower (rate = 10^5 $M^{-1}s^{-1}$) but are present in higher cellular concentrations[94]. In addition to GSH, the cell also possesses two proteins that can reverse disulfide formation. Thioredoxin (Trx) and glutaredoxin (Grx) recognize protein disulfides and undergo two consecutive thiol-disulfide exchange reactions resulting in reduction of the protein and oxidation of Trx or Grx. Other defense mechanisms involve agents that minimize the availability of pro-oxidants (e.g. transferrins, albumins, haptoglobins, haemopexin, heme-oxygenases and metallothionein) and proteins that protect biomolecules against oxidative damage by other mechanisms, e.g. chaperones.

The levels and composition of antioxidant defenses differ from tissue to tissue and cell type to cell type (possible even between cells of the same type) in a given tissue. When RS are generated *in vivo*, many antioxidants come into play. Their relative importance depends upon: which RS is generated, how it is generated, where it is generated, and what target of damage is measured. For example, when primary cultures of rat neurons are exposed to H_2O_2 or $NO^{\bullet}$, α-tocopherol appears to be a protective antioxidant. By contrast, α-tocopherol provides little protection against damage by $O_2^{\bullet-}$[95]. If the oxidative stress is the same, but a different damage target is measured, different outcomes result. For example, exposure of human blood plasma to gas-phase cigarette smoke causes lipid peroxidation of plasma lipids, which is inhibited by ascorbate. However, ascorbate does not protect against damage to plasma proteins by cigarette smoke, as measured by the carbonyl assay.

Antioxidants have been defined as any substance that delays, prevent or removes oxidative damage to a target molecule[74,96]. However, it is important to note that "antioxidants" can stimulate oxidative damage *in vivo*. For example, ascorbic acid and α-tocopherol have been shown to increase oxidative DNA damage in different models[97,98] (see also section 1.7.2).

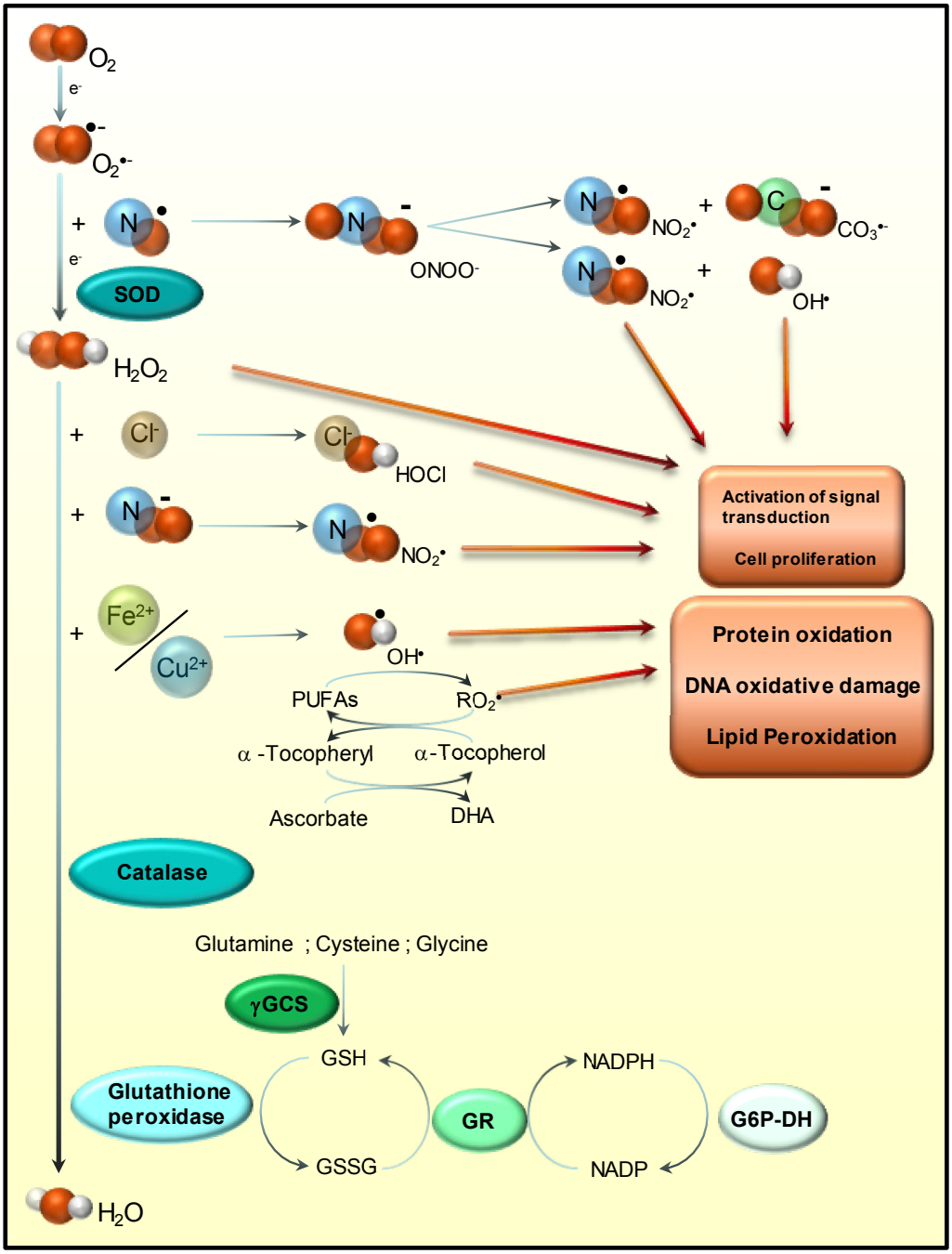


Figure 1.4: Radical species and antioxidant enzymes.

1.5.5 ROS and mechanisms of maintenance of "redox homeostasis"

Free radicals and reactive non-radical species exist in low, but measurable concentrations in the cells[74,99]. Their concentrations are determined by the balance between their rates of production and their rates of clearance by various antioxidants. Thus each cell is characterized by a particular concentration of electrons (redox state) stored in many cellular constituents and the redox state of a cell and its modulation determines cellular functioning[100].

In recent years, the term "redox state" has not only been used to describe the state of a redox pair, e.g. GSSG/2GSH, Asc¢AcsH− and others, but also to describe more generally the redox environment of a cell[100,101]. The redox state of a cell is kept within a narrow range under normal conditions, similar to the manner in which a biological system regulates its pH. Under pathological conditions, the redox state can be altered to lower or higher values.

The intracellular "redox homeostasis" or "redox buffering" capacity is instantiated primarily by GSH and Trx. The GSSG/GSH ratio represents the major cellular redox buffer and, therefore, is a representative indicator of the cellular redox environment[100,102]. Generally, a more reducing environment (maintained by elevated levels of GSH and Trx in the cell) stimulates proliferation and a slight shift towards a mildly oxidizing environment initiates cell differentiation. A further shift towards a more oxidizing environment in the cell leads to apoptosis and necrosis. While apoptosis is induced by moderately oxidizing stimuli, necrosis is induced by an intense oxidizing effect[103-105].

1.5.6 Redox signal transduction

The term redox signaling is used to describe a regulatory process in which the signal is delivered through redox reactions. Cysteine residues in signaling proteins function as "redox sensors" through the reversibility of the thiol/disulfide redox couple. Cysteine oxidation can, therefore, act as a switch, allowing a protein's conformation or activity to be modulated in an analogous manner to tyrosine phosphorylation in kinase signaling cascades. Redox signaling requires that the steady state of "redox balance" is disturbed either by an increase in ROS formation or a decrease in the activity of

antioxidant systems. An increase in free radicals leads to a temporary shift of the intracellular redox state toward more oxidizing conditions that represents the physiological basis for redox regulation. The low concentrations of ROS present during signaling events guarantee that the local increase in ROS will only have a significant oxidative effect on targets in close proximity to the source, where the effective ROS concentration is fairly high. This may provide a structural basis for a redox signaling network, where the local concentration of ROS can be regulated by the respective contributions of the generating systems and the scavenging activities of antioxidants.

Signaling mechanisms that respond to changes in the thiol/disulphide redox state involve; (i) transcription factors AP-1 and NF-κB, (ii) bacterial OxyR, (iii) Keap1 and Nrf2, (iv) protein tyrosine phosphatases, (v) Src family kinases, (vi) JNK and p38 MAPK signaling pathways, (vii) insulin receptor kinase activity, and others[102,106-109]. Under pathological conditions, however, substantially high concentrations of RS may lead to permanent changes in signal transduction and gene expression, which is typical of disease states.

There are, therefore, innumerable possibilities for sites of ROS activity in signaling pathways. However, which of these are functional *in vivo* is currently the subject of investigation. Moreover, elements of these signaling pathways may vary significantly among different cell types.

1.6 Molecular mechanism of cellular response to As_2O_3

The clinical effectiveness of As_2O_3 in APL has stimulated research activities aiming at understanding its mechanisms of action. Although many questions remain to be resolved, *in vivo* and *in vitro* data suggested that partial differentiation of APL cells and induction of apoptosis is likely to constitute the cellular basis of the effects of As_2O_3.

1.6.1 As_2O_3-induced differentiation in APL cells expressing the PML fusion protein.

Initial clinical observations showed that during intravenous infusion of As_2O_3 in APL, a gradual reduction of leukemic promyelocytes occurred and a large amount of myelocyte-like cells and degenerative dying cells with condensed nuclei losing cytoplasm

appeared in both the bone marrow and peripheral blood. These observations supported the idea that As_2O_3 could induce differentiation. It was then demonstrated that As_2O_3 therapy induced a progressive decrease in the proportion of cells expressing CD33, an antigen associated with primitive myeloid cells, along with an increase in the proportion of cells expressing CD11b, an antigen restricted to mature myeloid elements[110].

In vitro effects of pharmacologic concentrations of As_2O_3 on fresh APL cells and the APL cell line NB4 were then studied[111]. The results demonstrated that As_2O_3 exerts dual effects on these cells; at low concentrations (0.1 to 0.5μM), As_2O_3 promotes partial differentiation, while at higher doses (>0.5μM), it induces apoptosis[110,112,113].

The unique response of APL to As_2O_3 implies the importance of intrinsic properties of APL cells. Interestingly, both wild-type PML and PML-RARα fusion protein have been shown to be major targets of As_2O_3 treatment. PML is a tumor suppressor involved in complex functions including growth arrest and apoptosis induction. Normally, PML is located in the nucleus within a specific organelle named the PML nuclear body (NB). In APL cells, due to the heterodimerization of PML/RARα with wild-type PML, PML NBs are disrupted into a nuclear microspeckle pattern, with loss of PML functions[114,115]. A surprising finding was that 0.1 to 2μM As_2O_3 targets PML and PML-RARα to NBs, followed by degradation of the PML-RARα fusion protein[110,116-119]. The relief of the transcriptional repression of PML-RARα may pave the way for the operation of physiological RA signaling in granulocytic differentiation, particularly at the stage of promyelocytes. Although the response of APL cells to As_2O_3 has been linked to degradation of the PML/RARα fusion oncoprotein, exogenous expression of PML-RARα protein does not confer sensitivity to As_2O_3 suggesting that PML-RARα is not principally responsible for the sensitivity of APL cells to As_2O_3[120].

1.6.2 Mechanism of action of As_2O_3.

A second mechanism contributing to the therapeutic response of As_2O_3 is the induction of apoptosis, which occurs in APL cells (with a concentration of >0.5μM) and, moreover, in other malignant cell lines. Among the most studied apoptotic effects of As_2O_3 are the induction of oxidative stress, the depolarization of the mitochondrial

membrane, the release of cytochrome c, as well as activation of stress-related signaling pathways.

1.6.2.1 ROS generation by As_2O_3

Oxidative damage plays an important role in the cytotoxic effects of arsenic, as it does for other metals, such as iron, copper, nickel, chromium, cadmium, lead, and mercury[121]. Arsenic perturbs the cellular redox state through various mechanisms which involve the generation of ROS and reactions with cellular antioxidant systems.

In a wide variety of cell lines, treatment with As_2O_3 leads to a rapid accumulation of intracellular $O_2^{\bullet-}$ and H_2O_2 and associated formation of $OH^{\bullet}$[122-124]. In fact, the ability of As_2O_3 to increase the level of H_2O_2 has been correlated with its apoptotic activity[123]. Moreover, H_2O_2-resistant Chinese hamster ovary (CHO) cells are cross-resistant to As_2O_3, and CHO cells deficient in antioxidant enzyme are hypersensitive to As_2O_3[125,126]. In contrast, the effect of As_2O_3 on $NO^{\bullet}$ depends on the cell type and dose of arsenical tested[127-129]. Another indication that As_2O_3 induces oxidative stress through ROS generation comes from experiments where treatment with various antioxidants, free radical scavengers, or inhibitors of ROS-producing enzymes reverted As_2O_3-mediated damage[122,123]. To address the question of how As_2O_3 exerts its effects, our lab developed arsenic-resistant subclones. These cells were generated by constant culture in the presence of As_2O_3 at concentrations that were slowly increased over time. Once a population of cells was obtained that could be sustained in 1μM As_2O_3, single clones were selected by plating in methylcellulose. The two clones that were selected for further expansion, AsR2 and AsR3, are now routinely cultured in the presence of 2μM As_2O_3. Both clones are approximately 10-fold less sensitive to As_2O_3 than their parental cell line, NB4, and they have increased intracellular reduced GSH allowing for effective elimination of free radicals[120,130]. In addition, these cells accumulate 60% less arsenic than the parental NB4 cells due to an increased expression of ABCC1 (Mrp1), a known complex involved in the transport of arsenic[131]. As a result, As_2O_3-induced ROS do not accumulate sufficiently to initiate apoptosis in As_2O_3-resistant cells.

The precise mechanism by which As_2O_3 induces ROS is not clear and appears to be dependent on the cell type and dose of arsenical tested. However, in several

experimental models, among them leukemic cells, mitochondria are considered the major source of cellular ROS generated after As_2O_3 treatment. This conclusion is supported by several lines of evidence. First, incubation of cells with As_2O_3 caused a substantial inhibition of respiration as evidenced by a marked decrease of O_2 consumption in the As_2O_3-treated cells. Release of ROS seems to be a primary event, since it occurred three hours after drug exposure and before apoptosis became apparent (>24hours). Furthermore, analysis of complex activities using specific substrates suggests that As_2O_3 decreases complex I function. Second, the inability of As_2O_3 to induce a significant increase of ROS and apoptosis in the mitochondrial respiration-defective HL-60 cells (C6F/ρ^-) further suggests that the mitochondrial respiration function is important for the mechanism of action of As_2O_3[132].

Recently, NAD(P)H oxidase and NO synthase isozymes have also been proposed to be involved As_2O_3–mediated ROS generation. As_2O_3 treatment induces upregulation of expression of virtually all components of the NADPH oxidase complex[133], and As_2O_3 does not increase ROS in leukemic cells depleted of the $p47^{PHOX}$ or the $gp91^{PHOX}$ subunit of this enzyme. In addition, As_2O_3 induces translocation of Rac1[134] and Cdc42[135] in cultured cells, thereby enhancing $O_2^{\bullet-}$ production. However, we now have preliminary data that NADPH oxidase, although activated, does not play a significant role in As_2O_3-induced apoptosis (see Chapter 5).

The role of RNS in As_2O_3 toxicity has been less studied. It is known that NO synthase isozymes generate high levels of $NO^{\bullet}$, with minimal $O_2^{\bullet-}$ from their substrate, L-arginine, under normal conditions. However, uncoupling of NO synthase isozymes, resulting in decreased $NO^{\bullet}$ production and concomitant reduction of O_2 to $O_2^{\bullet-}$, does occur under unusual circumstances (e.g., decrease in substrate or cofactor 5,6,7,8-tetrahydrobiopterin (BH4))[136,137]. Interestingly, significant reduction of BH4 levels, but not of L-arginine, is seen following prolonged exposure of rabbits to inorganic arsenate (As^V)[138]. The arsenic-mediated loss of BH4 could be due to its destruction by $O_2^{\bullet-}$ [139], generated through the upregulation of NAD(P)H oxidase.

1.6.2.2 Reaction with sulphydryl groups

The biological effects of As_2O_3 have been also proposed to be mediated by reactions with vicinal sulfhydryl groups of cysteine residues present on cell proteins. This reaction results in the formation of stable ring structures or in the oxidation of critical cysteine amino acids within the catalytic sites of different enzymes. For example, in the pyruvate and α-ketoacid dehydrogenases, the reduced dithiol form of the lipoyl prosthetic group, which is bound covalently to the enzyme, possesses a vicinal thiol group that is inactivated upon As_2O_3 treatment. Components of the electron transport chain in different membranes could also be inactivated by the same mechanism. Monoamine oxidase is another example of As_2O_3-targeted protein. In addition, As_2O_3 causes oxidation of the sulfhydryl moieties of Sp1, inhibiting its DNA-binding activity[140]. Recently, it was demonstrated that As_2O_3 irreversibly inhibits mammalian TrxR with an IC(50) of 0.25 μM by binding both the N-terminal redox-active dithiol and the C-terminal selenothiol-active site of reduced TrxR[141].

1.6.2.3 Mitochondrial role in As_2O_3-induced apoptosis

The fact that As_2O_3 induces apoptosis in a variety of cell types suggests that some common mechanisms should be involved. It is well established that mitochondria play a major rate-limiting role in apoptosis. Various pro-apoptotic signals converge on the mitochondria and trigger depolarization of the mitochondrial membrane potential (ΔΨm) and opening of the permeability transition pore (PTPC). The PTPC is a multiprotein complex formed at the contact site between the inner and outer mitochondrial membranes that plays key role in the control of apoptosis. Bcl-2 and Bcl-$_{XL}$, both well known to have anti-apoptotic properties, can inhibit opening of purified pores reconstituted into liposomes, suggesting a role of PTPC opening in inducing apoptosis[142,143].

As do most chemotherapeutic drugs, As_2O_3 can also induce an early ΔΨm collapse in intact APL cells[144], malignant lymphocytes[117] and carcinoma cell lines[145,146] (Figure 1.5). Moreover, the As_2O_3-resistant clones of NB4 (AsR2 and AsR3) did not show decreased ΔΨm upon As_2O_3 treatment, in contrast to its NB4 parental cell line (Diaz *et al.*, unpublished results). On isolated mitochondria, As_2O_3 induces PTPC opening, and promoted the purified, reconstituted mitochondrial permeability transition

in vitro[142]. As_2O_3-induced ΔΨm collapse and apoptosis have been related to dithiol oxidation or cross-linking, which has been shown to be associated with a higher probability of PTPC opening. Alternatively to the direct effects on PTPC, As_2O_3 could disrupt mitochondria through the generation of ROS. These data support that mitochondria, in particular the PTPC, are important targets of As_2O_3.

The opening of the PTCP allows the influx of water and small molecules, resulting in swelling of the mitochondrial matrix with the eventual rupture of the outer mitochondrial membrane. As a consequence, pro-apoptotic factors are released from mitochondria into the cytosol, among them cytochrome c and AIF. Once released to the cytosol, cytochrome c can activate caspases[147,148], and AIF translocates to the nucleus promoting chromatin condensation and partial DNA degradation[149]. Multiple reports have described the release of cytochrome c and activation of caspases by As_2O_3[123,150-152]. In fact, loss of ΔΨm by As_2O_3 have been invariably associated with activation of caspases, although depending on the caspase implicated, this activation have been described either as a result of or as a requirement for mitochondrial membrane depolarization. The activation of caspase-9 and caspase-3 with the subsequent cleavage of its specific substrate such as poly (ADP-ribose) polymerase (PARP) has been observed in most cells treated with As_2O_3[123,150,153,154]. However, in some cell lines, As_2O_3-induced apoptosis is independent of caspase-3 activation[117,118]. These *in vitro* results have been supported *in vivo* by examining the activation of caspases in bone marrow mononuclear cells from an APL patient treated with As_2O_3 for 11 weeks. An upregulation of procaspase-3 and active caspase was observed by Western blotting suggesting that the observations made upon As_2O_3 treatment *in vitro* accurately reflect those mechanisms which contribute to the *in vivo* clinical response to As_2O_3[30].

The extrinsic pathway of apoptosis has been demonstrated to play a role in As_2O_3-mediated cell death. In NB4 cells, caspase-8 is activated by As_2O_3 in a GSH-dependent manner[130]. Inhibition of caspase-8 blocks not only the activation of caspase-3, but also the loss of ΔΨm. It was then shown that caspase-8 activation induced cleavage of the pro-apoptotic protein Bid to its active form tBid, which mediated $\Delta\Psi_m$ loss by acting as a Bax agonist. Bax oligomerization resulted in insertion into the mitochondrial membrane,

mediating cytochrome c release and subsequent activation of the mitochondrial apoptotic pathway.

Recently, the p53 status of the cell has been proposed to dictate whether the intrinsic or extrinsic pathway is preferentially induced by As_2O_3. Using a panel of myeloma cells, it has been shown that caspase 9 is the primary caspase activated by As_2O_3 in cells expressing wild type p53, while those expressing p53 mutants or that were p53 null preferentially activated caspase 8[155]. This observation therefore provides a mechanism to account for the apparent cell-type specific effects of As_2O_3 on caspase activation.

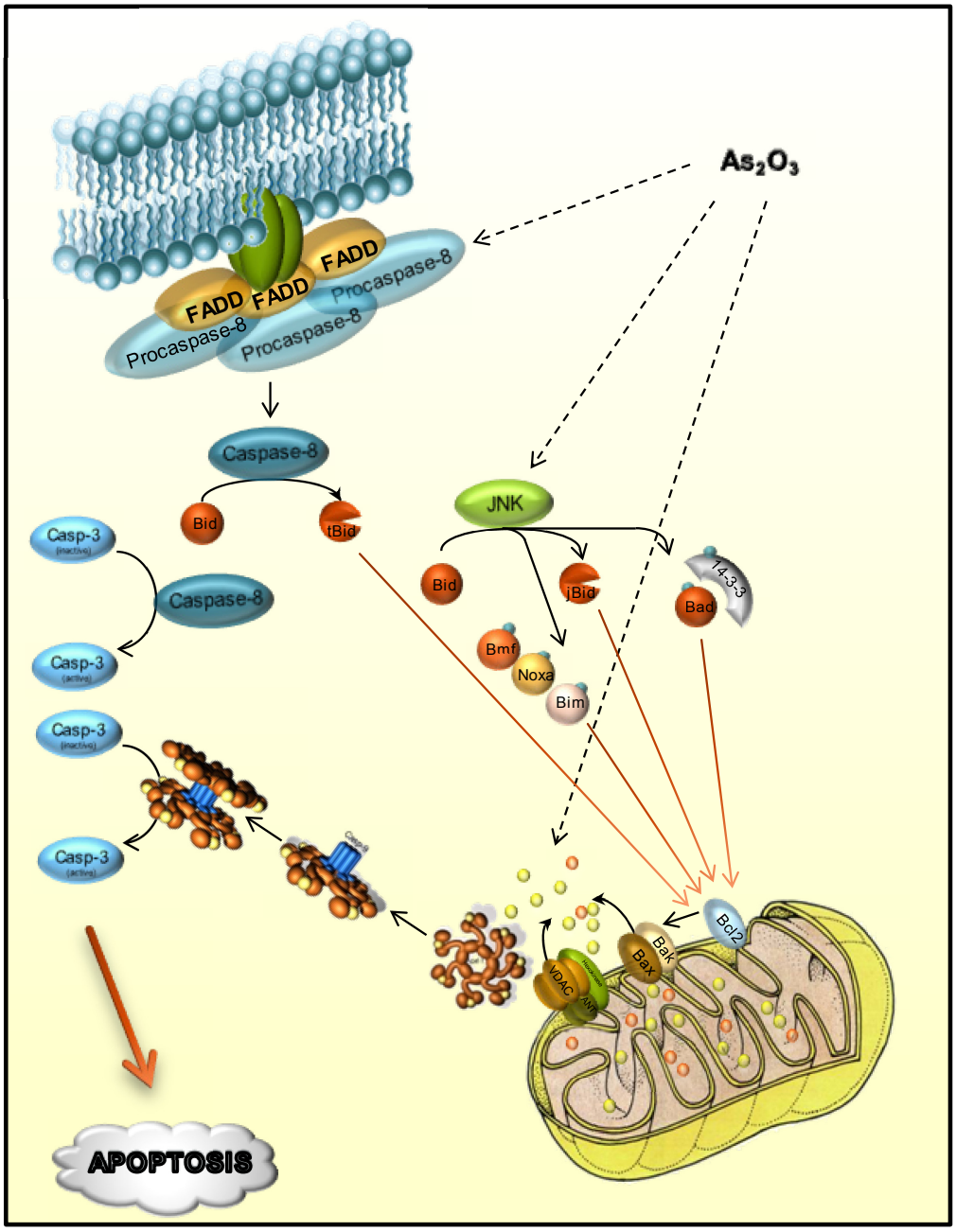


Figure 1.5: Mitochondrial role in As_2O_3-induced apoptosis.

1.6.3 Activation of signal transduction pathways by As_2O_3

Lines of evidence obtained from very different model systems point to the ability of As_2O_3 to induce cytotoxicity through the alteration in the function of several enzymes and signaling molecules. Recently, efforts have been made at identifying those pathways essential for the induction of apoptosis (Figure 1.6).

The c-Jun N-terminal kinase (JNK), or stress-activated protein kinase, is an important member of the mitogen-activated protein kinase superfamily (MAPK), which have been related to As_2O_3-mediated apoptosis in different experimental models. As with all MAPK family members, activation of JNK occurs as a result of its phosphorylation by the upstream kinases SEK1 (MKK4) and SEK2 (MKK7). Our lab and others have demonstrated that As_2O_3 induces JNK1, and to a lesser extend JNK2 activation in NB4 cells[156-161]. However, in the arsenic-resistant subclones of these cells (AsR2 and AsR3 cells), JNK was not activated even at doses that elicit significant activation in NB4 cells. The role of JNK in As_2O_3-mediated apoptosis was elucidated by comparison of the effects of As_2O_3 on cell proliferation in the SEK1 knockout cells and wild-type cells. Results from our lab clearly show that when the JNK pathway cannot be activated, As_2O_3-induced apoptosis is inhibited. In addition, NB4 cells treated with dicumarol (a JNK inhibitor) showed resistance to As_2O_3-induced apoptosis[162]. Additional supporting evidence has been obtained using the NKM-1 cell line, which was established from a patient with acute myeloid leukemia. In this cell line, As_2O_3 treatment was also accompanied by sustained JNK activation, and As_2O_3-mediated cell death was decreased by the addition of a JNK inhibitor[163]. In JB6 cells, a mouse epidermal cell line, activation of JNK was shown to be essential for induction of apoptosis by As_2O_3, as expression of a dominant negative mutant of JNK nearly completely abrogated As_2O_3-induced apoptosis[159]. As_2O_3-mediated JNK activation has also been demonstrated in various cervical cancer cells[151], in the chronic myelogenous leukemia (CML) cell lines K562 and KCL22[164], in U-937 human promonocytic leukemia cells[165], in ILKM-3, U266 and XG-7 multiple myeloma cell line[166] among others. Although in a majority of cell lines JNK activation is required for sensitivity to As_2O_3, others suggest that JNK activation by As_2O_3 is an incidental finding, with little significance for the induction of apoptosis. In HepG2 cells, for example, Kang *et al.* reported that, while treatment with antioxidants

could inhibit As_2O_3-induced apoptosis, similar treatments had no effect on As_2O_3-induced JNK activity[167]. In contrast, JNK was not activated after As_2O_3 exposure in HL60 cells. Therefore, while indirect, this evidence suggests that JNK activation may not be a significant effect of As_2O_3 treatment in all cell types. Although As_2O_3 have also been shown to induce p38 MAPK in CML- or APL-derived cell lines and in primary cells, inhibition of p38 MAPK activation by pharmacological inhibitors (SB203580) or downregulation of p38 MAPK by siRNA significantly increased JNK activation, growth inhibition and apoptosis induced by As_2O_3[168,169].

At present, a number of signaling pathways have been defined as the molecular mechanisms of As_2O_3-mediated JNK activation; one of them involves the MAPKKK ASK1. ASK1 is a ubiquitously expressed MAPKKK that activates both JNK and p38 by phosphorylating and activating respective MAPKKs (SEK1, SEK2)[170]. Yan *et al*. have demonstrated that As_2O_3 activates ASK1 in leukemic cells at relatively low concentrations, which is accompanied by ROS accumulation and is inhibited by the antioxidant N-acetyl-l-cysteine (NAC)[171]. In other experiments, As_2O_3 was reported to induce ASK1 recruitment to nuclear bodies in APL cells[172]. Our laboratory has hypothesized that ASK1 activation is important in JNK phosphorylation and As_2O_3-mediated apoptosis. Using genetic approaches, we have demonstrated that ASK1 is required for As_2O_3-induced apoptosis; $ASK1^{-/-}$ MEFs cells are less susceptible to As_2O_3-induced apoptosis than wild-type $ASK1^{+/+}$ cells. Importantly, in the $ASK1^{-/-}$ cells JNK activation is dampened (unpublished results). Thioredoxin is an important cellular redox regulatory protein considered as the internal inhibitor of ASK1[173,174]. The activity of ASK1 depends on the redox status of Trx, as only the reduced form of Trx, not the oxidized form, is capable of binding to ASK1 and blocking its kinase activity. Thioredoxin reductase (TrxR), reduces Trx and favors ASK1 inactivation. In NB4 cells, inhibition of TrxR by Au^{III} enhances As_2O_3-induced apoptosis (unpublished results), suggesting that the thioredoxin–ASK1 system serves as the molecular switch that converts a redox signal to kinase activation and apoptosis. Experiments aiming at elucidating the role of Trx isoforms and Trx inhibitory proteins in As_2O_3-mediated ASK1 activation are ongoing.

How MAPKKK proteins upstream to JNK are activated upon As_2O_3 treatment is unclear. Some evidence implicates Ras and the Rho family of GTPases, including Rho, Rac, and Cdc42[135,157,164]. However, the complexity of this system, together with the possible redundancy of related proteins, makes delineating the events that initiate JNK activation a difficult task, and further work will be required to completely elucidate the mechanism of As_2O_3-mediated JNK activation.

In addition to activated pathways leading to cell death, survival signals are inhibited by As_2O_3. AKT, a serine/threonine protein kinase, mediates the phosphoinositide 3-kinase (PI3K)-induced cell survival signals by phosphorylation and inactivation of several pro-apoptotic proteins including BAD[175], caspase-9[176], and members of the forkhead (FKHR) family of transcription factors[177,178], rendering them inactive. AKT also negatively regulates the MAP kinase pathways required for As_2O_3-induced apoptosis. AKT phosphorylates ASK1 and SEK1 and inhibits their function[179,180]. Our lab has recently demonstrated that As_2O_3 decreases not only AKT activity, but also total AKT protein. Decreased AKT expression further correlates with JNK activation as well as with sensitivity to As_2O_3[181].

Other signaling pathways involved in As_2O_3-mediated JNK activation have been studied and are listed below. (i) There is evidence that Src kinase is another redox-sensitive pathway implicated in JNK activation[182]. As_2O_3-mediated Src induction leads to JNK activation[183-185]. Currently, the exact signaling pathway from Src activation to JNK activation remains elusive. One possibility is the role of Gab1, a docking protein downstream of epidermal growth factor receptor signaling. Upon H_2O_2 exposure, Gab1 is phosphorylated and binds to Src homology 2-containing protein-tyrosine phosphatase, a critical step leading to specific activation of JNK[186]. UV-induced JNK activation has been found to follow such a pathway[187]. (ii) Another interesting signaling pathway in As_2O_3-mediated JNK activation is the role of glutathione-S-transferase π (GSTπ). The monomeric form of GSTπ binds to the C-terminal fragment of JNK and suppresses its kinase activity[188]. In NB4 cells treated with As_2O_3, GSTπ oligomerization, dissociation of the GSTπ–JNK complex and JNK activation were detectable after 24 h and were followed by an increase of the apoptotic rate starting at 72 h. Neither GSTπ polymerization nor JNK activation was found in AsR2 cells that showed a very low

apoptotic rate[189]. Further studies demonstrated that GSTπ is increased in several As_2O_3-resistant cell lines[190-193] and undetectable in As_2O_3-sensitive cells[53,123]. In addition, As_2O_3 inhibited cell proliferation of DU-145 and PC-3 cells (both cells express GSTπ), but not of LNCaP cells (which lack GSTπ expression)[194]. GSTπ, as a component of the As_2O_3 detoxification pathway, may also inhibit As_2O_3-induced apoptosis by decreasing intracellular arsenic levels. It is worth mentioning that the effect of GSTπ on JNK is independent of the ASK1–SEK1/2 pathway. (iii) Recently, another route was reported leading to As_2O_3-induced JNK activation, independent of the ASK1–SEK1/2 pathway. As_2O_3-induced JNK activation requires the interaction of JNK with receptor-interacting protein (RIP) and tumor necrosis factor receptor-associated factor 2 (TRAF2) to form a signaling complex at the cell membrane lipid rafts[195]. In fact, the disruption of raft structures make cells resistant to the effects of arsenite[161], because the signals necessary to initiate the apoptosis cascade cannot be properly transduced.

Taken together, it is believed that As_2O_3-induced ROS and oxidative stress are capable of modulating JNK activation via multiple signalling pathways. Figure 1.6 summarizes various pathways involved in JNK activation mediated by ROS from either exogenous or endogenous sources.The next important question is how activated JNK leads to apoptosis. Currently many studies have suggested that mitochondria are the main site of action for JNK in apoptosis.

It has been found that primary fibroblasts prepared from $JNK1^{-/-}JNK2^{-/-}$ and from $SEK1^{-/-}SEK2^{-/-}$ embryos exhibit marked defects in stress-induced apoptosis[196,197], which was associated with failure to release mitochondrial apoptogenic factor proteins, including cytochrome c. Indeed, micro-injection experiments demonstrated that the mutant cells did not exhibit defects in apoptosis when cytochrome c was directly injected into the cytoplasm[197]. Moreover, in $Bax^{-/-}Bak^{-/-}$ fibroblasts, activated JNK was not able to cause the release of mitochondrial cytochrome c and conformational changes in Bax/Bak observed in wild-type cells were not found in $JNK^{-/-}$ cells[198]. As a consequence, apoptosis was impaired. Moreover, mitochondrial translocation of JNK occurs in As_2O_3-treated cells[199]. These studies establish that mitochondria are a primary target of pro-apoptotic signaling by JNK.

It is believed that the Bcl-2 family proteins may provide a molecular link between JNK and the mitochondrial apoptotic machinery. In fact, several Bcl2-like proteins have been proposed to mediate the effects of JNK on cell death. As_2O_3-induced apoptosis is associated with upregulation of Bax[152]. In addition, Bax is considered to be phosphorylated and activated by JNK, although the site of phosphorylation has not been identified[200]. Bax has also been reported to be sequestered by 14-3-3 protein[201] and recently, it has been described that four of the seven isoforms of 14-3-3 are phosphorylated by JNK resulting in the dissociation of Bax from inactive 14-3-3 complexes[202]. Thus, 14-3-3 protein phosphorylation represents one mechanism that may contribute to JNK-mediated regulation of the pro-apoptotic activity of Bax. JNK-mediated phosphorylation of 14-3-3 proteins may also lead to the release of other proapoptotic proteins[203]. The anti-apoptotic proteins Bcl2, Bcl-XL and Mcl-1 are phosphorylated by JNK *in vitro* and this phosphorylation may suppress the antiapoptotic functions of these proteins[151,199]. Furthermore, the BH3-only protein Bid can be proteolytically processed in a caspase-independent, but JNK-dependent manner in cells exposed to As_2O_3[204]. JNK-dependent processing of Bid generates a novel form (jBid). However, the mechanism employed by JNK to regulate Bid processing is unknown and the structure of jBid has not been defined. In addition, the BH3-only protein Bad is phosphorylated by JNK in cells treated with As_2O_3[205]. This phosphorylation has been reported to lead to either increased[206] or decreased[207] Bad-mediated apoptotic activity in transfection assays, although the significance of the pro-apoptotic phosphorylation of Bad by JNK has been questioned[208]. The BH3-only proteins Noxa, Bmf and Bim are necessary for As_2O_3-induced cell death in myeloma[152,209]. These proteins are also phosphorylated by JNK and the phosphorylated forms increase apoptosis[210-212]. Furthermore, the expression of pro-apoptotic Bim can also be transcriptionally induced by JNK-dependent AP-1 activity, leading to JNK-dependent apoptosis[213-215]. Further studies are required to define whether any of these proposed mechanisms are relevant to JNK-stimulated apoptosis *in vivo*.

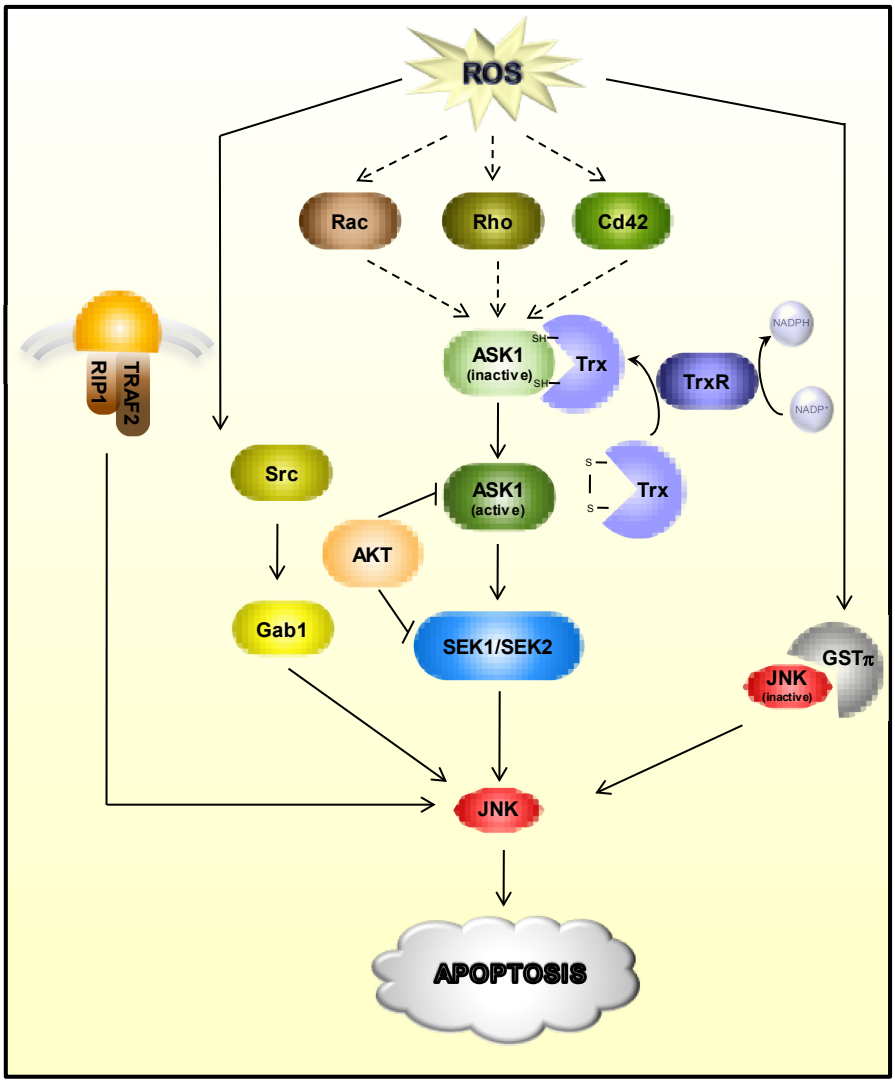


Figure 1.6: Signaling pathways of JNK activation mediated by As_2O_3.

One of the main routes involving JNK activation is via the ASK1–SEK1/SEK2–JNK module. In addition, many other ASK1-independent pathways have been identified, including (i) the Src–Gab1 pathway, (ii) the GSTπ pathway, and (iii) the RIP–TRAF2 and membrane lipid raft pathway. The exact roles of these pathways in ROS-induced JNK activation are believed to be cell-type and stimulus dependent.

1.7 Combination therapy with As_2O_3

Following the discovery of As_2O_3 as a new and promising treatment for various types of leukemia, particularly APL, several studies have investigated the use of As_2O_3 in the treatment of solid cancers, including neuroblastoma, head and neck cancer, gastric cancer, prostate cancer and renal cell carcinoma[153,216-219]. However, most of the studies showed that the *in vitro* cytotoxic effects of As_2O_3 in solid tumor cells could not be compared with those seen in APL. Even though As_2O_3 was reported to induce apoptosis or growth inhibition in various cell lines tested, the As_2O_3 concentrations required were higher than those in hematologic malignancies and thus not clinically achievable without the risk of As_2O_3-mediated side effects. Consequently, a search for agents suited to increase As_2O_3 efficacy in less sensitive solid tumors was initiated[220,221]. Because of the many pathways involved in mediating the effects of As_2O_3, the potential exists for synergism with a great variety of compounds to provide enhanced therapeutic benefits.

1.7.1 Increasing As_2O_3 activity by adding a differentiation inducer (ATRA)

As mentioned in section 1.4.1, ATRA plus chemotherapy is the current standard approach for newly diagnosed APL patients, and although new clinical trials have demonstrated the efficacy of As_2O_3 treatment in these patients, As_2O_3 is mainly used in advanced APL. Therefore, the first obvious combination treatment that emerges from these results is the one that explore the potential effects of ATRA+ As_2O_3 combination.

Although some reports on the joint effects of these two drugs in APL cells were controversial, mostly on some differentiation parameters obtained from *in vitro* culture systems[222], several lines of evidence support a strong synergistic effect between ATRA and As_2O_3[223-228]. These drugs would be expected to synergize, based mainly on their molecular mechanisms. In non-APL cells, ATRA binds to RARs, activating transcription of target genes, whereas As_2O_3 alters the traffic of PML proteins, enhancing their nuclear body association as well as their apoptotic properties[229,230]. In addition, in APL cells, both drugs degrade PML/RARα[229,231]. The synergism could also be predicted based on the fact that little cross-resistance between them has been observed; in cell lines resistant to As_2O_3, the addition of ATRA restored the responsiveness to As_2O_3-induced differentiation and apoptosis[232]. These findings suggest the existence of distinct but

convergent mechanisms of action[223,233]. *In vivo* effects of this combination have proven to be achievable. In a murine transplantation model of APL, the administration of As_2O_3 or ATRA alone only prolonged survival, whereas the concomitant use of As_2O_3 and ATRA led to the clearance of the leukaemia and long-term relapse-free survival[224]. In another transgenic mouse model of APL, As_2O_3 and ATRA gave significantly longer survivals than either drug alone[234].

Based on these findings, several clinical trials were designed to analyze whether the potential clinical benefit in combining ATRA and As_2O_3 could be reflected either in a higher quality of the complete remission (CR) or in a better disease-free survival. In a prospective clinical trial, the effects of the ATRA and As_2O_3 combination with those of ATRA or As_2O_3 mono-therapy were compared in remission induction and maintenance therapy of newly diagnosed APL patients. Although CR rates in As_2O_3, ATRA, and As_2O_3+ATRA groups were high (≥90%), the time to achieve CR differed significantly, with that of the combination group being the shortest one. Other studies have shown that ATRA+As_2O_3 combination achieve better results than either of the two drugs used alone in terms of the quality of CR and status of the disease-free survival[235]. The successful implementation of the ATRA and As_2O_3 synergistic targeting therapy model has prompted research groups to investigate the effect of this combination on human hepatoma, breast cancer, and lung cancer cells in an attempt to find a better combination therapy for solid tumors. Although no clinical trials have been designed, ATRA could synergistically increase As_2O_3–mediated growth inhibition and apoptosis in HepG2, Hep3B, MCF-7 and AGZY-83-a cells as well as in two RARα-expressing human T-cell lymphotropic virus type-I-transformed cells[236].

1.7.2 Combination therapy through modulation of glutathione redox system

The intracellular glutathione redox system modulates both the anti-proliferative and pro-apoptotic effects of As_2O_3, and represents the best characterized mechanism of As_2O_3 response[192,237-241]. Due to the multiple functions of glutathione, this molecule may modulate As_2O_3 toxicity by different mechanisms. GSH behaves as a powerful reducing agent, which protects protein thiols from oxidative damage by forming transient mixed disulfides, is an efficacious scavenger of ROS, can become oxidized and provide

electrons for antioxidant enzymes such as GSH peroxidase (which reduce H_2O_2 to H_2O), and can complex with trivalent arsenicals forming a transient $As(GS)_3$ molecule[241] (which renders arsenic more easily excreted through drug efflux pumps)[239]. Importantly, tumor cells appear to be more susceptible to intracellular oxidation than their normal counterparts (see section 1.8). Hence, the modulation of the GSH dependent redox state might represent a useful approach to increase the clinical efficacy of As_2O_3.

Davison *et al.*[120] examined the basis for APL cell sensitivity to As_2O_3. Their data demonstrate that malignant cells that are the most sensitive to As_2O_3-induced apoptosis appear to be those with the lowest levels of intracellular GSH. NB4 cells have been shown to express less GSH and GSH-associated enzymes than many other cells, such as HL60 and su-DHL-4 lymphoma cells, which are less sensitive to As_2O_3 than NB4 cells[192,242-244]. The AR subclones of NB4 cells (AsR2 and AsR3) were developed to exhibit resistance to As_2O_3 in our lab. In keeping with another arsenic-resistant cell lines, both clones feature a marked increase in GSH content[120].

Therefore, strategies to reduce intracellular GSH levels have been utilized in an attempt to enhance susceptibility to As_2O_3-induced apoptosis. BSO was developed as an anticancer drug candidate that depletes GSH by inhibiting γ-glutamyl-cysteine synthetase, the rate-limiting enzyme of GSH synthesis[245], and it was shown that a wide range of cancer cells with increased GSH levels can be sensitized to As_2O_3 by treatment with BSO[240]. Almost all cancer cell lines including those resistant to As_2O_3 (hepatocellular carcinoma, prostate, breast, lung, colon, cervix, bladder, and kidney cancers), become sensitized to As_2O_3 by the addition of BSO. Despite the therapeutic potential of this GSH-depleting agent, it is known that the synergistic effects observed may not be selective for cancer cells. Furthermore, BSO itself has not been successfully developed for clinical use and toxicities such as moderate myelosuppression, leukopenia and thrombocytopenia[246-248] limits its widespread use. Therefore, new agents that could safely lower GSH levels were investigated, among them ascorbic acid (AA). Ascorbic acid is widely heralded as an antioxidant[249]. However, increasing evidence reveals that AA can also act as an oxidizing agent and deplete intracellular GSH levels[250,251]. AA is rapidly oxidized to monohydroascorbyl and, further, to dehydroascorbic acid (DHA), which is then transported into the cell. Inside the cell, AA is regenerated from DHA via

glutaredoxin, which converts GSH to GSH disulfide. This depletes intracellular GSH and increases cellular susceptibility to oxidative stress[252,253]. In addition, *in vitro* AA cytoxicity depends on its interaction with free transition metal ions leading to the generation of H_2O_2 and $OH^{\bullet}$ [74,254]. Clinically relevant doses of AA have been proven to deplete intracellular GSH, increase H_2O_2 production, and potentiate As_2O_3 cytotoxicity in promyelocytic leukemia cells (NB4 and HL-60), B cell lymphoma DHL-4 cells, epithelial mammary carcinoma line T47D[240], cutaneous T cell lymphoma cell lines (HuT-78, SeAx, and Myla)[255], in peripheral blood mononuclear cells from patients with Sezary syndrome[255], in 4 MM cell lines (8226/S, 8226/Dox40, U266, and U266/Bcl-xL.)[256], and in freshly isolated leukemic cells from patients with various subtypes of AML (AML-M1, -M2, -M3, -M4 and -M5a)[220].

Although the combined effect of As_2O_3 and AA increased the survival time of mice injected with P388D-lymphoma cells, no additional effect was observed *in vivo* when murine T-cell prolymphocytic leukemia (T-PLL) were used[257]. Recent studies in rats, however, indicate that AA ameliorated As_2O_3 induced toxicity[258-261]. Nevertheless, as a follow-up to the restricted preclinical observation, several clinical trials have been conducted. A Phase I study found that As_2O_3+AA combination had acceptable toxicity. In this study, no alterations in the pharmacokinetics of As_2O_3 after co-administration of AA were found and clinical efficacy was demonstrated in heavily pre-treated myeloma patients (two patients achieved partial responses and four patients had stable disease)[262]. Although fewer clinical trials have been conducted in patients with solid tumors, the efficacy and toxicity of As_2O_3+AA was evaluated in patients with refractory metastatic colorectal carcinoma. Unfortunately, there was a lack of clinical response as monitored by CT scan and severe toxicity in all the enrolled patients occurred. Thus, As_2O_3+AA therapy did not produce desirable outcome in this group of heavily pre-treated patients. The lack of clinical response and pronounced side effects in this trial is intriguing but not without precedence. A similar study involving renal cell carcinoma[263], metastatic melanoma[264] and refractory germ cell malignancies[265] also did not produce any clinical response. Some explanations for these clinical results could come from studies stating that *in vitro*, AA cytoxicity depends on its interaction with free transition metal ions in culture media leading to the generation of H_2O_2, to an extent that varies with the medium

used to culture the cells and these reactions are unlikely to occur *in vivo*. In an elegant study designed to circumvent the *in vitro* pro-oxidant effects of AA, it was demonstrated that loading the cells with AA by incubation with DHA resulted in prominent, dose-dependent protection of As_2O_3 treated cells. In addition, there is little evidence that AA acts as a pro-oxidant *in vivo* largely because transition metals in plasma and tissues are sequestered by metal binding proteins, such as transferrin and ceruloplasmin, and are not available to generate free radicals[96,266-268]. Recently, it was shown that AA does not act as a pro-oxidant towards lipids and proteins in human plasma exposed to both transition metal ions and H_2O_2[269] and concentrations of AA as high as 1 mM resulted in no measurable H_2O_2 generation[270]. Consequently, the pro-oxidant and cytotoxic properties of AA are thought to be an *in vitro* artifact[267,271].

1.7.3 Combination therapy through intracellular ROS generation

Increasing intracellular ROS levels is a suitable approach to enhance As_2O_3-induced apoptosis in malignant cells. Compared with other leukemia cells that are less sensitive to As_2O_3, NB4 cells contain lower levels of glutathione peroxidase and catalase (which results in reduced redox buffering) and relatively higher levels of intracellular H_2O_2[123,144]. NB4 cells become resistant to As_2O_3 after administration of catalase[272] or selenite, which increases glutathione peroxidase activity, both resulting in a decrease in cellular H_2O_2 levels[123]. Therefore, it is suggested that down-regulating H_2O_2 metabolizing enzymes, or the biochemical pathways that activate them, could be therapeutically useful in altering the cytotoxicity of As_2O_3. In fact, agents such as mercaptosuccinic acid and aminotriazole, which increase H_2O_2 levels by inhibiting glutathione peroxidase and catalase, have been successfully used to augment As_2O_3-induced apoptosis in the human monocytic-like leukemic U937 cells and in the acute myeloid leukemia (AML)–derived cell line HL-60[123,240].

Recently, it has been demonstrated that the incorporation of polyunsaturated fatty acids (PUFAs) into cellular membranes sensitize tumor cells to ROS-inducing anticancer agents both *in vitro* and *in vivo*[273-276]. The ability of PUFAs to undergo lipid peroxidation has been implicated as being of pivotal importance for this effect. In fact, it was found that docosahexaenoic acid strongly increases ROS generation and As_2O_3-mediated

apoptosis in HL-60,[277], SK-BR-3 (breast cancer), SKOV-3 (ovarian cancer), HT-29, SW-620, LS-174T (colon cancer), PC-3 (prostate cancer), SK-N-DZ, SH-SY5Y, SK-N-BE(2), SK-N-AS and IMR-32 (human neuroblastoma)[278] and in one primary melanoma cell line[279]. However, no *in vivo* studies have addressed the effects of this combination in tumor-bearing animals.

In addition, emodin, a natural anthraquinone derivative, has a semiquinone structure able to induce the generation of intracellular ROS. Co-administration of low doses of emodin (0.5–10μM), with As_2O_3, enhanced As_2O_3-induced cytotoxicity in HeLa cells[280]. Yang *et al*. performed an *in vivo* study and showed that emodin made the EC/CUHK1 cell-derived tumors more sensitive to As_2O_3 with no additional systemic toxicity and side effects[281]. These results suggested an innovative and safe chemotherapeutic strategy that uses natural anthraquinones as ROS generators to increase the susceptibility of tumor cells to As_2O_3 therapy.

1.7.4 Combination therapy targeting As_2O_3-activated signalling pathways.

Another important aspect of As_2O_3-induced apoptosis is the signaling pathways that are activated upon As_2O_3 treatment as discussed in section 1.6.3.

Trx inhibition has been explored as a strategy to increase As_2O_3 toxicity. Alteration of Trx1 from its reduced form to oxidized form *in vivo* by 2,4-dinitrochlorobenzene (DNCB), a specific inhibitor of TrX reductase, sensitizes the human hepatoma cell line $HepG_2$ to As_2O_3-induced apoptosis[282].

AKT negatively regulates the MAP kinase pathways required for As_2O_3-induced apoptosis. Therefore, strategies to inhibit AKT function have been developed in an attempt to increase As_2O_3-mediated apoptosis. AKT phosphorylates SEK1 and ASK1 and inhibits their functions[179,180]. Oxidative stress initiates the dissociation of AKT from JIP1, a scaffolding protein, which facilitates the activation of the ASK-SEK-JNK pathway[283]. AKT binding to JIP1 negatively regulates this signal transduction pathway, thus a decrease in AKT as a result of oxidative stress could enhance JNK signaling. In fact, treatment with pharmacologic inhibitors of PI3K (LY294002 and wortmannin) or an Akt inhibitor (Akt_i5) potentiated JNK activation and increased the apoptotic action of As_2O_3

in myeloid leukemia cells[284,285] and in NB4 cells[181]. However, *in vivo* experiments have not been performed in tumor-bearing animals using AKT inhibitors.

1.7.5 Combination therapy with other compounds

In human myeloid leukemia cells that express Bcr-Abl, As_2O_3 treatment reduces Bcr-Abl levels and induces apoptosis. These effects appear to be independent of Bcr-Abl kinase activity, because they occur even when cells are pretreated with STI571 (Gleevec), a tyrosine kinase inhibitor specific for the Bcr-Abl tyrosine kinase. Significant enhancement of apoptosis as well as greater reductions in all levels of Bcl-X_L and AKT activity were observed with combination STI-571 and As_2O_3 therapy[286]. This finding is supported by data derived from bone marrow cells of two patients with chronic myelogenous leukemia (CML), one in chronic phase and the other in blast-crisis phase of the disease[287].

The combination of As_2O_3 with IFN-α has been demonstrated to induce cell cycle arrest and apoptosis[288,289] in adult T-cell leukemia cell lines, as well as clinically in patients[56,288]. Synergy between As_2O_3 and IFN-α was demonstrated using a colony forming unit-granulocyte/macrophage assay with mononuclear cells from six patients in the chronic phase of CML. Positive results from this study prompted additional investigation of these agents in combination, although the results have not been published yet.

Other compounds have been added to As_2O_3 to increase its toxicity in different malignant cell lines with varying effects. These include HSP90 inhibitors[290], TRAIL[155,291], dithiothreitol[292], 19-Nor-1,25$(OH)_2D_2$, a novel, noncalcemic vitamin D analogue[293], 2-methoxyestradiol, a new anticancer agent currently in clinical trials[294], cyclic AMP[295], atrazine, a potent endocrine disruptor that inhibits phosphodiesterase[296], RWJ-241947 (MCC-555), a unique peroxisome proliferator-activated receptor-gamma ligand with antitumor activity[297], the proteasome inhibitor bortezomib[45,298], and phytosphingosine[299]. A detailed discussion is not warranted based on the lack of preclinical data.

1.8 Cancer and oxidative stress modulation.

Therapeutic selectivity is one of the major issues in cancer chemotherapy. Strategies to improve therapeutic selectivity rely largely on our understanding of the biological differences between cancer and normal cells, and on the availability of therapeutic agents that target biological events critical for cancer cells but not essential for normal cells.

1.8.1 Intrinsic ROS stress in cancer cells

Growing evidence suggests that cancer cells, compared to normal cells, are under increased oxidative stress associated with oncogenic transformation and alterations in metabolic activity[300-304]. Increased oxidative stress in malignant cells could result from: (i) more ROS formation with unaltered antioxidant defenses, (ii) unaltered RS formation with decreases in antioxidant defenses, or (iii) a combination of the above.

In vitro studies showed constitutive generation of H_2O_2 in seven human cancer lines[305]. *In vivo* experiments suggest that malignant tumors do generate more ROS than normal cells[300,306-308]. Cells from prostate cancer patients[309] and liver cancer patients[310] contain mutations in mitochondrial DNA that may increase $O_2^{\bullet-}$ generation in the mitochondria although the mechanism of $O_2^{\bullet-}$ production appears to be the same in tumor and normal mitochondria. Chronic lymphocytic leukemia cells freshly taken from patients showed increased ROS production compared with normal lymphocytes[294], as did B-cell lines from patients with Burkitt's lymphoma associated with Epstein–Barr virus infection[311] and malignant B-cells from patients with hairy cell leukemia[312]. The accumulation of oxidative products of DNA, proteins, and lipids in tissues has also been examined as an indication of ROS production. DNA oxidative products, and lipid peroxidation products have been detected in various cancer tissues including renal cell carcinoma, mammary ductal carcinoma, colorectal adenocarcinomas, and blood samples from leukemia patients or individuals with familial adenomatous polyposis[313-319].

Increased ROS stress in cancer cells is likely to impact the expression of antioxidant enzymes, such as SODs, Catalase, GPx, GST, amongst others. Decreased MnSOD activity was observed in H6 hepatoma cells compared to normal mouse liver and reduced MnSOD activity was also observed in colorectal carcinomas[320,321]. Low activities

of CuZnSOD, catalase and GPx1 are also often reported in transformed cell lines[322-324]. However, there is no clear pattern of major decreases in MnSOD or other antioxidant enzymes in freshly obtained human cancerous tissue. Indeed, SOD expression and activity are sometimes elevated in mesothelioma, neuroblastoma, melanoma, stomach, ovarian and breast cancer[325-328]. Probably what is important *in vivo* is not the level of any one antioxidant enzyme, but the balance of ROS production and overall antioxidant defense in malignant cells.

1.8.2 Mechanisms of increased ROS stress in cancer cells

Despite the prevalent ROS stress observed in a wide spectrum of human cancers, the precise mechanisms responsible for such stress remain to be defined. Several potential mechanisms have been considered. First, the ability of oncogenes, such as *c-myc* and *ras*, to induce ROS has been demonstrated[329,330]. The oncogene *c-myc* increases ROS generation, induces DNA damage, and mitigates p53 function. Transformation of rat fibroblasts by TGFβ and EGF induces $O_2^{\bullet-}$ generation[331]. The oncogenic *RAS2* (val19) allele, which causes constitutive activation of the cAMP-PKA pathway, has been demonstrated to increase ROS production and promote oxidative protein damage[330]. In addition, *src*- or *ras*-transformed oncogenic fibroblasts produce $O_2^{\bullet-}$ through the membrane-associated NADPH oxidase. $O_2^{\bullet-}$ has demonstrated to be important for the maintenance of the transformed state and induction of cellular proliferation[83,304,332,333]. Second, mutations of mtDNA are likely to affect the activity of its encoded components of the respiration complexes leading to malfunction of the mitochondrial respiratory chain. Mitochondrial DNA mutations are frequently detected in cancer cells[303,334-337]. There seems to be a correlation between mtDNA mutations and increased ROS contents in primary leukemia cells isolated from patients[338]. The metabolic rate of cancer cells is in general higher causing an increase in the ATP expenditure to sustain their biochemical functions associated with uncontrolled cell growth and proliferation. This energy demand places a further stress on the mitochondrial respiration chain, and is likely to contribute to increased ROS generation. Interestingly, the Ras oncogenic signal seems to be able to uncouple the electron flux through the electron transport chain, and ATP generation. Ras expression have been associated with alterations in the redox state of the semiquinones

wich increases the probability of $O_2^{\bullet-}$ generation[330,339]. Certain types of chronic inflammation such as ulcerative colitis, chronic hepatitis, and *Helicobacter pylori* infection have been associated with a high risk of cancer development[340,341]. Cytokines at the inflammatory site mediate the recruitment of mast cells, neutrophils, and activated macrophages, which subsequently release free radicals as a consequence of the respiratory burst. The released ROS at the inflammatory site can damage DNA, protein, and lipids, and the progression of this chronic inflammation is one important mechanism that leads to malignant transformation. Another suggested mechanism for increased ROS stress in cancer cells is a decrease in the expression or the activity of antioxidant enzymes that may cause ROS accumulation. As mentioned above, in certain cancer cells, the levels of SOD, especially the expression of MnSOD, seem to be reduced compromising the mitochondrial ability to eliminate $O_2^{\bullet-}$.

1.8.3 Consequences of increased ROS in cancer cells

The increased amounts of ROS in cancer cells can induce various biological responses, such as transient growth arrest and adaptation, stimulation of cellular proliferation, promotion of mutations and genetic instability, alterations in cellular sensitivity to anticancer agents, and apoptosis or necrosis[342]. The actual outcomes depend on the cellular genetic background, the type and level of the specific ROS present, time of exposure, and the quantity of cellular antioxidant defenses that remove ROS and also on the activities of cellular repair systems that clear oxidative damage (Figure 1.7)

1.8.3.1 Adaptation

Cells usually tolerate mild to moderate oxidative stress, which often results in increased synthesis of antioxidant defenses (and/or other defenses such as heat shock proteins) in an attempt to restore redox homeostasis[343,344]. The mobilization of the redox-buffering systems, among them the TrX and GSH, may be considered as the first line of cellular adaptation to ROS stress. In some cellular models, the upregulation of antioxidant enzymes such as SOD, catalase, and peroxidases is another important adaptation, which provides a more sustainable protection against increased ROS stress. In some cases, mild oxidative stress can elevate defenses sufficiently to protect cells against

more severe oxidative stress applied subsequently (e.g. in ischemic preconditioning). However, under sustained ROS stress conditions, a pro-oxidant state is favored and cells may exhaust ROS-buffering capacity.

1.8.3.2 Enhanced cell proliferation

It is recognized that ROS and RNS may serve as second messengers in cellular signaling transduction pathways. As a consequence of the abnormal oxidative state of cancer cells, the regulation of the redox dependent signaling pathways is dysfunctional and may promote cellular growth by increasing the rate of cellular proliferation and/or inhibiting apoptosis[304,333,345].

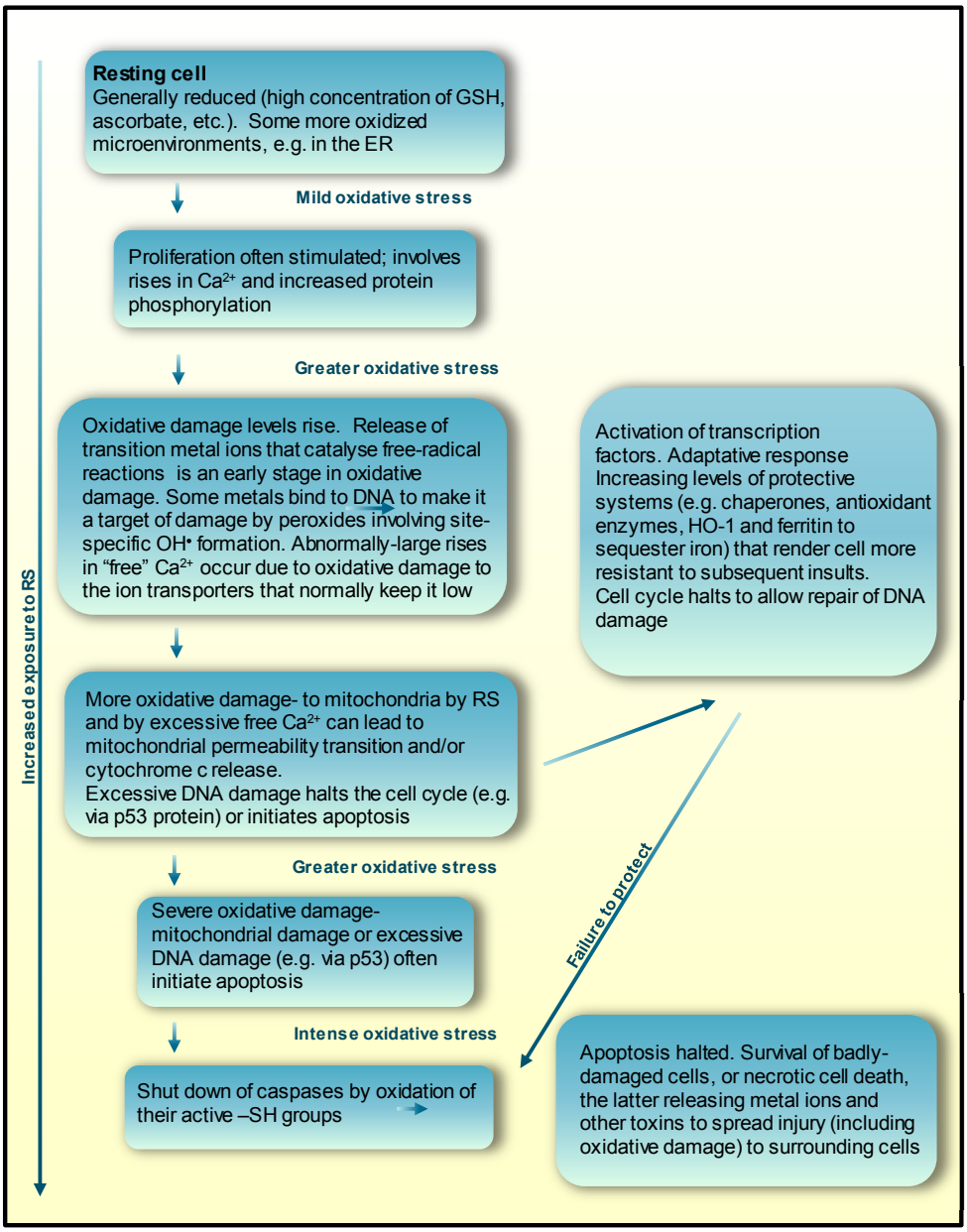


Figure 1.7: How cells respond to increasing exposure to reactive species.

(Adapted from How cells respond to oxidative stress from 'Free Radicals in Biology and Medicine' by Halliwell, B. and Gutteridge, J. M. C, 2007)

The mechanisms responsible for stimulation of cell proliferation are likely to involve interaction of ROS with specific receptors and signaling molecules such as protein kinases and transcription factors. For example, AP-1 (activator protein-1) is highly sensitive to changes in redox environment due to a cysteine residue in the DNA binding domain[346]. *In vitro* reducing agents, such as DTT, have been shown to activate AP-1, while oxidation of the cysteine residue with alkylating agents inhibits DNA binding[347], suggesting that this protein may be redox sensitive *in vivo*. AP-1 controls several genes essential for cell cycle progression including cyclin D1 and p53[348]. However, AP-1 activation is not simply a proliferative switch, as it also controls genes for apoptosis such as the Bcl-2 family member Bim[349]. The outcome of AP-1 activation is, therefore, dependent upon the activation of other signaling pathways. One of these may be the activation of NF-κB, which governs genes that suppress apoptosis including the caspase inhibitors cIAP1 and cIAP2.

Growth factors and cytokines transmit proliferative signal through the Ras/MAP kinase cascade. Ras contains a redox sensitive cysteine residue (C118) that has been shown to modulate its activity[350]. Raf-1, a downstream effector of Ras, is the initiating kinase in the MEK-ERK cascade and also contains cysteine-rich domains susceptible to oxidation. Raf-1 regulates cell survival and apoptosis through MEK/ERK-dependent pathways. However, Raf-1 also elicits cellular responses in a MEK/ERK-independent manner such as activation of cell survival protein (Bcl2), cell cycle progression, and differentiation and inhibition of pro-apoptotic proteins (ASK1 and MST2).

Oxidation of p53 protein at cysteine residues has been shown to negatively affect its ability to bind DNA containing the p53 promoter element and thus compromises its capacity to regulate the expression of downstream genes[351,352]. Thus, it is possible that ROS may compromise the cell-cycle regulatory function of p53 and contribute to uncontrolled cell proliferation.

1.8.3.3 Cellular injury and cell death

As discussed in section 1.5.3, excessive production of ROS may inflict damage to various cellular components including DNA, protein, and lipid membranes[353]. The response to oxidative injury may be reversible; for a variable period the cell may be in an

altered redox state which does not lead to cell death, and then returns to "normal". However, when oxidative damage levels rise, senescence or initiation of cell death is observed.

Cell death has been subdivided into regulated and unregulated mechanisms. Apoptosis, a form of regulated cell death, reflects a cell's decision to die in response to cues and is executed by the cellular machinery. Unregulated cell death (often called necrosis) is caused by overwhelming stress that is incompatible with cell survival. However, emerging evidence suggests the existence of multiple non-apoptotic, regulated cell death mechanisms, among them, autophagic cell death, necroptosis and poly(ADP–ribose) polymerase-1 (PARP1)-mediated necrotic death[354]. Although apoptosis may be the preferred type of physiological cell death, the option to die by apoptosis might not always be available under *in vivo* conditions. Situations that involve an imbalance of ROS-generation and ROS-detoxification, limited energy metabolism or a lack of proper protein synthesis might restrict the ability of cells to activate apoptotic cell death. Under such circumstances, cells might choose to die through one of the alternative cell death pathways.

1.8.4 Therapeutic implications of ROS modulation in malignant cells

The sustained oxidative stress in malignant cells has been postulated to result in a unique biochemical characteristic with significant therapeutic implications. Due to the increased intracellular ROS levels in malignant cells, it is reasonable to speculate that, compared to normal cells, malignant cells would be more dependent on redox-buffering systems and antioxidant enzymes to reduce their oxidative stress. Therefore, the addition of exogenous ROS or the inhibition of antioxidant mechanisms might push the malignant cells to an oxidative stress status beyond a threshold, leading to death[355,356]. This would predict that cancer cells should be more sensitive to drugs that either cause further ROS generation or impair cellular ability to eliminate ROS. The hypothesis is supported by recent studies demonstrating that human leukemia cells are more sensitive than normal lymphocytes to 2-methoxyestradiol (2-ME), a novel anticancer agent that causes ROS accumulation by inhibiting all isoforms of SOD[302,357]. Similar results were obtained in human ovarian cancer cells compared to normal ovarian epithelial cells[294]. Interfering

with the pro-oxidant–antioxidant balance in cancers has risks, as any compound that induces ROS would eventually be also toxic to normal cells when used in high concentrations.

In addition, if malignant cells are under oxidative stress, and that stress contributes to malignancy (e.g enhanced cell proliferation and apoptosis suppression), another independent therapeutic strategy may entail enhancement of the redox buffering system by administration of potent antioxidants which might have anti-cancer effects. The increase in scavenging mechanisms should decrease ROS signaling and depress tumor growth. In fact, information on the potential role of antioxidant nutrients in the prevention of cancers has accumulated over the past several decades[358,359]. Large intervention trials addressing the role of supplementation with antioxidant micronutrients in cancer prevention were first launched in the 1980s. However, results of five large intervention trials published between 1993 and 2000 showed conflicting results. The Physicians' Health Study (PHS)[360] and Women's Health Study (WHS)[361] prevention trials did not find a protective effect of antioxidant supplementation on cancer incidence, the Alpha Tocopherol and Beta Carotene Lung Cancer Prevention Study (ATBC)[362] and the Carotene and Retinol Efficacy Trial (CARET)[363] found a deleterious effect, and the Supplementation en Vitamines et Minéraux Antioxydants (SU.VI.MAX)[364] concluded that antioxidant supplementation lowered the total cancer incidence only in men. Therefore, it is now accepted that antioxidant supplementation may have a beneficial effect upon cancer incidence only in healthy subjects who are not exposed to cancer risk and who have a particularly low baseline antioxidant status. High doses of antioxidant supplementation may be deleterious in subjects in whom the initial phase of cancer development has already started, and they could be ineffective in well-nourished subjects with adequate antioxidant status.

Accordingly, the appropriate application of ROS-inducing drugs and antioxidants seems to be critically important in designing proper strategies for both prevention and treatment of malignant disorders. The trend now in cancer treatment is to target therapies based on the gene expression, cell signalling and proteomic profiles of a tumour. Perhaps the cellular oxidative stress status could also predict the sensitivity of a tumor cell to an ROS-inducing agent. Indeed, levels of gene expression for antioxidant defense enzymes

and levels of other proteins related to cellular redox balance have been purported to constitute a redox signature score that seemed to be predictive of outcome in patients with diffuse large B-cell lymphoma[365]. The development of such a score by combining parameters of oxidative damage with those of redox state, DNA-repair activity and antioxidant defense might be a useful therapeutic tool.

CHAPTER 2

Trolox selectively enhances arsenic-mediated oxidative stress and apoptosis in APL and other malignant cell lines

2.1 Preface

Prior to this project, only a few compounds were used to increase As_2O_3 toxicity and it was well established that As_2O_3 could mediate ROS generation. Therefore, it was hypothesized that compounds with antioxidant properties would protect cells from As_2O_3-mediated toxicity. However, in 2001, Grad *et al*. demonstrated that in myeloma cells, ascorbic acid behaved as a pro-oxidant when combined with As_2O_3. In order to discover new combination therapies, we tested different antioxidants for their ability to enhance As_2O_3-mediated cell death. Trolox, a vitamin E derivative with potent antioxidant activity, melatonin, a hormone secreted by the pineal gland, and resveratrol, a phytoalexin found in dietary plants including grapes and peanuts were tested in a variety of malignant cells. This study led us to the identification of trolox as a potent enhancer of As_2O_3-mediated cell death and to propose new potential uses for this combination in hematological malignancies.

2.2 Abstract

Although arsenic trioxide (As_2O_3) is an effective therapy in acute promyelocytic leukemia (APL), its use in other malignancies is limited by the toxicity of concentrations required to induce apoptosis in non-APL tumor cells. We looked for agents that would synergize with As_2O_3 to induce apoptosis in malignant cells, but not in normal cells. We found that trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid), a widely known antioxidant, enhances As_2O_3-mediated apoptosis in APL, myeloma and breast cancer cells. Treatment with As_2O_3 and trolox increased intracellular oxidative stress, as evidenced by HO-1 protein levels, JNK activation, and protein and lipid oxidation. The synergistic effects of trolox may be specific to As_2O_3, as trolox does not add to toxicity induced by other chemotherapeutic drugs. We explored the mechanism of this synergy using electron paramagnetic resonance and observed the formation of trolox radicals when trolox was combined with As_2O_3, but not with doxorubicin. Importantly, trolox protected non-malignant cells from As_2O_3-mediated cytotoxicity. Our data provide the first evidence that trolox may extend the therapeutic spectrum of As_2O_3. Furthermore, the combination of As_2O_3 and trolox shows potential specificity for tumor cells, suggesting it may not increase the toxicity associated with As_2O_3 monotherapy *in vivo*.

2.3 Introduction

Arsenic has been used as a therapeutic agent for more than 2,400 years. Until the 1930s, arsenic was used as a treatment for patients with chronic myelogenous leukemia. More recently, the use of arsenic in leukemia has resurfaced after reports from China that arsenic induced a high remission rate in acute promyelocytic leukemia (APL), including those who were resistant to therapy with all-trans retinoic acid[1,2].

The activity of arsenic (As_2O_3) in APL is in part related to the disappearance of the PML-RARα fusion protein, the gene product of the chromosomal translocation t(15,17) characteristic of APL, and the induction of differentiation[3,4]. As_2O_3 can also induce apoptosis through a variety of mechanisms, which appear to be independent of PML-RARα degradation[5]. In addition to causing mitochondrial toxicity[6], impairing microtubule polymerization[7], and deregulating a number of proteins and enzymes through binding to sulfhydryls groups[8-10], considerable evidence suggests that As_2O_3 induces the accumulation of reactive oxygen species (ROS) and subsequently, induces oxidative stress[11,12]. Indeed, the intracellular redox status has been shown to be important in predicting whether a cell will respond to arsenic[11,13].

Recently it has been shown that As_2O_3 stimulates apoptosis in additional malignant cells including acute myeloid leukemia, chronic myeloid leukemia, myeloma and various solid tumor cells[14-17]. However, higher concentrations of As_2O_3 are required to induce apoptosis in non-APL tumor cells, suggesting that higher, more toxic doses might be needed for clinical efficacy. Clinical trials are currently testing arsenic in the treatment of lymphoma and myeloma[18], but clear evidence of clinical benefit has, thus far, been largely restricted to patients with APL. Therefore, the sensitization of the resistant tumor cells to As_2O_3 could expand its therapeutic spectrum.

Different compounds have been reported to enhance As_2O_3-mediated apoptosis[19-21]. Recently ascorbic acid (AA), a key antioxidant molecule, was reported to augment the toxicity of As_2O_3 *in vitro*[22,23]. However, there is some evidence that the toxicity of ascorbate is due to ascorbic acid-mediated production of hydrogen peroxide, to an extent that varies with the medium used to culture the cells[24].

Trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid) is a hydrophilic vitamin E analogue lacking the phytyl tail, with enhanced antioxidant capacity due to its

increased cell permeability. It provides protection against oxidative reactions in aqueous solutions[25] and against cisplatin-induced apoptosis in renal proximal tubular epithelial LLC-PK1 cells[26]. There is also evidence that trolox inhibits DNA damage formation induced by singlet oxygen in human lymphoblast WTK-1 cells[27] and protects red blood cells during photodynamic treatment[28].

In spite of a large and consistent literature documenting anti-oxidant effects of trolox in several different experimental models, here we report that this compound can enhance As_2O_3-mediated cytotoxicity in APL, myeloma and breast cancer cells. We show an increase in intracellular oxidative stress when trolox and As_2O_3 are combined, leading to caspase 3 activation and apoptosis in leukemic cells. We extend previous reports that trolox does not enhance the cytotoxicity of other chemotherapeutic agents to cancer cells and provide evidence that the enhancement of apoptosis by As_2O_3 may be limited to malignant cells.

2.4 Experimental Procedures

4.1.1 Cell lines

The arsenic trioxide-resistant APL cell line, NB4-M-AsR2 (AsR2), was generated by culturing NB4 cells in the presence of As_2O_3 at concentrations that were gradually increased over time[13]. NB4 (provided by Dr. M Lanotte), AsR2 and multiple myeloma IM9 (ATCC) were maintained in RPMI 1640 media. MCF-7 and MDA-231 were obtained from ATCC and maintained in alpha MEM. T47D (ATCC) was cultured in D-MEM/F12. All media were purchased from Life Technologies, Inc and supplemented with 10% fetal bovine calf serum (FBS). AsR2 was routinely grown in RPMI containing 2μM As_2O_3. In experiments examining the response of AsR2, the cells were first washed thoroughly to remove As_2O_3, and then cultured 24 hours in media alone prior to initiating the experiment. All cells were grown in a humidified chamber at 37^o C with a 5% CO_2 environment.

4.1.2 Growth Assays

NB4, IM9 and AsR2 cells were seeded at 1x10^5 cells/ml in 24-well plates. Cells were treated with various concentrations of As_2O_3 or doxorubicin, alone or in combination with 100μM trolox for six days. Viable cells were counted by trypan blue exclusion on day 1, 3 and 6. All cells were maintained at a density lower than 1x10^6 cells/ml through dilution as required, and media +/- treatment was replaced every third day. MCF-7, MDA-231 and T47D were seeded in 24-well plates at a density of 4000 cells/well. The next day, fresh media containing As_2O_3 +/- trolox was added. On the days indicated, cells were fixed in 10% trichloroacetic acid and subsequently stained with sulforhodamine B (SRB). Bound SRB was solubilized in 10mM unbuffered Tris and optical density was measured at 570 nm in a microplate reader.

4.1.3 Propidium Iodide Staining

Quantitation of apoptotic cells was performed as previously described[29]. Cells were treated, washed in buffer (PBS/ 5% FBS/ 0.01 M NaN_3) at 4^o C, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was

measured on a Becton-Dickinson FACS Calibur. Cells undergoing DNA fragmentation and apoptosis (those in which PI fluorescence was weaker than the typical G_0-G_1 cell cycle peak) were quantified using CellQUEST software.

4.1.4 Annexin V staining

Cells were stained with Annexin-V-FITC and Propidium Iodide in binding buffer according to the manufactures recommendations (BD Pharmigen, San Diego, CA). The fluorescent signals of FITC and PI were detected by FL1 at 518 nm and FL2 at 620 nm, respectively, on a FACScan (Becton Dickson, San Jose, CA). Apoptotic cells (Annexin V positive/ PI negative) were quantified using the CellQUEST software.

4.1.5 Western Blotting and immune kinase assays

Cell extracts were washed with cold PBS and resuspended in 0.1 ml lysis buffer (5mM NaH_2PO_4, 1mM DTT, 10% glycerol, 1mM PMSF, 10μg/ml each aprotinin and leupeptin, pH 7.4) at 4°C. Extracts were centrifuged at 14,000 rpm at 4°C, and supernatants were transferred to fresh tubes. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada). To detect HO-1 or PARP, 50μg of protein was added to an equal volume of 2x sample buffer and run on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad), stained with Ponceau S in 5% acetic acid to ensure equal protein loading, and blocked with 5% milk in PBS containing 0.5% Triton X-100 for 1 hour at room temperature. The membrane was hybridized overnight at 4°C with antibody against PARP (1:1000; Oncogene) or 3 hours with an antibody against HO-1 (1:1000; StressGen). Following three washes with PBS and 0.5% Triton X-100, blots were incubated with a goat anti-rabbit antibody (1:10,000; PharMingen) for one hour at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). Immunostaining for β-actin was used to confirm equal protein loading. Immune complex kinase assays for c-jun kinase activity were performed as we have previously described[30].

4.1.6 Caspase-3 Activity Assay

Activation of caspase-3 was detected using a fluorescent caspase-3 inhibitor, Red-DEVD-FMK (Oncogene Research Products, San Diego, CA), which irreversibly binds to activated caspase-3 in apoptotic cells. Cells were treated for two days and harvested into microcentrifuge tubes. The cells were incubated with 1μl of Red-DEVD-FMK for 1 hour at 37^{o}C in 5% CO_2. Subsequently, cells were washed twice, resuspended and analyzed by flow cytometry, using the FL-2 channel.

4.1.7 Protein Carbonyls

Oxidized and reduced BSA were prepared and its carbonyl content was quantitated by a colorimetric carbonyl assay described previously[31]. NB4 cells were treated for 3 days with As_2O_3, trolox or the combination. Protein samples were adjusted to 4mg protein/ml. The standards and protein samples were incubated with 3 volumes 10mM 2,4-dinitrophenylhydrazine (DNP) in 6 M guanidine-HCl, 0.5 M potassium phosphate, pH 2.5 for 45min at room temperature (mixing every 10-15min). Aliquots of cell proteins and standards were diluted in PBS and adsorbed to a 96-well immunoplate by incubation overnight at 4°C. After washing with PBS, non-specific sites were blocked with 0.1% Tween 20 in PBS for 1.5 hours at room temperature. After further washing with PBS the wells were incubated with biotinylated anti-DNP antibody (Molecular Probes, 1:10000 dilution) for 1 hour at 37°C. Wells were washed and incubated with streptavidin-biotinylated horseradish peroxidase (Amersham International, 1:3000 dilution), After further washing, o-phenylenediamine/peroxide solution was added. The reaction was stopped after 7 min with 2.5 M sulfuric acid and the absorbance was read with a 490 nm filter. A six point standard curve of reduced and oxidized BSA was incubated with each plate.

4.1.8 Quantification of 8-iso PGF α

NB4 cells were treated for 3 days with As_2O_3, trolox or the combination. Cells were washed twice with PBS containing 0.005% BHT, and 10μg/ml indomethacin. The intracellular and membrane bound 8-iso $PGF_{2\alpha}$, a specific marker for lipid peroxidation,

was measured using a competitive ELISA kit from Cayman Chemical Company following the manufacturer instructions.

4.1.9 Detection of trolox phenoxyl radicals and measurement of intracellular GSH

Electronic spin resonance spectroscopy reactions contained 0.02mM As_2O_3, 1mM Trolox, 5% (v/v) DMSO and 0.2μg/ml doxorubicin. Following the final addition of As_2O_3, reaction mixtures were transferred immediately to a quartz ESR flat-cell positioned and pre-tuned within the cavity of a Bruker ESP 300 spectrometer using a rapid delivery device[32] and recording commenced using the following instrument settings: modulation frequency, 100kHz; centre field, 3471.50G; sweep width, 50.0G; modulation amplitude, $9.51x10^{-1}$G; receiver gain, $6.30x10^{4}$; scan time, 20.97s; time constant, 10.24msec; power, 20mW. Spectra was simulated using WinSIM program available for use at the NIEHS/NIH website (http://epr.niehs.nih.gov/)[33]. Intracellular reduced GSH levels were assessed enzymatically with glutathione reductase as previously reported[13].

4.1.10 Peripheral blood mononuclear cell purification and Colony Forming Unit Assay

Peripheral blood mononuclear cells (PBMC) were obtained from two healthy normal donors after obtaining informed consent and were collected into tubes containing 7.2mg K_2EDTA. The blood was diluted 1:3 in PBS, layered onto an equal volume of Ficoll-Plaque™ PLUS (Amershan Biosciences, Piscataway, NJ) and centrifuged at 1500 rpm for 30 minutes. The mononuclear cell layer was collected and washed twice in PBS. Methylcellulose media was prepared by combining IMDM, 30% FBS, 1% bovine serum albumin, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 0.1U penicillin, 0.1ug/ml streptomycin, granulocyte-macrophage colony-stimulating factor (GM-CSF; 10ng/ml), interleukin-3 (IL-3; 10ng/ml0) and erythropoietin (EPO; 3U/ml). PBMC were seeded in this media at a concentration of 300,000 cells/ml and treated with or without As_2O_3, trolox or the combination. Cultures were performed in triplicate in $35mm^2$ dishes and

incubated at 37^{o}C in 4% CO_2. Colonies derived from colony-forming units–erythrocyte (BFU-E) and CFU-GM were counted on day 7 and 13.

4.1.11 Statistical Analysis

The significance of data was determined using Prism version 3.0. Analysis of variance followed by Newman-Keuls post-tests were used to determine if cell treatments produced significant changes.

4.2 Results

4.2.1 Trolox significantly enhances the inhibitory effects of As_2O_3 on APL, multiple myeloma and breast cancer cells.

We examined the effects of As_2O_3 and trolox, both separately and in combination, on the growth of different cell lines. Figure 2.1A shows that treatment of NB4 cells for six days with 0.5 or 1 μM As_2O_3 reduced viable cell number by 25%±4.7 and 70%±5.6 of control, respectively. 100μM trolox alone had no effect on cell number at any time point. However, if the cells were treated with 0.5 or 1 μM As_2O_3 and 100μM trolox in combination, 57% ±3.5 and 97% ±4.2 reductions of cell number were observed. In all cases, trypan blue positive cells were less than 3%. A difference was also seen between As_2O_3 and As_2O_3+trolox after 72 hours, with 1μM As_2O_3 decreasing cell number by 30% and the combination by 50% ($p<0.001$). We next determined whether trolox could sensitize arsenic-resistant cells. We used an NB4-derived, arsenic-resistant subclone (AsR2), which has an IC_{50} value roughly 10-times higher than its parental NB4 cell line[13] and the multiple myeloma IM9 cell line, which is also less sensitive to As_2O_3 than NB4 cells[34]. An enhancing effect of trolox on As_2O_3-mediated growth inhibition was observed in both cell lines (Fig 2.1 B and C), although trolox did not restore the sensitivity to lower concentrations of As_2O_3 in the highly resistant AsR2 cell line (data not shown). Some solid tumor cells have been shown to be more resistant to As_2O_3 than APL cells, so we tested the combined effect of As_2O_3 and trolox in breast cancer cell lines. As shown in Table 1, As_2O_3-mediated cytotoxicity was enhanced by trolox in all tested cell lines.

4.2.2 Trolox enhances As_2O_3-mediated apoptosis in As_2O_3 sensitive and resistant malignant cells.

To evaluate whether the growth inhibitory effect observed upon combined treatment of As_2O_3 and trolox in NB4, AsR2 and IM9 cells was due to the induction of apoptosis, cells were treated for 48 hours, subsequently stained with PI and analyzed by flow cytometry. As shown in Figures 2.2A and B, trolox enhanced As_2O_3-mediated apoptosis in the cell lines studied, at all concentrations of arsenic tested, while trolox alone had no effect on the apoptotic rate. To confirm an enhanced induction of apoptotic death, FITC-labeled Annexin V, which detects phosphatidylserine residues appearing on

the external surface of early apoptotic cells, was used. Consistent with the increase in the $subG_0$ subpopulation after PI staining, trolox augmented the percentage of cells positive for Annexin V (Figure 2.2B lower panels). To further confirm the induction of apoptosis by the combination of As_2O_3 and trolox, we evaluated caspase 3 activation and PARP cleavage. Trolox significantly enhanced the percentage of cells with activated caspase 3 (Figure 2.2C) and cleaved PARP (Figure 2.2D). These results support the hypothesis that the combined treatment with As_2O_3 and trolox induced apoptosis in NB4 cells in a dose dependent fashion. Similar results were obtained with AsR2 and IM9 cells (data not shown).

We then asked whether trolox could enhance the induction of apoptosis by other cytotoxic agents that induces ROS. The anthracycline doxorubicin has been shown to generate genotoxic stress in a different number of cell types[35,36]. 1-β-D-Arabinofuranosylcytosine (AraC) is a nucleoside analog used in the treatment of acute myelogenous leukemia[37,38]. Etoposide causes single and double-strand DNA breaks when incubated with cells[39,40]. We examined the possibility that the combination of trolox and doxorubicin, AraC or etoposide might increase cell growth inhibition and apoptosis in NB4 cells. As shown in Figure 2.2E, these compounds induced apoptosis in a dose-dependent manner. However, no additional increase of cellular apoptosis was observed when the cells were co-treated with trolox. Trolox also had no effect on sensitivity to doxorubicin or Ara C in AR2 and IM9 cells (data not shown).

4.2.3 The combination of As_2O_3 and trolox results in increased cellular oxidative stress.

Oxidative damage has been postulated to be a key mechanism by which arsenic initiates the apoptotic process. Because trolox potentiates As_2O_3-induced apoptosis, it is possible that the combination treatment increases cellular oxidative stress. Therefore, we determined whether As_2O_3 affected various markers for oxidative stress and whether trolox could augment this effect. Heme oxygenase-1 (HO-1), which is the rate-limiting enzyme for heme degradation and has been widely described as a stress responsive protein[41], was not detected when trolox was used alone (Figure 2.3A). However, the

combined treatment markedly enhanced As_2O_3-mediated HO-1 induction in all the cell lines tested, suggesting that this combination increased the cellular oxidative stress.

To document oxidative damage to cellular components, we analyzed lipids and proteins isolated from NB4 cells treated with As_2O_3 or As_2O_3+trolox for 3 days. Proteins carbonyls are generated by a variety of mechanism and are sensitive indices of oxidative injury[42]. Isoprostanes are chemically stable prostaglandin-like compounds that are produced independent of the cyclooxygenase (COX) enzyme by free radical-catalyzed peroxidation of arachidonic acid (AA) *in situ* in membrane phospholipids[43]. F2-isoprostanes are a reliable marker of lipid peroxidation in vivo[44,45]. Figures 2.3 B and C show that As_2O_3 alone induces protein oxidation and, to a lesser extent, lipid peroxidation. Oxidative damage to both proteins and lipids was found to be significantly higher when trolox and As_2O_3 where combined. Similar results were obtained in AR2 and IM9 cells (data not shown).

4.2.4 The cytotoxic effects observed when trolox and As_2O_3 are combined are not due to generation of extracellular H_2O_2.

Several reports have demonstrated that ascorbic acid (AA), a known antioxidant compound, enhances As_2O_3-induced cytotoxicity in multiple myeloma cells. Clement *et al.*[24], reported that ascorbate-mediated killing in HL60 cells depends on the levels of H_2O_2 produced by the reaction of AA within the cell culture medium, and direct addition of H_2O_2 to the cells reproduced these results. Further, degradation of extracellular H_2O_2 by the addition of catalase, which catalyzes the decomposition of H_2O_2 to H_2O and O_2, blocked any additional toxicity from AA[24]. They concluded that the extracellular H_2O_2 generated plays a major role in the synergy observed in vitro by As_2O_3 and AA. We asked whether the synergy observed between trolox and As_2O_3 was influenced by the generation of extracellular H_2O_2. If so, we would expect that the addition of catalase could block the generation of extracellular H_2O_2 and consequently decrease the apoptotic rate. Therefore, we treated NB4 cells for three days with As_2O_3, trolox, AA and catalase as indicated in Figure 2.4. The addition of catalase (500U/ml) prevented the induction of apoptosis by H_2O_2, suggesting that even a very large extracellular production of H_2O_2 by As_2O_3 and trolox could be blocked. Catalase significantly blunted the synergy of As_2O_3

with AA, confirming previous reports[22]. In contrast, the addition of catalase did not protect cells treated with As_2O_3+trolox.

2.5.5 Trolox enhances As_2O_3 -mediated c-jun terminal kinase (JNK) activation.

It has been demonstrated that JNK is activated in response to oxidative stress[46,47]. We have reported that JNK activation is necessary for As_2O_3-induced apoptosis of NB4 cells[30]. Therefore, we asked whether the activation of JNK in NB4 cells treated with As_2O_3 and trolox for 16 hours might play a role in the synergistic effect of these compounds. We used an immune complex assay with GST-c-jun as an exogenous substrate. Figure 2.5A shows that a 24 hour treatment of NB4 cells with as little as 0.5uM As_2O_3 induces significant JNK activation leading to phosphorylation of c-jun. As expected, higher As_2O_3 concentrations increased JNK activation. Consistent with the idea that trolox enhances As_2O_3-mediated oxidative stress, we observed a further increase in JNK activity when cells are co-treated with As_2O_3 and trolox. As expected, the arsenic resistant cell line AsR2 cells showed little activation of JNK following treatment with As_2O_3, even at doses sufficient to elicit robust activation of NB4 cells (Figure 2.5B). However, when trolox was added to the media, a considerable JNK activation was observed which correlated with apoptotic induction.

2.5.6 As_2O_3 induces the formation of trolox phenoxyl radicals.

Electron paramagnetic resonance (EPR) is an important tool in experimental studies of systems containing unpaired electrons. We used EPR to directly assay the generation of trolox radicals. As shown in Figure 2.6B, addition of trolox to reaction mixtures containing As_2O_3 resulted in the observation of an intense seven-line EPR signal. The g-value (3477.530 G), the relative intensities, and the splittings all confirm the presence of the trolox phenoxyl radical. It's identity is further confirmed by the simulated spectrum (Figure 2.6C), which is based on the published coupling constants for this radical[48]. This signal is not generated by trolox alone (Figure 2.6A) nor in the presence of doxorubicin (Figure 2.6D) suggesting the requirement of As_2O_3 and its hydration products for the formation of this radical.

2.5.7 Trolox does not potentiate As_2O_3 effects in non-malignant cells.

We sought to determine the effects of As_2O_3 combined with trolox in normal human hematopoietic colony forming cells and mouse embryonic fibroblasts. Normal human PBMCs were isolated, grown in methylcellulose, and treated with As_2O_3, trolox or the combination for 2 weeks. Figure 2.7A shows that 1μM of As_2O_3 inhibited CFU-E by approximately 62%, but had minimal effect on CFU-GM or BFU-E colony formation. Treatment with trolox alone did not inhibit colony formation and trolox did not enhance As_2O_3-inhibition of CFU-GM, BFU-E or CFU-E. Mouse embryonic fibroblasts were treated with different concentrations of As_2O_3 for three days, stained with PI and analyzed by flow cytometry. Interestingly, trolox significantly decreased As_2O_3-mediated apoptosis at all doses studied (Figure 2.7B).

2.6 Discussion

The induction of apoptosis by As_2O_3 has been linked to the accumulation of free radicals and subsequent induction of oxidative stress. Intracellular oxidative status has been shown to be important for As_2O_3 sensitivity, and strategies to alter the redox environment may allow normally As_2O_3-resistant cells to become susceptible to As_2O_3-mediated apoptosis.

In part because of differences in cellular redox environments, As_2O_3 is less active in most malignant cells than in APL[49], prompting a search for agents that enhance As_2O_3 efficacy. One such agent is the widely used antioxidant, ascorbic acid. AA has been shown to potentiate As_2O_3-mediated cytotoxicity in HL60 cells, as well as in su-DHL-4 [22], 8226/S and U266 cells[23]. There is some evidence that the time at which AA is administered in relation to exposure to oxidative stress has an impact on AA pro-oxidant capacity *in vitro*[50]. Co-treatment of Chinese hamster ovary AS52 cells with AA and a radical generating system (RGS) resulted in a significant increase in cell death compared to treatment with RGS alone[50]. However, when AS52 cells were pre-treated for 24 hours with AA and then challenged with RGS, the cells were protected. Ascorbate-generated H_2O_2 may be responsible for the enhancement of As_2O_3 cytotoxicity in vitro, because the latter is attenuated by co-administration of catalase. Furthermore, the propensity for AA to generate H_2O_2 *in vitro* appears to be substantially influenced by the composition of the culture media[24,22]. The effects of AA on arsenic-mediated cytotoxicity *in vivo* are conflicting. No additional benefits were observed by combining As_2O_3 with ascorbic acid in the treatment of murine T cell leukemia[51]. On the other hand, the combined effects of AA and As_2O_3 increased the survival time of BDF1 mice injected with P388D1 lymphoma cells[22].

On the basis of the ascorbate experience, we evaluated various "antioxidant" compounds for their ability to enhance As_2O_3-mediated apoptosis. Our initial experiments performed with the human APL cell line, NB4 revealed that trolox had a strong potentiating effect on As_2O_3-mediated growth inhibition and apoptosis. In contrast, melatonin and resveratrol, compounds reported to have antioxidant or pro-oxidant capacities depending on the cellular redox status[52,53], were not able to substantially enhance As_2O_3-mediated cytotoxicity (data not shown). NB4-derived AsR2, IM9

myeloma cells and a variety of breast cancer cells were all sensitized to As_2O_3 when trolox was added to the culture media, revealing a broad capacity of trolox to increase As_2O_3-mediated cytotoxicity in different tumor cells. We showed that the potentiation of growth inhibition by trolox involved induction of apoptotic cell death as monitored by caspase 3 activation and PARP cleavage. In contrast, trolox was not able to enhance apoptosis mediated by other common chemotherapeutic drugs (doxorubicin, AraC or etoposide). These data are consistent with previous reports that trolox does not influence doxorubicin-induced oxidative damage[54,55]. In addition, cisplatin, a major antineoplastic drug for the treatment of solid tumors[56], has been shown to induce apoptosis in LLC-PK1 cells that can be significantly inhibited by trolox co-treatment[26]. Trolox also prevented cisplatin-induced ototoxicity when applied locally in guinea pigs round windows[57].

Although the mechanism by which trolox enhances As_2O_3-mediated apoptosis in malignant cells remains unknown, here we provide evidence that trolox potentiates As_2O_3-induced oxidative stress. The HO-1 gene is highly sensitive to induction by oxidative stress, and HO-1 activity degrades cellular heme to products (biliverdin, bilirubin) with proven antioxidant properties[58]. Our findings that HO-1 protein is substantially up-regulated in tumor cell lines following treatment with As_2O_3 and trolox implicate oxidative stress as a killing mechanism in this system. Augmented levels of protein carbonyls and isoprostanes in these cells further support this conclusion. However, we could not find any differences in the intracellular GSH content upon As_2O_3 and trolox treatment in any of the cells studied (data not shown) suggesting that, unlike ascorbic acid, the synergy observed between trolox and As_2O_3 is not due to changes in GSH levels.

To our knowledge, there are only a few reports on the pro-oxidant capacity of trolox. Synergistic effects between selenite and trolox resulting in enhanced superoxide production and cytotoxicity have been reported. In a mechanism similar to that proposed for As_2O_3, cytotoxicity of selenium is believed to involve simultaneous thiol oxidation and superoxide production[59]. This is supported by data showing that only selenium compounds that generate superoxide can synergize with trolox. In addition, the combination of Cu^{2+} and trolox resulted in increased ROS generation and cytotoxicity in astrocytes[60]. Trolox exhibits pro-oxidant properties when Cu^{2+} is used to mediate low

density lipoprotein oxidation, but in the absence of Cu^{2+}, trolox shows an antioxidant capacity[61]. Thus, redox-cycling may be an important feature in the prooxidant mechanism of trolox.

Our EPR experiments suggest that As_2O_3 may bio-activate trolox to a potentially tumoricidal phenoxyl radical (trolox$^{\bullet}$). These observations suggest that As_2O_3 not only generates ROS, but also induces the formation of trolox$^{\bullet}$, which contributes to the intracellular pool of reactive oxygen species and consequently enhances cellular oxidative stress. Consistent with the hypothesis that trolox$^{\bullet}$ plays a role in the augmentation of As_2O_3-induced apoptosis, the combination of trolox and doxorubicin was not synergistic and did not generate the trolox radical. Therefore, trolox may be useful to increase As_2O_3-mediated cytotoxicity but it may not be effective when combined with other chemotherapeutic drugs. These observations do not preclude the possibility that other mechanisms may also be involved. Plasma membrane CoQ reductase (PMQR), the enzyme responsible for reducing trolox$^{\bullet}$ to trolox, may be affected by As_2O_3, thereby augmenting cellular oxidative stress[62]. Experiments are in progress to elucidate the rates and mechanisms for the reactions between trolox and As_2O_3 under aerobic and anaerobic conditions and the potential role of PMQR in the generation of trolox$^{\bullet}$.

We have previously demonstrated that in SEK -/- murine embryonic fibroblast cells, the activation of c-jun N terminal Kinase in response to As_2O_3 is dampened. The reduced JNK response in these cells or in NB4 cells treated with dicumarol (a known JNK inhibitor) is associated with resistance to As_2O_3-induced apoptosis[30]. These results indicated that JNK activation is required for sensitivity to As_2O_3 in these cells. We observed that trolox enhances As_2O_3-mediated JNK activation, which is consistent with our previous conclusion that JNK is an important mediator in As_2O_3-induced oxidative damage and apoptosis. We treated $SEK^{+/+}$ and $SEK^{-/-}$ cells with the combination of As_2O_3 and trolox and analyzed the induction of apoptosis. As expected, $SEK^{-/-}$ cells were more resistant to As_2O_3 than $SEK^{+/+}$, and trolox alone had no effect on apoptosis in either the $SEK^{+/+}$ or $SEK^{-/-}$ cells. Interestingly, trolox protected these non-malignant cells from As_2O_3-mediated toxicity although care should be taken when extrapolating these data from murine to human cells.

Consistent with this finding, we found that trolox does not enhance cytotoxicity of As_2O_3 in colony forming assays using human hematopoietic peripheral blood mononuclear cells. We show here that the combination of As_2O_3 and trolox does not increase the As_2O_3-mediated reduction in CFU-E, CFU-GM and BFU-E. It has been also found that arsenic damage to supercoiled ϕX174 DNA and DNA in peripheral human lymphocytes in culture are inhibited by trolox[63]. Therefore, cytotoxic enhancement accruing from trolox exposure may be specific to tumor cells.

In this study, we provide preclinical evidence for the potential efficacy of As_2O_3 and trolox combination therapy in APL and other malignancies intrinsically less sensitive to As_2O_3 monotherapy. It is important to note that standard treatment of APL patients with 0.15 mg/kg/d As_2O_3 yields a maximum concentration of 1 to 2 μM As_2O_3 in the plasma [64,65], which is similar to the *in vitro* doses reported here. Given the low toxicity of trolox and As_2O_3 and considering the synergy we observed between the two drugs, our results justify the study of this combination in *in vivo* models of human leukemias and other malignancies.

2.7 Acknowledgements

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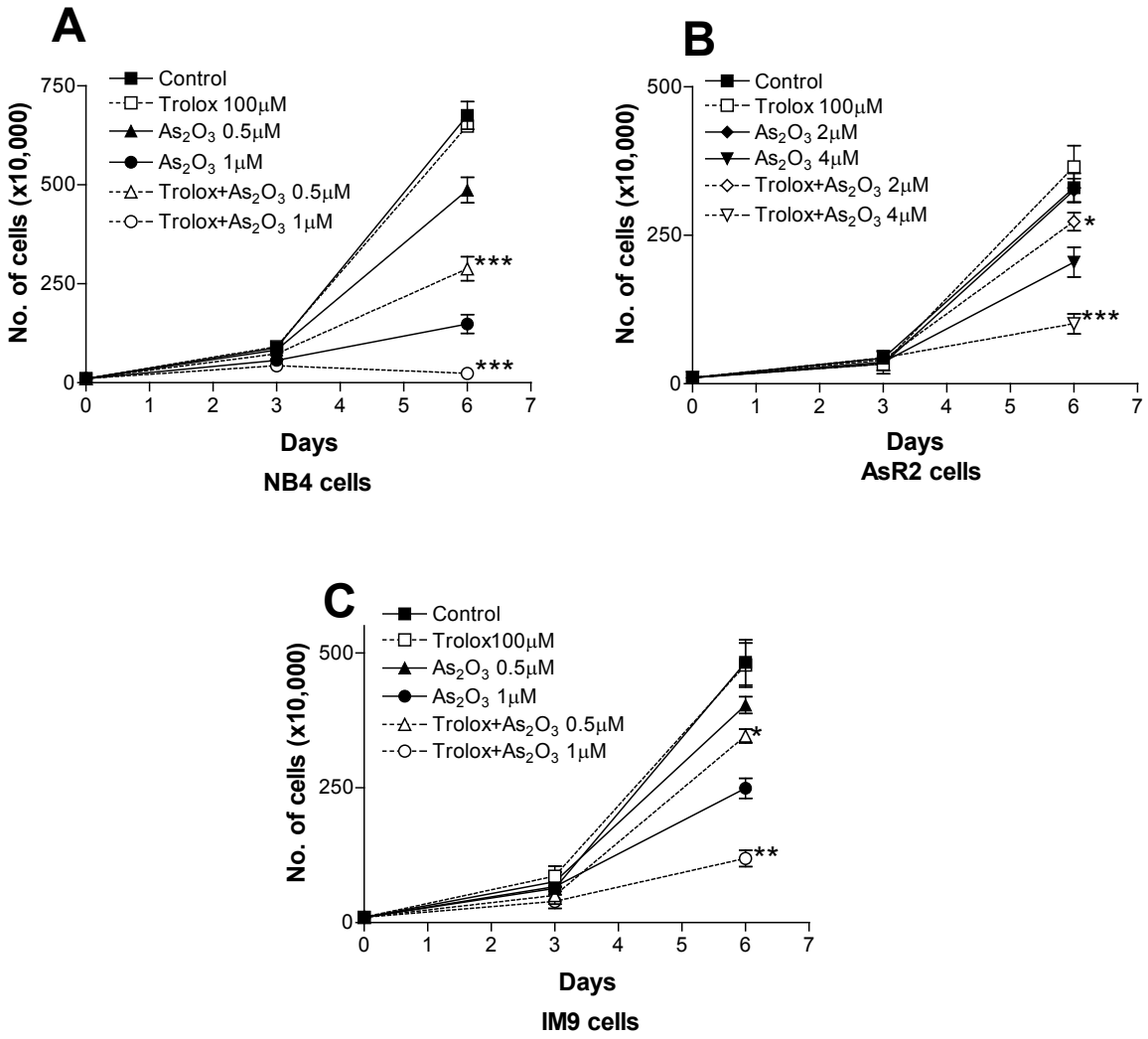

Figure 2.1: Trolox enhances As_2O_3-induced growth inhibition in NB4, AR2 and IM9 cells.

NB4 (A), AsR2 (B) and IM9 cells (C) were treated with trolox, As_2O_3 or the combination. Cell viability was evaluated on day 1, 3, and 6 using trypan blue exclusion. Values are the mean of three independent experiments each performed in triplicates. Standard deviation bars are shown. *, ** and *** indicate a significant difference $p<0.05$, $p<0.01$ and $p<0.001$, respectively from As_2O_3-treated cells.

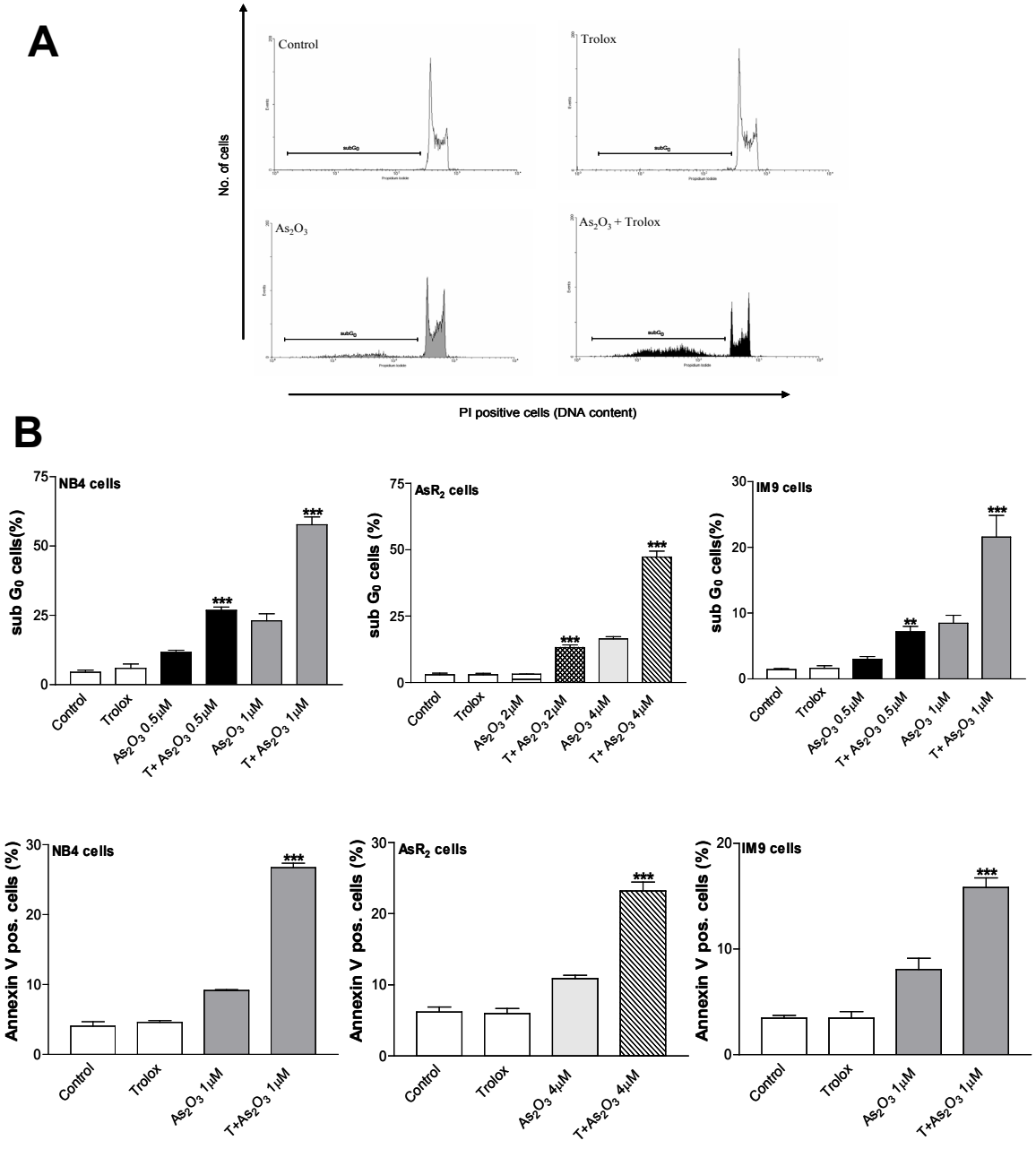

Figure 2.2: Trolox enhances arsenic-mediated apoptosis in NB4, AR2 and IM9 cells.

(A, B) NB4, AsR2 and IM9 cells were treated with As_2O_3 and trolox (T) for 48 hours. Apoptosis was detected by PI-staining. Flow cytometric histograms are shown in (A). Quantitation of PI-positive cells in a hypotonic fluorochrome solution was performed. Apoptotic cells were also stained with Annexin-V-FITC and Propidium Iodide in binding buffer and quantified (B). Each bar represents an average of three independent samples, and standard deviation bars are shown. Asterisks indicate significant differences from As_2O_3-treated cells (** $p<0.01$; *** $p<0.001$).

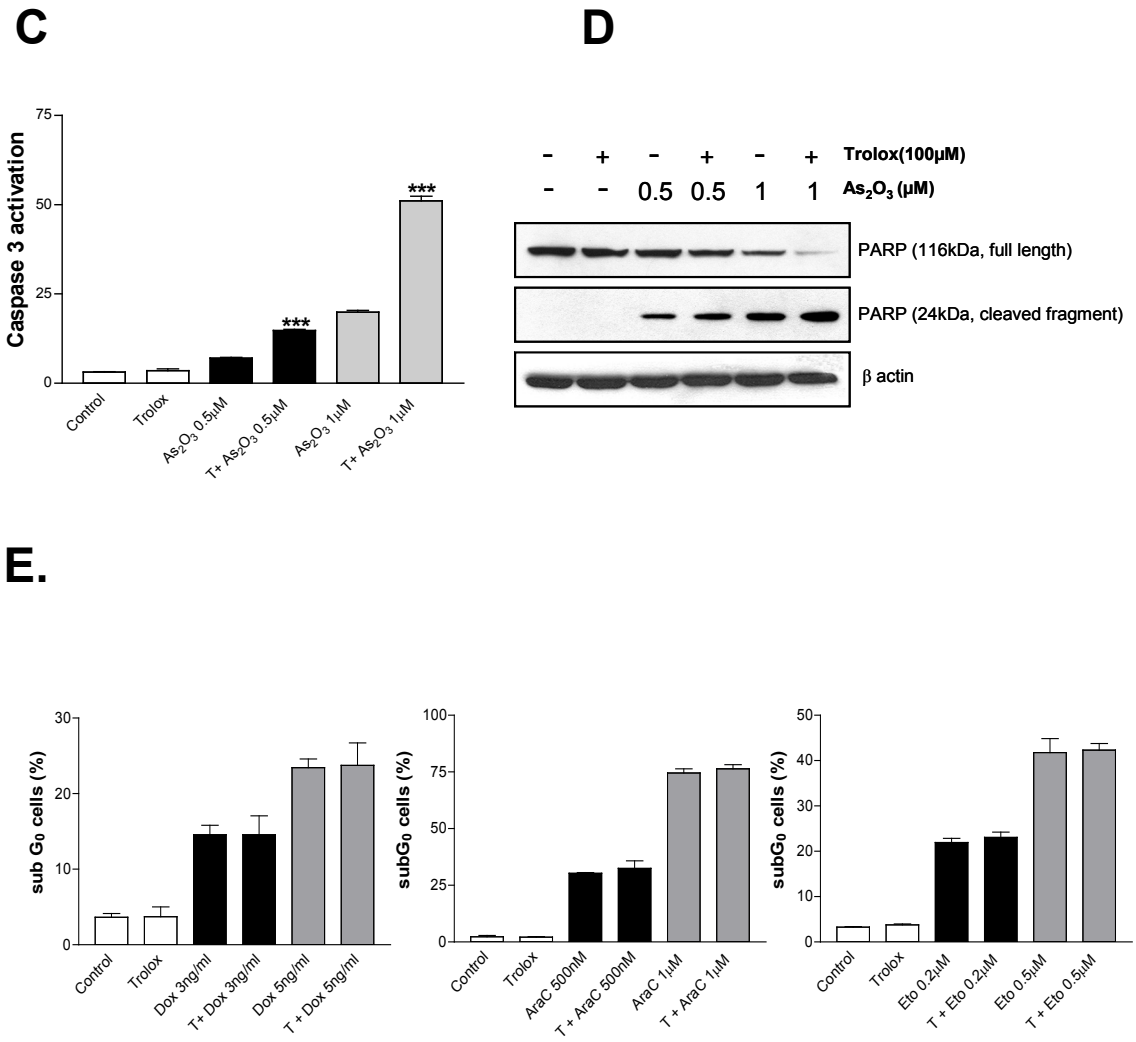

Figure 2.2: Trolox enhances arsenic-mediated apoptosis in NB4, AR2 and IM9 cells.

(C) Cells were treated as indicated for 48 hours. Caspase 3 activation was measured using Red-DEVD-FMK. Its binding to activated caspase 3 was analyzed by flow cytometry. Asterisks indicate significant differences ($p<0.001$) from As_2O_3-treated cells. (D) Western blotting was performed to determine PARP protein levels after 48 hours treatments. β-actin was used to show equal loading of lanes. Results are representative of three independent experiments each performed in duplicate. (E) NB4 cells were treated with doxorubicin, AraC or etoposide with or without trolox (T) for 48 hours. Apoptosis was detected by PI-staining as described above. Each bar represents an average of three independent samples, and standard deviation bars are shown.

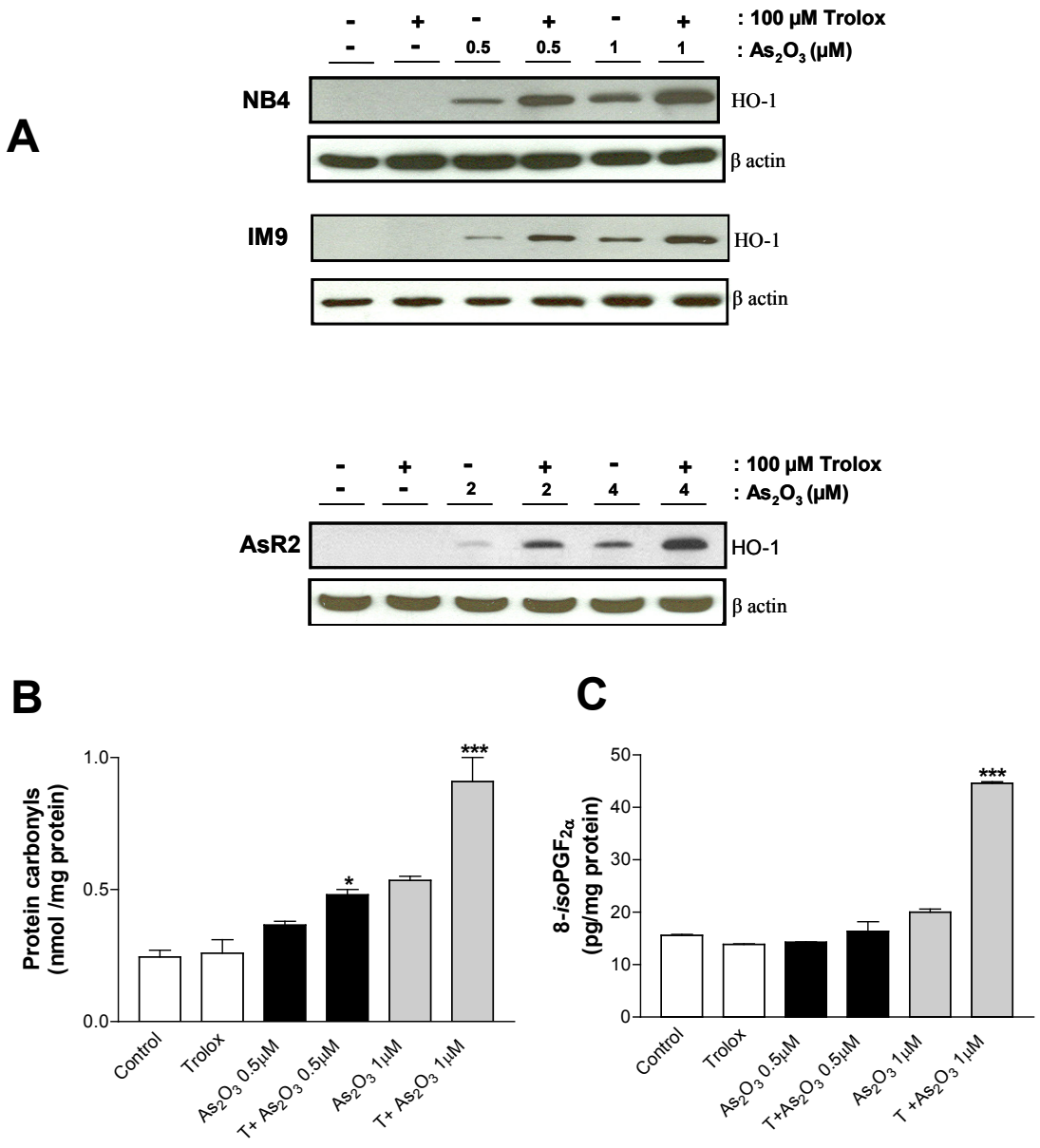

Figure 2.3: Trolox potentiates As_2O_3-mediated oxidative stress.

(A) NB4, IM9 and AsR2 cells were treated with As_2O_3 and trolox for 24 hours. Western blot was used to determine HO-1 protein levels. β-actin was used as a loading control. These data represent three independent experiments. (B) Protein carbonyl content was detected by ELISA in NB4 cells treated with As_2O_3 alone, trolox or the combination for 3 days with the concentrations indicated. Data depicted are representative of three independent experiments each performed in duplicate. Asterisks indicate significant differences from As_2O_3-treated cells. (* $p<0.05$; *** $p<0.001$). (C) 8isoPGF$_{2\alpha}$ was detected in whole cells extracts from NB4 cells treated with the indicated compounds for three days. Asterisks indicate significant differences from As_2O_3-treated cells ($p<0.001$).

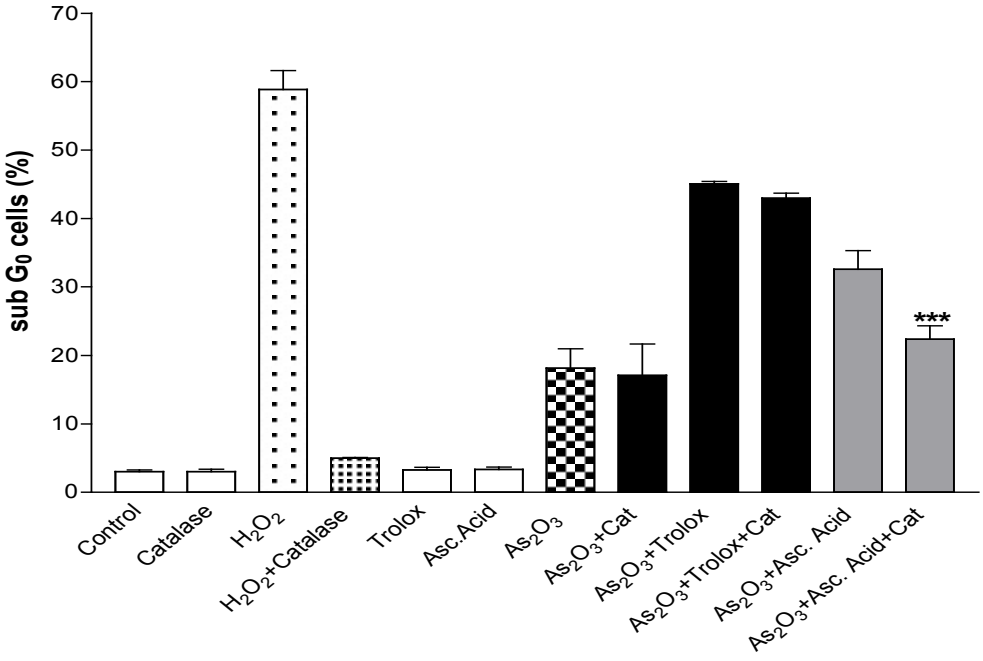

Figure 2.4: The synergistic effects of trolox on arsenic-mediated apoptosis are not related to extracellular H_2O_2 production.

Cells were treated with As_2O_3 (1μM) and trolox or ascorbic acid (100μM) for 48 hours. Catalase (500U/mL, Cat) was added as indicated to degrade the extracellular H_2O_2 generated. Apoptosis was detected by PI-staining, and quantitated by flow cytometric measurement of PI-positive cells. Each bar represents an average of three independent samples, and standard deviation bars are shown. Asterisks indicate significant differences from As_2O_3+AA-treated cells ($p<0.001$).

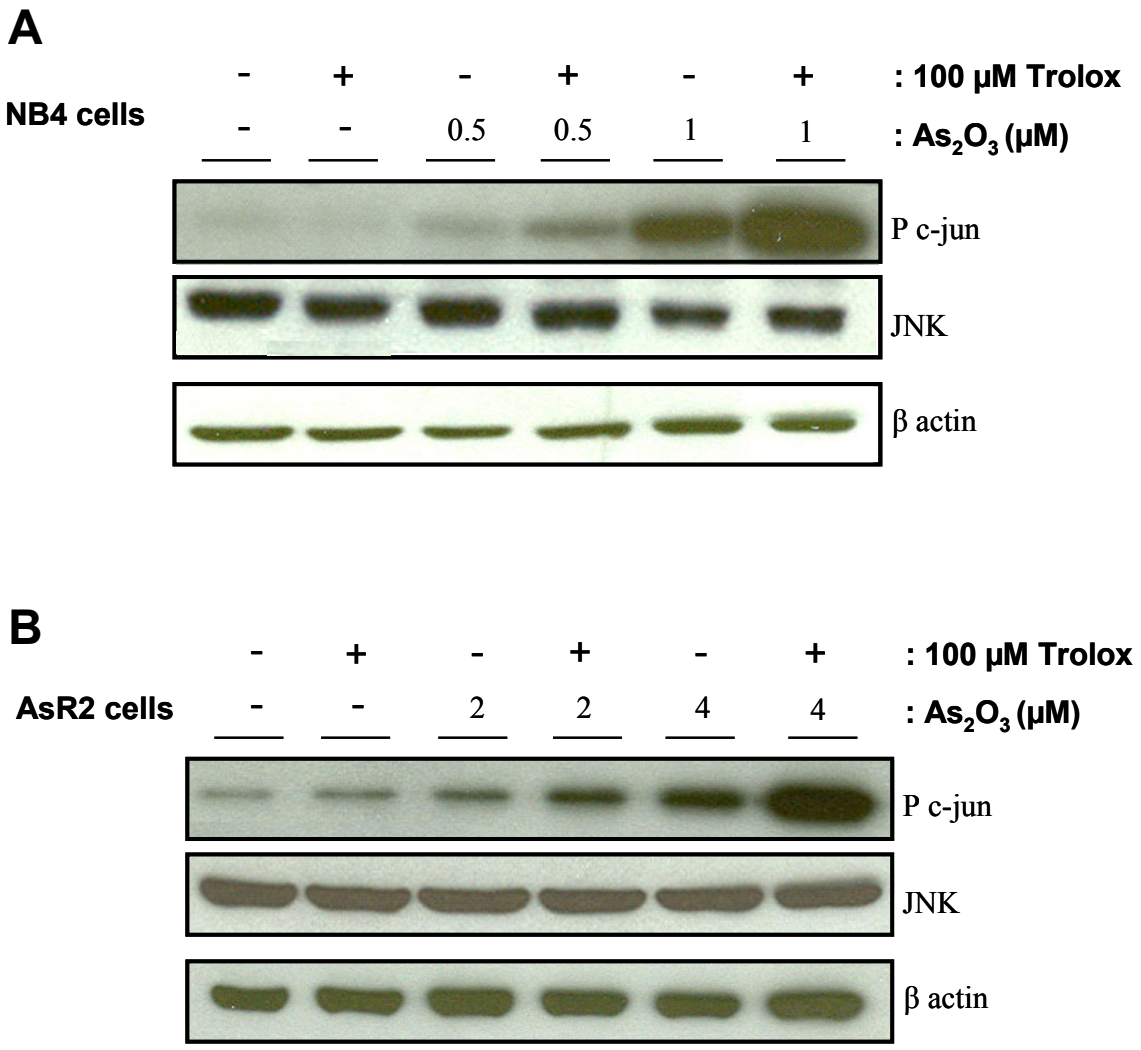

Figure 2.5: Trolox enhances As_2O_3-mediated JNK activation.

Immune complex kinase assays were performed to measure JNK activity with extracts from NB4 (A) or AsR2 cells (B) treated with As_2O_3 and trolox for 16 hours as described in materials and methods. Data depicted are representative of three independent experiments.

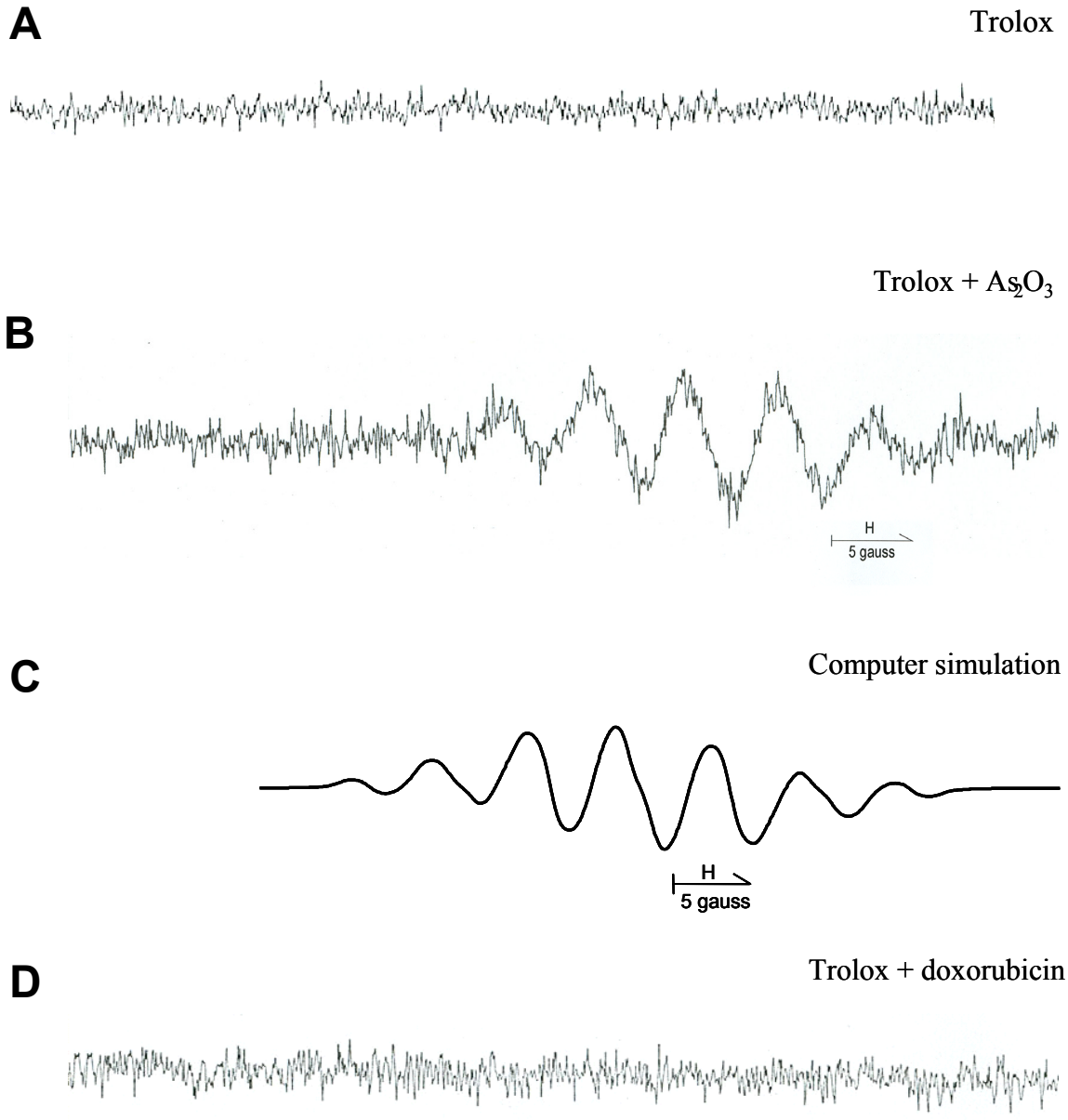

Figure 2.6: Electronic Paramagnetic Resonance detection of the trolox phenoxyl radical.

EPR spectra of trolox in the reaction system containing 1mM Trolox, 5% (v/v) DMSO (A) and As_2O_3 0.02mM (B) or 0.2μg/ml doxorubicin (C). (D) Computer simulation of spectrum in (B) obtained using the hyperfine splitting constants: a^H (CH_3) = 4.56 G; a^H (CH_3) = 4.86 G; a^H (CH_3) = 0.23 G; a^H (CH_2) = 0.37 G; $a^{H'}$ (CH_2) = 0.76 G.

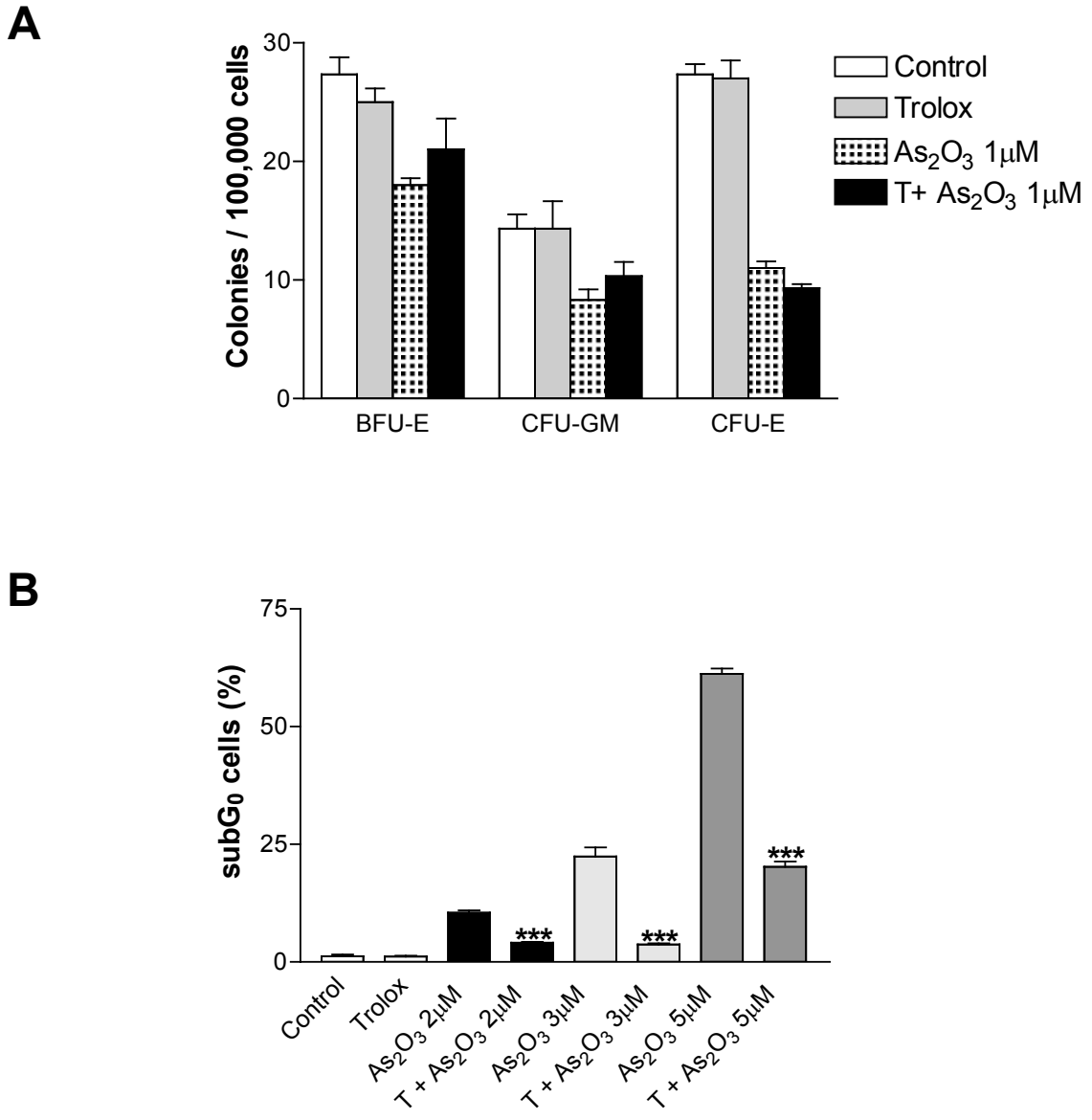

Figure 2.7: The synergistic effects of trolox on arsenic-mediated apoptosis are unique to cancer cells. (A) Normal human PBMC were isolated from three normal donors using a Ficoll gradient. Colony forming ability of PBMC treated with As_2O_3 and trolox was assessed by counting CFU-E, CFU-GM and BFU-E after 15 days. Results are representative of three independent experiments each performed in triplicate. (B) Mouse embryonic fibroblasts were treated with As_2O_3 with or without trolox for three days. Apoptosis was detected by PI-staining, and quantitated by flow cytometry measurement of PI-positive cells. Each bar represents an average of three independent samples. Asterisks indicate significant differences from As_2O_3-treated cells. ($p<0.001$).

Cell lines	IC_{50} As_2O_3	IC_{50} As_2O_3 + Trolox
MCF-7	2.07±0.02	1.02 ±0.09
T47D	3.22 ±0.07	1.56 ±0.03
MDA-MB-231	2.27 ±0.08	0.98 ±0.02

Table 2.1: Effect of trolox on As_2O_3 mediated growth inhibition in breast cancer cells.

Cells were treated with 1μM As_2O_3 and 100μM trolox for 3 days. Viable cell number was determined using the trypan blue exclusion method. IC_{50} indicates concentration of drug needed to inhibit 50% of cell growth. Values are the mean of three independent experiments.

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CHAPTER 3

Trolox enhances the anti-lymphoma effects of arsenic trioxide, while protecting against liver toxicity

3.1 Preface

Combinations of As_2O_3 with other agents have been explored to increase antitumor efficacy and the therapeutic index of As_2O_3. Our previous study identified trolox as a potent enhancer of As_2O_3-mediated cell death *in vitro*. This chapter describes the *in vivo* anti-cancer properties of this combination in lymphoma tumor-bearing mice and its ability to prolong survival and decrease metastatic spread. Moreover, we analyzed the effects of this combination on normal liver function.

3.2 Abstract

Arsenic trioxide (As_2O_3) is an effective therapy in acute promyelocytic leukemia (APL), but its use in other malignancies is limited by the higher concentrations required to induce apoptosis. We have reported that trolox, an analogue of α-tocopherol, increases As_2O_3-mediated apoptosis in a variety of APL, myeloma and breast cancer cell lines, while non-malignant cells may be protected. In the current study, we extended previous results to show that trolox increases As_2O_3-mediated apoptosis in the P388 lymphoma cell line *in vitro*, as evidenced by decrease of mitochondrial membrane potential and release of cytochrome c. We then sought to determine whether this combination can enhance antitumor effects while protecting normal cells *in vivo.* In BDF_1 mice, trolox treatment decreased As_2O_3-induced hepatomegaly, markers of oxidative stress, and hepatocellular damage. In P388 tumor-bearing mice, As_2O_3 treatment prolonged survival, and the addition of trolox provided a significant further increase in life span. In addition, the combination of As_2O_3 and trolox inhibited metastatic spread, and protected the tumor-bearing mice from As_2O_3 liver toxicity. Our results suggest, for the first time, that trolox might prevent some of the clinical manifestations of As_2O_3-related toxicity while increasing its pro-apoptotic capacity and clinical efficacy in hematological malignancies.

3.3 Introduction

Arsenic trioxide (As_2O_3), first used in traditional Chinese medicine, is highly effective in the treatment of patients with acute promyelocytic leukemia (APL)[1-3]. Although the precise mechanism of action of As_2O_3 in APL is unclear, *in vitro* studies reported that As_2O_3 leads to cellular redox status perturbation, cellular signaling modulation, differentiation, growth inhibition and apoptosis[4]. Clinically achievable concentrations, i.e. between 1 and 2 μM of As_2O_3, induce apoptosis and inhibit growth of various malignant cells, including multiple myeloma and human T lymphotropic retrovirus type I-associated adult T cell leukemia cells[5-7]. Recently, Rousselot *et al*. documented *in vivo* activity of arsenic in the treatment of multiple myeloma using a SCID mouse xenotransplantation model[8]. In another xenotransplantation model, As_2O_3 (3.75 mg/kg) induced a dramatic reduction of L540Cy Hodgkin tumors[9]. Subsequent *in vivo* studies characterized the effectiveness of arsenic in various hematological malignancies, and multiple phase I/II clinical trials are underway to evaluate its feasibility, safety and potential efficacy. Arsenic has also been tested in non-hematological cancer. Using an orthotopic prostate metastasis model, As_2O_3 alone provided a dose-dependent inhibition of both primary and metastatic lesions, although an increased survival rate was only obtained in the group treated with the combination of As_2O_3 and buthionine sulfoxamine (BSO), an inhibitor of γ-glutamyl cysteine synthase[10].

In spite of these and other studies showing sensitivity to arsenic treatment *in vitro* and *in vivo*[5-14], the degree of sensitivity has been consistently less than in APL cells, and clinical trials in different hematological malignancies and solid tumors have had mixed results[15-18]. These initial investigations suggest that arsenic trioxide, as a single agent, may have limited clinical activity outside APL. Therefore, combinations with other agents should be explored to increase antitumor efficacy and the therapeutic index of As_2O_3.

Several compounds have been reported to increase As_2O_3-mediated apoptosis *in vitro*[5,19,20]. BSO modulates the cellular glutathione system and can significantly potentiate the effects of As_2O_3, converting arsenic resistant cell lines to a sensitive phenotype[21,22]. Although *in vivo* effects have been reported for this combination[23], the observed additive toxicity may not be selective for cancer cells, and BSO itself has not been successfully

developed for clinical use. Ascorbic acid (AA), a key antioxidant molecule, augments the toxicity of As_2O_3 *in vitro*[24,25], but controversy exists regarding its mechanism of action[26], and its potential for utility in the clinic is under study.

We recently demonstrated that trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid), enhances the sensitivity of APL to As_2O_3 *in vitro.* We extended these results to NB4, an arsenic resistant subclone of NB4, the IM9 multiple myeloma cell line, and a variety of breast cancer cell lines[27]. In all these malignant cell lines, treatment with As_2O_3 and trolox increases intracellular oxidative stress, as evidenced by elevated heme oxygenase-1 (HO-1) protein levels, JNK activation, and protein and lipid oxidation.

Importantly, we found that trolox could protect non-malignant cells from As_2O_3-mediated cytotoxicity *in vitro*, suggesting it may diminish or overcome the adverse effects associated with As_2O_3 monotherapy *in vivo,* potentially increasing the therapeutic index. As a presumed antioxidant, trolox has been used to mitigate the toxic effects of several compounds in animal models. Trolox reduced liver necrosis in a model of hepatic ischemia-reperfusion in rats[28] and decreased streptozotocin-induced liver and kidney damage in mice[29].

In the present study, we addressed the effects of As_2O_3 and trolox on the viability of lymphoma P388 cells *in vitro* and in a mouse tumor model. We also investigated mechanisms underlying the pro-apoptotic properties of this combination, and its potential toxic effects in mice. We show that the combination of As_2O_3 and trolox decreases arsenic toxicity in non-tumor and tumor bearing mice, while increasing the survival time and limiting the metastatic spread in mice bearing P388 lymphoma cells.

3.4 Experimental procedures

3.4.1 Growth Assays

P388 cells (provided by Dr. Jing, Mount Sinai Medical Center, New York) were treated with various concentrations of As_2O_3 +/- 100μM trolox for six days. Viable cells were counted by trypan blue exclusion on days 1, 3 and 6. Logarithmic growth phase was maintained at a density lower than $1x10^6$ cells/ml through dilution as required, and media +/- treatment was replaced every third day.

3.4.2 Annexin V / Propidium Iodide staining

Cells were stained with Annexin-V-FITC and Propidium Iodide (PI) in binding buffer according to the manufacturer (BD Pharmingen, San Diego, CA) and analyzed on a FACScan (Becton Dickinson, San Jose, CA). Apoptotic cells (Annexin V positive/ PI negative) were quantified using the CellQUEST software (Becton Dickinson).

3.4.3 Detection of the Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ were determined with the J-aggregate-forming lipophilic cationic fluorochrome JC-1 (Molecular Probes, Eugene, OR). Cells were incubated with 2.5 mg/mL JC-1 for 15 min. Cells were washed 2x with PBS, resuspended in PBS, and analyzed on a FACScan. Data were analyzed and expressed as the ratio of mean florescence intensity (MFI) between FL2 (polarized, dimeric) and FL1 (depolarized, monomeric) fluorescence.

3.4.4 Preparation of S-100 Fractions and Assessment of Cytochrome c Release

Cells were harvested, washed with ice-cold PBS and resuspended in 5 volumes of buffer (75mM NaCl, 8mM Na_2HPO_4, 1mM NaH_2PO_4, 1mM EDTA, 350ug/ml digitonin, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10μmol/L aprotinin and 10μmol/L leupeptin). After 30 min on ice, the cells were centrifuged twice at 750g, 10 min at 4°C. Cytosolic S-100 fractions (supernatants) were obtained by centrifugation at 100,000g for 60 min at 4°C. Cytochrome c release into the S-100 fraction for each condition was assessed by Western blot analysis.

3.4.5 Western Blotting

Livers were disrupted by a Polytron homogenizer (Brinkmann, Westbury, NY). Debris was removed by centrifugation at 700×g for 15 min, followed by centrifugation of the supernatant twice at 14,400×g for 15 min and finally at 100,000×g for 1 hour at 4 °C. Proteins were separated and probed as described previously [27] with cytochrome c (1:500, BD Pharmigen), HO-1 (1:1000, Stressgen), and HSP70 (1:5000, Stressgen). Immunostaining for β-actin confirmed equal protein loading.

3.4.6 Cytochrome C Oxidase (CcO) activity and cellular ATP levels

Mouse liver mitochondria were isolated using a mitochondria isolation kit (Sigma). CcO activity was calculated based on the rate of oxidation of ferrocytochrome c (decrease in absorbance at 550 nm). Ferrocytochrome c concentrations were determined using a kit (Sigma), with values expressed as $\mu mol \cdot min^{-1} \cdot mg$ mitochondrial $protein^{-1}$. The intracellular ATP concentration was determined with a luminescent ATP detection kit (ATPLite; PerkinElmer Life Sciences) and was measured using a multiplate reader. ATP was calculated from a standard curve and was expressed as μM ATP/mg wet tissue.

3.4.7 *In vivo* toxicity experiments

All procedures conformed to the NIH guidelines for the care and use of laboratory animals and were approved by the McGill University Animal Care Committee. BDF_1 mice (Charles River Laboratories; Wilmington, MA) were randomly divided into eight groups of five mice. Each group received trolox (2.5, 10, 20 or 50 mg/kg), As_2O_3 (7.5 mg/kg) or the combinations of trolox and As_2O_3 i.p. every other day for a total of 14 injections. Animals were weighed every other day. One day after the last dose of arsenic, blood was collected by cardiac puncture. Serum was separated and total protein levels, glucose content, activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AKP) were assayed using commercially available kits. Mice were sacrificed by cervical dislocation. Liver was extracted and washed in ice-cold isotonic saline solution and weighed. Liver samples were fixed in 10% phosphate-buffer formalin (pH 7.4), embedded in paraffin, sectioned, stained with hematoxylin-eosin, and

examined with bright field microscopy by a pathologist. Lymphocyte infiltration and numbers of binucleated cells were quantified in 10 random 0.159mm^2 fields/sample.

3.4.8 *In vivo* anti-tumor experiments

P388 cells were injected i.p. in DBA/2 mice (Charles River Laboratories, Wilmington, MA). After 15 days, cells were collected from the peritoneum, washed and resuspended in PBS. For experiments, 0.1 mL containing 2x10^6 cells obtained from the ascites was inoculated i.p. in BDF_1 mice. Mice were randomly divided into six groups each with eight mice. After 24 hours, each group was given saline, As_2O_3 (7.5 or 10 mg/kg), and trolox (50 mg/kg) alone or in combination i.p. every other day for a total of 14 injections. The percentage increase in lifespan over control (ILS) was calculated as follows: ILS%= 5T/C% minus 100, where T is the test mean survival time, and C is the control mean survival time. Macroscopically visible lesions were counted in liver, stomach, pancreas and intestine by a pathologist blinded to the treatment groups. Sections of liver were stained for hematoxylin and eosin to verify that the counted visible liver lesions were indeed liver metastases.

3.4.9 Statistical Analysis

Significance was determined by analysis of variance followed by Newman-Keuls post-tests using Prism version 3.0 (GraphPad software, San Diego, CA). The combination index (CI), an indication of the interaction between two drugs, was determined by the formula: $a/A + b/B = 1$ where a is the IC_{50} of As_2O_3 + trolox at a concentration b; A is the IC_{50} of As_2O_3; and B is the IC_{50} of trolox. According to this formula, when $CI<1$ the interaction is synergistic, when $CI=1$, the interaction is additive, and when $CI>1$, the interaction is antagonistic[30].

3.5 Results

3.5.1 Trolox significantly enhances As_2O_3–induced apoptosis of murine lymphoma P388 cells.

Based on our finding of synergy in NB4, AsR_2, IM9 and a variety of breast cancer cells lines[27], we first investigated whether trolox would increase the *in vitro* efficacy of As_2O_3 in a lymphoma cell line for which there is an established animal model. As Figure 3.1A shows, treatment of P388 cells for six days with 2 and 4μM As_2O_3 reduced the viable cell number by 19.0% and 51.7% of control, respectively ($p<0.001$). Thus, P388 cells are less sensitive to As_2O_3 than some leukemic cell lines, in which 0.5μM and 1μM As_2O_3 are sufficient to induce a similar effect[32]. Trolox (100μM) alone had no effect on cell number at any time point. However, if the cells were treated with 2 or 4μM As_2O_3 and 100μM trolox in combination, 41.2% and 81.3% reductions in cell number were observed ($p<0.001$, when compared to As_2O_3 alone at either dose). In all cases, trypan blue positive cells were less than 3%. A difference was also seen at 72 hours, where addition of trolox enhanced the action of 4μM As_2O_3 by decreasing cell number 29% compared to As_2O_3 alone. The CI value was determined to be 0.619 documenting a synergistic interaction between As_2O_3 and trolox.

A variety of complementary techniques were then performed to analyze whether the observed growth inhibitory effects were the result of the induction of apoptosis in P388 cells. Using Annexin V/propidium iodide staining, we found that 24% of the cells treated with 2μM As_2O_3 were apoptotic after 48 hours (Figure 3.1B). This percentage was nearly doubled when trolox was added. Similarly, the addition of trolox to 4μM As_2O_3 increased apoptosis from 33.2% to 58.5% ($p<0.001$). Consistently, control and cells treated with trolox exhibited JC-1 orange fluorescence due to the formation of JC-1 aggregates, indicating that the mitochondria were polarized (Figure 3.1C). Exposure to As_2O_3 induced a very rapid decline in $\Delta\Psi m$, as revealed by complete loss of JC-1 orange fluorescence and a shift to JC-1 green fluorescence due to formation of JC-1 monomeres. Consistently, a dose-dependent decrease of $\Delta\Psi m$ was observed in the cells treated with the combination of As_2O_3 and trolox. As a consequence of the decreased mitochondrial membrane potential, cytochrome c may be released from the mitochondria, providing another marker of apoptosis. As shown in Figure 3.1D, cytoplasmic cytochrome c content

was increased when trolox and As_2O_3 were used in combination at both As_2O_3 doses. Thus, our data indicate that trolox increases As_2O_3-induced apoptosis in P388 cells.

3.5.2 Trolox decreases As_2O_3-mediated toxicity *in vivo* in BDF_1 mice.

In our previous work, we demonstrated that trolox decreases cytotoxicity of As_2O_3 in mouse embryonic fibroblasts[27]. In addition, we have since determined that trolox protects the non-tumorigenic murine hepatocyte AML cells from As_2O_3 toxicity *in vitro* (data not shown), suggesting that synergistic toxicity of the combination could be specific to tumor cells. To test this hypothesis *in vivo*, we first conducted toxicological studies to define the maximum tolerable dose of trolox in BDF_1 mice.

Trolox treatment was well tolerated and not toxic at the doses studied (2.5, 10, 20, and 50 mg/kg), as indicated by assessment of body weight over the course of the study; the average body weight did not differ significantly from the control animals in any treatment group. The higher dose approaches its maximum solubility limit in 300μL, the maximum volume that can be injected in mice intraperitoneally.

Liver damage has been reported to be a marker of arsenic toxicity in different experimental animals[31,32]. Therefore, we asked whether trolox could affect As_2O_3-associated hepatocellular damage *in vivo*. Mice were randomly divided into groups of five mice and treated with two doses of trolox and a dose of As_2O_3 (7.5mg/kg) reported to be moderately toxic[24]. None of the animals exhibited discomfort or obvious distress throughout the duration of the experiment. No significant differences in weight were observed in any of the treated groups compared to control. As depicted in Figure 3.2A, moderate hepatomegaly was observed in the As_2O_3-treated group. The average liver weights in the control and trolox groups were quite similar, with an average of 1.07±0.14g, while in the As_2O_3-treated group, average liver weight was increased to 1.53±0.35g ($p<0.05$). However, in the groups treated with the combination of As_2O_3 and trolox, the hepatomegaly was abrogated, with an average liver weight of 1.10±0.1g ($p<0.05$ As_2O_3 vs. As_2O_3+trolox).

When hepatocellular injury occurs, the associated plasma membrane leakage can be detected biochemically by assaying aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in serum. Figures 3.2B and C show that both enzymatic

activities were increased in the As_2O_3-treated group by 4.2 and 3.5-fold compared to the control group, respectively. However, in the animals treated with the combination of As_2O_3 and either 10 or 20 mg/kg trolox, a significantly decreased induction of AST and ALT activities was observed ($p<0.05$). The activity of alkaline phosphatase, an indicator of cholestasis, was not significantly affected by any of the treatments (data not shown), suggesting that As_2O_3 can induce a direct injury to the hepatocytes without blocking bile excretion. We did not observe any change in glucose or total protein levels in any of the groups (data not shown) or any fulminant hepatic failure, perhaps due to the short duration of the experiment.

Histopathological analysis of liver samples demonstrated a cell injury pattern in the As_2O_3-treated group (Figure 3.3A) characterized by hepatocellular degeneration, inflammatory infiltrates composed of fibrinous exudates and polymorphonuclear leukocyte aggregates, and areas with focal necrosis. A marked increase in binucleated cells was observed, suggesting regeneration of hepatocytes after acute toxicity. We tested whether the addition of trolox would modify this cellular pattern of toxicity. Lymphocyte foci and binucleated cells were counted in randomly selected fields by a pathologist blinded to the treatment groups. A significant decrease in lymphocyte infiltration and binucleated cells was observed after treatment with As_2O_3 and trolox compared to As_2O_3 alone (Figure 3.3B and C). These results indicate that trolox significantly protects hepatocytes from As_2O_3–mediated toxicity.

3.5.3 Trolox decreases As_2O_3-mediated oxidative stress, and ameliorates the As_2O_3-mediated decrease in cellular metabolic rate in BDF_1 mice.

Oxidative damage may be a key mechanism by which arsenic mediates its toxic effects. Because trolox decreases As_2O_3-induced liver toxicity, we hypothesized that the addition of trolox reduces hepatocellular oxidative stress induced by As_2O_3. HO-1, an oxidative stress-responsive protein[33], was not detected in the liver of control animals or animals treated with trolox alone, but was markedly induced by As_2O_3. The addition of trolox significantly decreased As_2O_3-mediated HO-1 induction in all the animals. (Figure 3.4A). The 70 kilodalton heat shock proteins (HSP-70 family) are important for protein folding and help to protect cells from stress. HSP-70 expression has been used as an

indicator of As_2O_3 exposure in different experimental models[34]. As depicted in Figure 3.4B, HSP-70 protein levels were enhanced in the livers of As_2O_3-treated group, while they were decreased to near basal levels when the animals were treated with the combination of As_2O_3 and trolox. The HSP70 antibody recognizes the inducible form of HSP-70 (HSP-72) and the constitutive form HSP-73, explaining the basal levels in the control and trolox treated groups.

Thus, having established that As_2O_3 induces liver oxidative stress, and that the addition of trolox significantly restores hepatocellular redox homeostasis, we further analyzed whether the hepatocellular metabolic rate was affected by this combination. Sulfhydryl groups in many enzyme systems react with arsenicals, which may result in a block of the Krebs cycle, interrupting oxidative phosphorylation, which in turn causes marked depletion of ATP stores[7]. The activity of cytochrome c oxidase in the liver is considered to be a good metabolic marker for functional activity of cells. Therefore, we asked whether As_2O_3 could reduce CcO activity and ATP stores and whether trolox might play a role in the restoration of the basal levels. Figure 3.4C shows that the hepatic CcO enzymatic activity of animals treated with As_2O_3 was decreased by 74.2%, while this activity was only decreased by 48.6% with the combination of As_2O_3 and trolox ($p<0.05$). As predicted, As_2O_3 treatment induced a 63.5% decrease in liver ATP levels, while the animals treated with As_2O_3 and trolox only showed a 32.2% reduction ($p<0.001$) (Figure 3.4D). These results again show protective effects of trolox on arsenic-mediated liver toxicity.

3.5.4 Trolox increases As_2O_3-mediated antitumor effects in BDF_1 mice bearing lymphoma P388 cells while protecting against liver toxicity.

On the basis of the *in vitro* potency and favorable *in vivo* toxicity profiles, As_2O_3 and trolox were evaluated for *in vivo* antitumor efficacy in mice bearing P388 murine lymphoma tumors. The dose selection for As_2O_3 (7.5 and 10 mg/kg body weight) was based on the relatively low toxicity seen in our initial study of non-tumor bearing mice. Trolox was given at 50 mg/kg, which was not toxic in our preliminary results but approached the maximum solubility. As shown in Figure 3.5A, As_2O_3 treatment prolonged survival, with median survival times of 20 and 18 days for 7.5mg/kg and

10mg/kg As_2O_3, respectively, as compared to 14 days for controls ($p<0.001$). The median survival time for animals treated with the combination of As_2O_3 and trolox was further prolonged to 24.5 and 22 days compared to As_2O_3 alone ($p<0.001$). Treatment with 7.5mg/kg As_2O_3 provided a 46.4% increase in life span (ILS) (Figure 3.5B). When this dose was combined with trolox, we observed a 73.5% ILS. Animals treated with 10mg/kg As_2O_3, experimented an increased in life span of only 28.6% ($p<0.001$). We observed moderate weight loss and lethargy in these mice (data not shown), suggesting that this dose is toxic. However, the addition of trolox doubled the increase in lifespan of 10mg/kg As_2O_3 alone without evidence of increased toxicity.

The effects of the combination of As_2O_3 and trolox on tumor metastases were profound. At the time of sacrifice or death for tumor progression, metastases were present in all of the eight (100%) saline-treated control animals as seen macroscopically and in histological sections (Figure 3.5C and data not shown). Treatment with 7.5 and 10mg/kg As_2O_3 reduced the number of animals with metastases to 62.5% and 37.5% respectively. The incidence of metastases was significantly decreased to 37.5% and 12.5% when trolox was combined with 7.5 mg/kg and 10mg/kg As_2O_3 ($p<0.001$). We examined livers from tumor-bearing mice to analyze whether trolox could also modulate As_2O_3-mediated oxidative stress and its effects on the metabolic rate of these animals. We found that the non-treated tumor-bearing animals had a higher baseline of liver oxidative stress than non-tumor bearing mice, as demonstrated by an increase in HO-1 protein levels (first lane of Figure 3.6A, compared to Figure 3.4A). Interestingly, treatment with trolox alone decreased the hepatic oxidative stress. As_2O_3 treatment caused some further increase in HO-1 expression at both 7.5mg/kg and 10mg/kg (Figure 3.6A and data not shown). However, addition of trolox significantly decreased HO-1 protein levels at both doses of As_2O_3 studied. Similarly, HSP70 expression was consistently increased by As_2O_3, and this effect was reduced by trolox (Figure 3.6B). We then explored the effects of the As_2O_3 and trolox combination on hepatic metabolic rate using assays of CcO and ATP as shown before. Consistently, CcO activity (Figure 3.6C) and ATP stores (Figure 3.6D) were markedly reduced in the animals treated with As_2O_3, but significantly restored in the animals treated with the combination of As_2O_3 and trolox. In addition, serum ALT levels were less elevated when As_2O_3 was given with trolox (data not shown). These results

show that although tumor-bearing mice have baseline liver damage, consistent with our data for non-tumor bearing mice, trolox protects the liver from arsenic-mediated toxicity.

3.6 Discussion

Although activity in many malignant cell lines requires concentrations of As_2O_3 that are not clinically achievable, our previous work identified trolox as a compound that might have a dual role depending on the cellular microenviroment. In malignant cells, trolox synergizes with As_2O_3 to increase its toxicity while in non-malignant cells trolox decreases As_2O_3-mediated cellular damage. This is the first report showing that the combination of As_2O_3 and trolox *in vivo* targets malignant cells and limits cancer metastases, while decreasing damage to normal cells.

In this study, we used the mildly As_2O_3-resistant P388 lymphoma cell line to assess the potential synergy of As_2O_3 and trolox *in vitro* and *in vivo.* We found that the combination of As_2O_3 and trolox enhanced growth inhibition and apoptosis of P388 cells. These data support a synergistic effect of trolox in enhancing As_2O_3 toxicity consistent with previously published work in NB4, AsR_2, IM9, MCF7, T47D and MDA-231 cells[27]. These *in vitro* effects provided the rationale for experiments using the P388 lymphoma cell line to determine how trolox would affect As_2O_3 activity and toxicity *in vivo*. We first performed *in vivo* studies with As_2O_3 and trolox alone or in combination to investigate whether trolox would affect arsenic toxicity in BDF_1 mice. We observed As_2O_3-induced liver toxicity, which has been previously reported to involve tissue necrosis and other histological and biochemical, changes in several animal models[35]. In these non-tumor bearing mice, we found that trolox protects normal hepatic cells from As_2O_3 toxicity. This is the first study to report *in vivo* protective effects of trolox on arsenic-induced hepatotoxicity, although several studies have demonstrated its efficacy in preventing toxicity of other metals[36]. In addition, studies have shown that a trolox derivative (U-83836E) appeared to be beneficial in reducing lipid peroxidation products and in partially preventing the decrease in glutathione and antioxidant enzymes induced by methanol in liver, serum[37], and brains[38] of rats. Lower doses of trolox than used in the present study clearly reduced methylmercury-induced toxicity in rats[39]. These studies suggest that trolox may decrease toxicity associated with a broad spectrum of compounds.

Based on our *in vitro* results and the favorable *in vivo* toxicity profile, we evaluated As_2O_3 and trolox for *in vivo* antitumor efficacy in the peritoneal P388 murine

lymphoma model. This model was used to test the combination of ascorbic acid and arsenic, which also was reported to be effective *in vitro* in various cell lines. In addition, this model has been used to test the effects of different antineoplastic drugs[40-42]. Trolox doses ranging from 2mg/kg and 100mg/kg have been used in rats and rabbits[43-45]. The intraperitoneal route was chosen in an attempt to avoid the potential confounding factors on As_2O_3 absorption through the gastrointestinal tract and to compare parenteral arsenic effects with previous reports in the literature[46,47]. We showed that the addition of trolox to As_2O_3 given to mice bearing P388 lymphoma cells significantly increased their survival time. It has been reported, using the same model, that ascorbic acid enhanced antitumor properties of As_2O_3[24]. Dai *et al.* observed an increase in survival time with the combination of AA and As_2O_3, although they used a lower dose of As_2O_3 (5mg/kg), which did not increase the lifespan of the mice as a single agent. Using 7.5mg/kg As_2O_3, we obtained a significant prolongation of survival that was markedly improved by the addition of trolox. Most notably, our studies show the potential for As_2O_3 and trolox given as a combination to limit metastases. It remains critical to reconcile these two opposite effects of the As_2O_3 and trolox combination, i.e., a cooperative action of As_2O_3 and trolox against lymphoma growth and metastases with the concomitant protection by trolox of normal cells *in vivo*. Our data suggest that trolox may behave as a prooxidant in cancer cells exposed to As_2O_3, while having antioxidant properties in normal cells.

Therapeutic enhancement by antioxidants is counterintuitive to the apparent role of ROS in apoptosis. However, the pro- or antioxidant effects of many redox-active compounds may vary substantially as a function of the cellular redox microenvironment and models employed. For example, it has been proposed that the selectivity of certain chemotherapeutic agents to cancer cells may be due, in part, to the relatively low concentrations of antioxidant enzymes documented in some malignant cells[48,49]. It is noteworthy that normal cells in general are more efficient in eliminating ROS than malignant cells. Furthermore, a specific ROS or its intracellular localization could be the critical determinant of cell death or survival. Finally, it has been demonstrated that vitamin E[50] may modulate tumor cell permeability by altering levels of lipid peroxidation in surface membranes, raising the possibility that trolox differentially augments As_2O_3 uptake by malignant cells.

Our observation of reduced toxicity of As_2O_3 *in vivo*, delayed death in mice bearing P388 tumor cells, and reduction of metastatic spread in this model, suggests that the combined use of As_2O_3 and trolox may increase the therapeutic index of arsenic and possibly decrease the development of secondary tumors in patients with advanced lymphoma and other malignancies.

3.7 Acknowledgements

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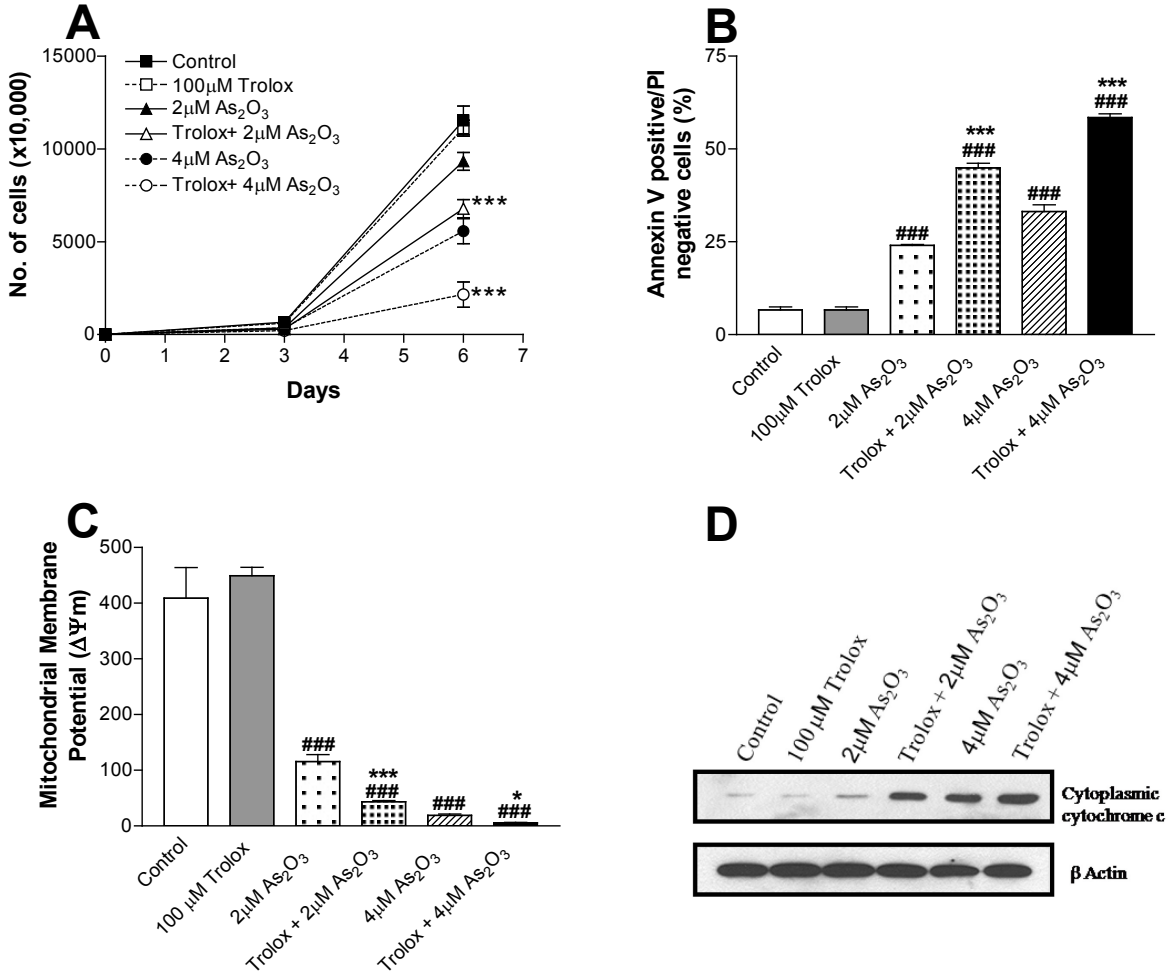

Figure 3.1: Trolox enhances As_2O_3-mediated growth inhibition and apoptosis in murine P388 lymphoma cells.

(A) P388 cells were treated with media (■), trolox (□), 2 μM As_2O_3 (▲), 4μM As_2O_3 (•) and the combination of trolox with 2μM As_2O_3 (Δ) and 4μM As_2O_3 (○).Viable cells were counted by trypan blue exclusion on day 1, 3 and 6. Bars denote standard deviations. Asterisks indicate significant differences ($p<0.001$) from As_2O_3-treated cells. (B) P388 cells were treated with As_2O_3 and trolox for 48 hours. Apoptosis was detected by AnnexinV-FITC and PI staining. Apoptotic cells (Annexin V positive/ PI negative) were quantified using the CellQUEST software. (C) Ratios of mean fluorescence intensity (JC-1 orange fluorescence and JC-1 green fluorescence) were calculated to determine changes in ΔΨm. (D) S-100 fractions were isolated and cytochrome c release into the S-100 fractions for each condition was assessed by Western blot analysis. Asterisks indicate a significant difference (*$p<0.05$, ***$p<0.01$) from As_2O_3-treated cells. Number signs indicate a significant difference ($p<0.001$) from controls.

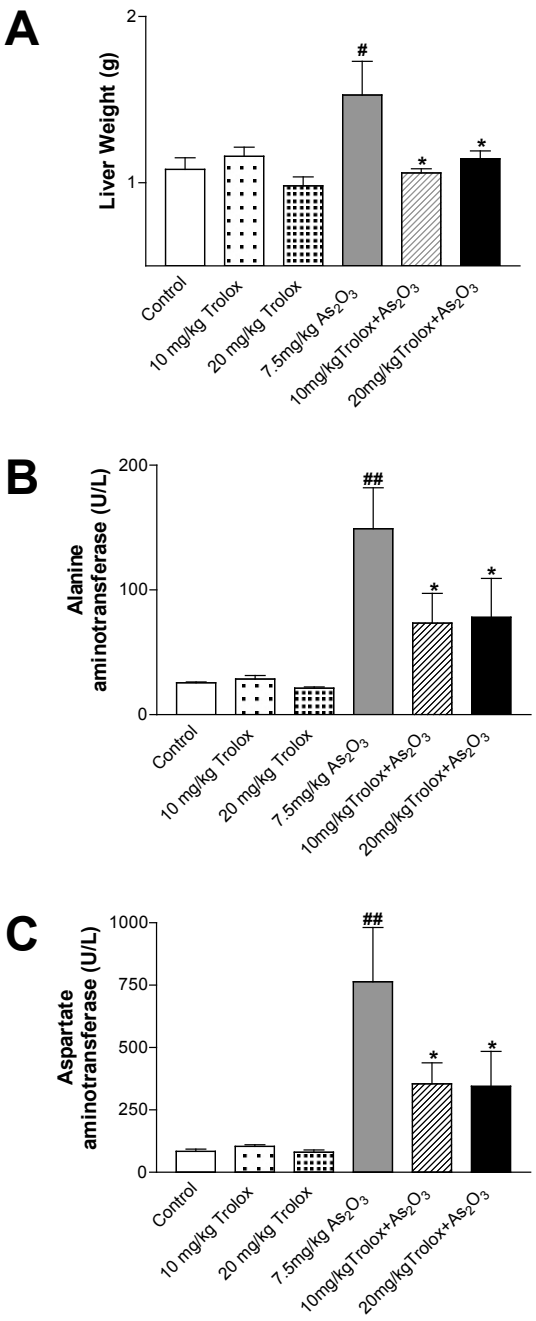

Figure 3.2: Trolox decreases As_2O_3-mediated liver toxicity *in vivo*.

Animals were treated as indicated in Materials and Methods section. One day after the last dose of arsenic, animals were sacrificed and the livers were weighed (A). Blood was collected by cardiac puncture. Serum activities of alanine aminotransferase (B) and aspartate aminotransferase (C) were assayed using commercially available kits in all the animals. Asterisks indicate a significant difference ($p<0.05$) from As_2O_3-treated group. Number signs indicate a significant difference ($^{\#}p<0.05$, $^{\#\#}p<0.01$) from control group.

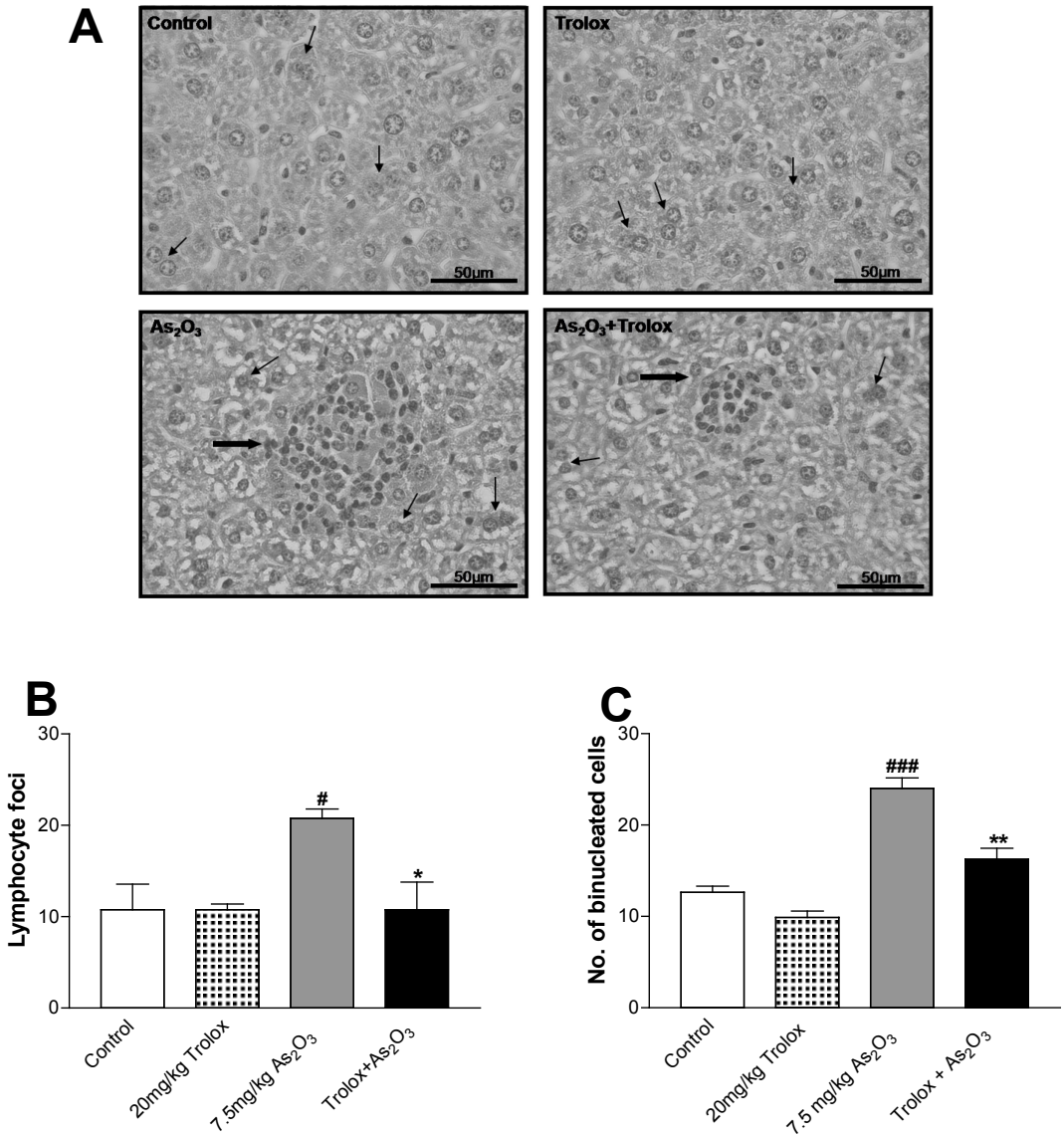

Figure 3.3: Trolox modulates As_2O_3 effects on liver morphology.

(A) Photomicrographs (40x) of the liver samples from animals treated with saline solution (Control), 50mg/kg trolox (Trolox), 7.5mg/kg As_2O_3 (As_2O_3), and the combination of As_2O_3 and trolox (As_2O_3+Trolox). Inflammatory infiltrates composed of fibrinous exudates and polymorphonuclear leukocytes are depicted using big arrows. Representative examples of binucleated cells, an indication of hepatocellular regeneration following a toxic treatment, are shown using small arrows. Quantification of lymphocyte foci (B) and binucleated cells (C) are also shown. Asterisks indicate significant differences (*$p<0.05$, **$p<0.01$) from As_2O_3-treated group. Number signs indicate significant differences ($^{\#}p<0.05$, $^{\#\#\#}p<0.001$) from control group.

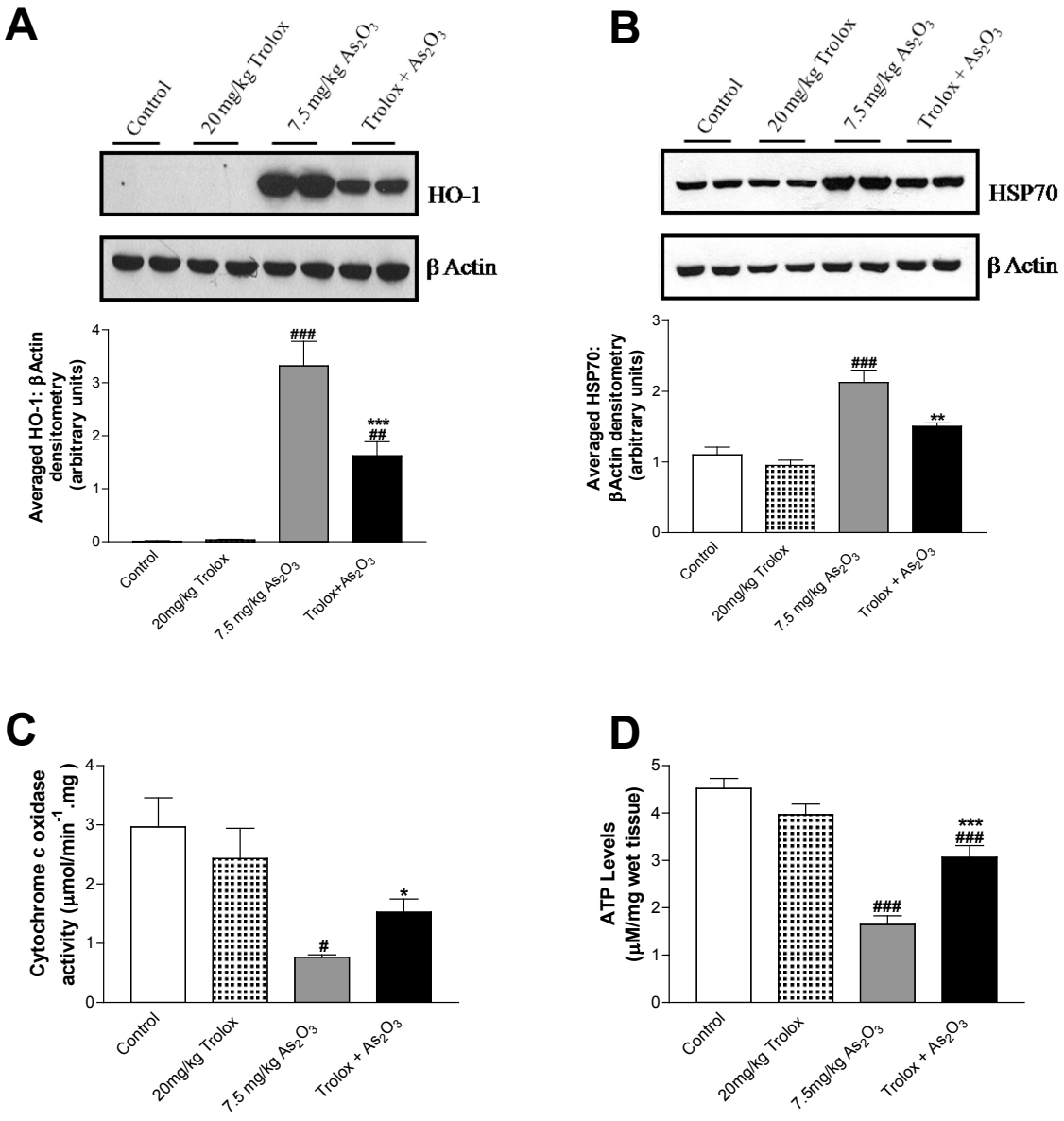

Figure 3.4: Trolox protects mice against As_2O_3-mediated oxidative stress and blocks As_2O_3-mediated decrease in hepatic metabolic rate.

Animals were treated as indicated in Materials and Methods section. Western blotting was performed to determine total cellular HO-1 (A) and HSP-70 (B) protein levels with β-actin as loading control in liver samples from all the animals. Densitometric analyses of blots from 6 animals were performed. (C) Mitochondria from liver were extracted and cytochrome c oxidase activity was assayed. (D). ATP concentrations in hepatocytes were measured with a luminescent ATP detection kit. Asterisks indicate significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$) from As_2O_3-treated group. Number signs indicate significant differences ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$) from control group.

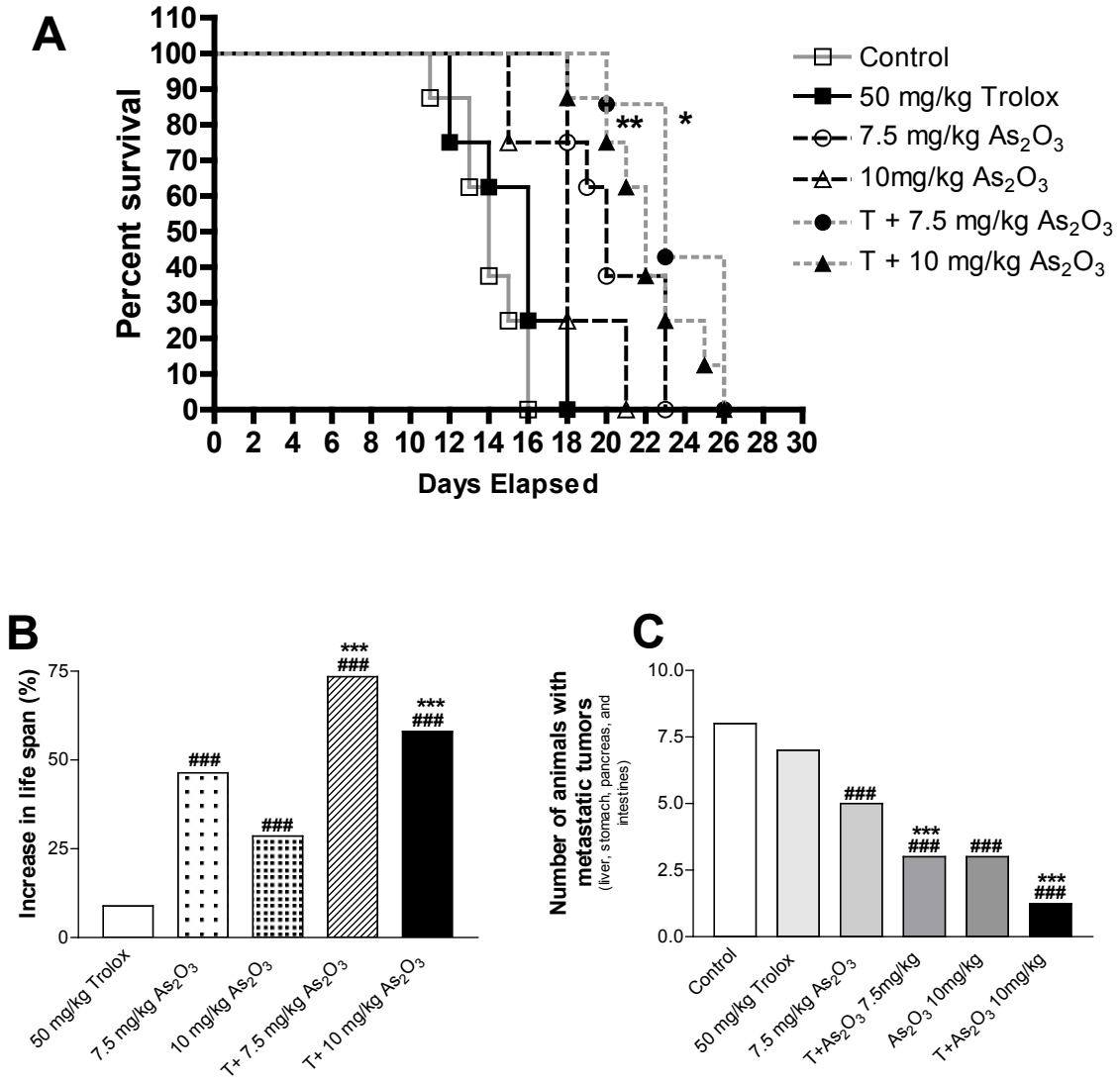

Figure 3.5: Trolox increases As_2O_3 antitumor effects in BDF_1 mice.

Animals bearing P388 lymphoma cells were treated as indicated in Materials and Methods section. Animal deaths were tabulated and Kaplan-Meyer curves were generated to depict percent survival (A). Increase in life span of treated animals relative to controls was calculated (B). Macroscopically visible lesions were counted in liver, stomach, pancreas and intestine in all the animals. Asterisks indicate significant differences from As_2O_3-treated groups (*$p<0.05$; **$p<0.01$, ***$p<0.001$). Number signs indicate significant differences ($p<0.001$) from controls.

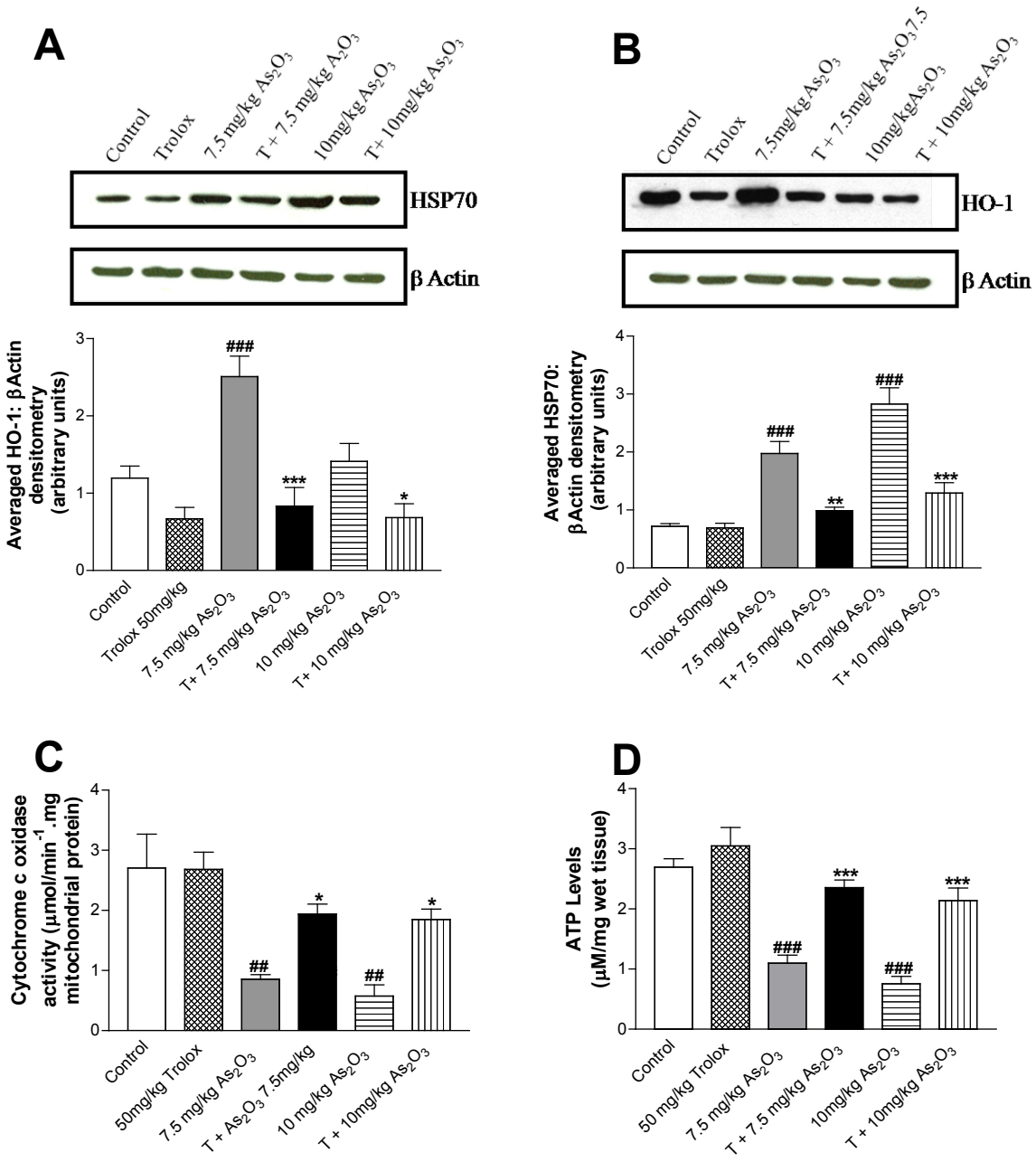

Figure 3.6: Trolox protects tumor-bearing mice against As_2O_3-mediated toxicity.

Animals were treated as indicated in Materials and Methods section. Western blotting was performed in liver samples from all the animals to determine HO-1 (A) and HSP-70 (B) protein levels with β-actin as loading control. Densitometric analyses of blots were performed using eight animals per group. (C) Mitochondria from liver were extracted and cytochrome c oxidase activity was assayed. (D). ATP concentrations in hepatocytes were measured with a luminescent ATP detection kit. Asterisks indicate significant differences (*$p<0.05$, ***$p<0.001$) from As_2O_3-treated group. Number signs indicate significant differences ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$) from control group.

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CHAPTER 4

Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by As_2O_3 and trolox.

(This chapter contains original, unpublished data)

4.1 Preface

Following the characterization of the *in vitro* and *in vivo* effects of As_2O_3 and trolox combination therapy, we decided to extend our study and investigate the pro-apoptotic properties of this combination in cancer and normal cells. This chapter describes a model to analyze, under isogenic conditions, the effect of oncogene expression on trolox and As_2O_3 toxicity, and partially explore the cellular redox status induced by the combination of As_2O_3 and trolox in this model.

4.2 Abstract

The understanding of the biological difference between cancer and normal cells constitutes the basis for designing strategies to improve therapeutic selectivity. An effective chemotherapeutic agent should target biological events critical for cancer cells but not essential for normal cells. In the present study we show that the increase in reactive oxygen species characteristic of oncogenically transformed cells can be exploited to selectively kill cancer cells using the combination of As_2O_3 and trolox. Oncogenic transformation of mouse embryonic fibroblasts causes elevated ROS generation and decreased total antioxidant capacity rendering the malignant cells highly sensitive to As_2O_3 and trolox, which causes severe ROS accumulation preferentially in the transformed cells and effectively decreases their redox buffering capacity.

4.3 Introduction

A major drawback to current chemotherapy is the difficulty in selectively targeting tumor cells. An ideal anticancer agent would be toxic to malignant cells while having minimum toxicity in normal cells. However, the vast majority of anticancer agents or combination therapies in clinical use do not account for this therapeutic selectivity. Therefore, strategies to develop novel selective drugs largely rely on our understanding of the biological disparity between cancer and normal cells.

Evidence suggests that one common biochemical change in cancer cells is the increased oxidative stress associated with oncogenic transformation, alterations in metabolic activity, and increased generation of ROS[1,2]. High levels of ROS can cause severe cellular damage, depending on the levels of ROS generated and the duration of the exposure[3]. The dose- and time-dependent effects of ROS and the fact that cancer cells are under elevated intrinsic oxidative stress may provide a unique opportunity to selectively kill malignant cells based on their vulnerability to further ROS insults.

Recently, several compounds have been described to increase ROS or decrease antioxidant buffering capacity of tumor cells, resulting in increased cytotoxicity[4,5]. For example, leukemic cells with high ROS are more sensitive to the SOD inhibitor, 2-methoxyestradiol[6]. Emodin, a natural anthraquinone derivative, which generates ROS, has been shown to enhance the cytotoxicity of As_2O_3 only in malignant cells via increased ROS generation and ROS-mediated inhibition of survival pathways[7]. Furthermore, we have demonstrated that trolox, a hydrophilic vitamin E analogue lacking the phytyl tail, potentiates As_2O_3-induced cell death in a variety of malignant cell lines. Importantly, the same concentrations of As_2O_3 and trolox that effectively kill leukemia cells do not induce significant toxicity in normal lymphocytes[8]. We also showed *in vivo* that trolox enhances As_2O_3 antitumor effects while protecting the tumor-bearing mice from As_2O_3–mediated liver toxicity[8].

Nevertheless, it is still unclear how the differences in cellular redox status can switch trolox properties as an antioxidant in normal cells to a pro-oxidant in cancer cells. Moreover, it is unknown whether the intrinsic oxidative stress in cancer cells lowers the threshold to this ROS-generating combination.

The objective of the current study was to investigate the biochemical basis for the selective anticancer activity of the combination of As_2O_3 and trolox. We demonstrated that oncogenic transformation leads to significantly higher basal levels of intracellular superoxide ($O_2\cdot^-$) and lower total antioxidant capacity. This intrinsic redox imbalance likely renders transformed cells more dependent on antioxidant defenses for survival and contributes to the increased sensitivity of these cells to As_2O_3 and trolox -induced apoptosis.

4.4 Experimental procedures.

4.4.1 Cell lines

MEFs and MEFs transfected with the oncogenes E6 and E7 (MEFs-E6/E7) were obtained from Dr. Al Moustafa and maintained in DMEM. All media were purchased from Life Technologies, Inc and supplemented with 10% fetal bovine calf serum (FBS). Cells were grown in a humidified chamber at 37^{o} C with a 5% CO_2 environment.

4.4.2 Growth Assays

MEFs and MEFs-E6/E7 were seeded in 24-well plates at a density of 4000 cells/well. The next day, fresh media was added and cells were treated with various concentrations of As_2O_3 alone or in combination with 100μM trolox for six days. Viable cells were counted by trypan blue exclusion on day 1, 3 and 6. Media +/- treatment was replaced every third day.

4.4.3 Propidium Iodide Staining

Quantitation of apoptotic cells was performed as previously described[8]. Cells were seeded in 6-well plates at a density of 1x 10^5 cells/well. The next day, fresh media was added and cells were treated with various concentrations of As_2O_3 alone or in combination with 100μM trolox for 48 hours. Cells were trypsinized, washed in buffer (PBS/ 5% FBS/ 0.01 M NaN_3) at 4^{o} C, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was measured on a Becton-Dickinson FACS Calibur. Cells undergoing DNA fragmentation and apoptosis (those in which PI fluorescence was weaker than the typical G_0-G_1 cell cycle peak) were quantified using CellQUEST software.

4.4.4 Western Blotting

Cell were scraped, washed with cold PBS and resuspended in 0.1 ml lysis buffer (5mM NaH_2PO_4, 1mM DTT, 10% glycerol, 1mM PMSF, 10μg/ml each aprotinin and leupeptin, pH 7.4) at 4°C. Extracts were centrifuged at 14,000 rpm at 4°C, and supernatants were transferred to fresh tubes. Protein concentration was determined with

the Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada). For HO-1 detection, 50μg of protein was added to an equal volume of 2x sample buffer and run on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad), stained with Ponceau S in 5% acetic acid to ensure equal protein loading, and blocked with 5% milk in PBS containing 0.5% Triton X-100 for 1 hour at room temperature. The membrane was hybridized overnight at 4°C with a rabbit-derived polyclonal antibody against HO-1 (1:1000; StressGen). Following three washes with PBS and 0.5% Triton X-100, blots were incubated with a goat anti-rabbit antibody (1:10,000; PharMingen) for one hour at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). Immunostaining for β-actin was used to confirm equal protein loading.

4.4.5 Hydroethidine staining

For the detection of superoxide ($O_2 \cdot^-$), cells were seeded at 1x 10^5 cells/mL in 24-well plates and treated for 24 hours with various concentrations of As_2O_3 alone or in combination with 100μM trolox. Cells were trypsinized, washed with PBS, and incubated with 10μM hydroethidine (HEt) for 30 minutes at 37°C. Subsequently, cells were washed in PBS. The fluorescent intensity of HEt was measured with a FACS Calibur and analyzed using CellQUEST software.

4.4.6 Total Antioxidant Capacity

Cells were seeded at 1x 10^5 cells/mL in 24-well plates and treated for 48 hours with various concentrations of As_2O_3 alone or in combination with 100μM trolox. The cellular total antioxidant capacity was measured using an Anti-Oxidant Assay Kit from Cayman Chemical Company following the manufacturer instructions. The assay relies on the inhibition of the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metamyoglobin. The amount of $ABTS^{\bullet+}$ was monitored spectrophotometrically, and the degree of suppression of absorbance caused by the antioxidants was proportional to their concentration, which was expressed as millimolar Trolox equivalents.

4.5 Results

4.5.1 Oncogenic transformation by E6/E7 and E6/E7/ErbB2 results in an imbalance in cellular redox homeostasis.

To understand differences in the response to As_2O_3 and trolox between normal and tumor cells and to test the hypothesis that oncogenic transformation causes increased ROS generation and renders the transformed cells vulnerable to further ROS stress, we used an isogenic model of malignant transformation. Normal murine embryonic fibroblasts (MEFs) were transfected with HPV 16 E6/E7 open reading frames using a recombinant retroviral system (MEFs-E6/E7). MEFs are not tumorigenic when inoculated in nude mice, while MEFs-E6/E7 formed tumors ($<0.5cm^3$) within 10 days and exhibited histopathology characteristic of carcinoma[9]. As shown in Figure 4.1A, the oncogenically transformed cells MEFs-E6/E7 have higher proliferation rates than the non-tumorigenic parental cell line, confirming the contribution of oncoproteins to cellular proliferation[10-12]. To analyze whether oncogenic transformation leads to an increase in basal levels of ROS, we monitored $O_2\cdot^-$ generation by hydroethidine staining. MEFs-E6/E7 cells exhibited a significant increase in basal ROS content compared to the parental MEFs (Figure 4.1B). The cellular defense mechanisms against ROS include redox buffering systems and various antioxidant enzymes. Because the intracellular levels of ROS are dependent on the balance between ROS generation and elimination, a decrease in the expression or the activity of antioxidant enzymes and/or redox buffering compounds may also cause ROS accumulation. To test this hypothesis, we measured the basal total antioxidant capacity in both cell lines. As shown in Figure 4.1C, MEFs-E6/E7 cells exhibited a significant decrease in the total antioxidant capacity compared to the parental MEFs.

4.5.2 Increased ROS generation and decreased total antioxidant capacity render the oncogenically transformed cells highly sensitive to As_2O_3.

We analyzed the cellular response to As_2O_3, a known ROS-inducing agent, in MEFs and MEFs-E6/E7 cell lines. Cells were treated with 5μM As_2O_3 for 48 hours and $O_2\cdot^-$ generation was measured by hydroethidine staining. Figure 4.2 shows that As_2O_3 caused a substantial increase in intracellular $O_2\cdot^-$ levels in MEFs-E6/E7 compared to

controls (22.8% vs 4.42%). In contrast, the parental cells were less sensitive to As_2O_3-mediated ROS generation (5.96% vs 1.80%), possibly due to their low basal ROS levels and higher antioxidant capacity. To determine whether the significant differences between MEFs and MEFs-E6/E7 vis-à-vis ROS accumulation in response to As_2O_3 correlated with the cytotoxic effects of this compound, we treated cells with 5μM As_2O_3 for 48 hours. Flow cytometry analysis of $subG_0$ populations showed that As_2O_3 effectively induced cell death in 26.22% of MEFs-E6/E7. However, MEFs cells were significantly less sensitive to As_2O_3 with only 15% undergoing apoptosis.

4.5.3 Trolox modulates differently As_2O_3 toxicity in normal and oncogenically transformed cells.

We then evaluated the effects of As_2O_3 and trolox, both separately and in combination, on MEFs and MEFs-E6/E7 apoptotic rate. Cells were treated, subsequently stained with PI, and analyzed by flow cytometry. Although 100μM trolox alone had no effect on apoptosis in any of the cell lines, the combination of As_2O_3 and trolox decreased substantially apoptosis in MEFs (15% in As_2O_3 alone vs 7.4% in controls) (Figure 4.3A). In contrast, the addition of trolox to 5μM As_2O_3 increased apoptosis from 20.7% to 60.8% in MEFs-E6/E7 (Figure 4.3B). We used an additional isogenic model to investigate *in vitro* the generality of the disparate responses to trolox plus As_2O_3 in normal compared to tumor cells. NIH-3T3 cells were transfected with the Ras oncogene and both cell lines were treated with As_2O_3 alone, trolox or the combination. We found that combination of As_2O_3 and trolox decreased apoptosis substantially in NIH-3T3 parental cells and increased apoptosis in the NIH-3T3-Ras transformed cells (data not shown). These results suggest that trolox protects wild-type cells from As_2O_3 toxicity, while enhancing transformed cell death induced by As_2O_3.

4.5.4 The modulation of As_2O_3-mediated toxicity involves changes in cellular oxidative stress.

We have previously demonstrated that oxidative stress is one of the mechanisms by which As_2O_3 and trolox initiate the apoptotic process. Because trolox potentiates

As_2O_3-induced apoptosis in transformed cells and protects non-transformed cells from As_2O_3 toxicity, it is possible that redox homeostasis in these cells is modulated differently by the combination treatment. Therefore, we explored whether and the extent to which trolox modulates As_2O_3-mediated generation of ROS in both cell lines. Figure 4.4A shows that in MEFs 5μM As_2O_3 increased the intracellular level of $O_2\cdot^-$ although no significant differences in the generation of this radical were observed at low dose of As_2O_3. Treatment with trolox significantly decreased As_2O_3-mediated $O_2\cdot^-$ production. However, in MEFs-E6/E7 the opposite effect was observed at both doses studied (Figure 4.4B). Trolox enhanced As_2O_3-mediated $O_2\cdot^-$ generation at all concentrations of arsenic tested. Interestingly, trolox alone decreased basal $O_2\cdot^-$ levels in both cell lines.

To further analyze the possibility that trolox modulates As_2O_3-mediated changes in cellular redox status, we measured the basal total antioxidant capacity in both cell lines upon treatment with As_2O_3, trolox or the combination. As shown in Figure 4.4C, As_2O_3 partially depletes cellular total antioxidant capacity in MEFs cells; total antioxidant capacity was partially restored when trolox was added to the As_2O_3-treated cells. The protective effect of trolox was completely inversed in MEFs-E6/E7 where the combination significantly decreased cellular total antioxidant capacity (Figure 4.4D).

In addition, we investigated the modulation of heme oxygenase-1 (HO-1), the rate-limiting enzyme for heme degradation that has been widely recognized as an oxidative stress responsive protein[13]. While the combined treatment markedly decreased As_2O_3-mediated HO-1 induction in wild-type MEFs cells, treatment with As_2O_3 and trolox in MEFs-E6/E7 markedly increased HO-1 protein levels (Figure 4.4E and F). Taken together, these results suggest that trolox differently modulates As_2O_3-mediated oxidative stress in non-tumorigenic cells and oncogenically transformed cells.

4.6 Discussion

The ROS stress in cancer cells is thought to play an important role in maintaining cancer phenotype due to their stimulating effects on cell growth and proliferation[14], genetic instability[15], and senescence evasion[16] and it has long been recognized as an unfavorable event associated with carcinogenesis and cancer progression. However, recent studies indicate that it is possible to use agents that promote cellular ROS accumulation to successfully kill cancer cells *in vitro*[17,18]. Because increased ROS generation is common in cancer cells with active metabolism under the influence of oncogenic signals, we hypothesized that the intrinsic ROS stress associated with oncogenic transformation would render the cells highly dependent on their antioxidant systems to neutralize the damaging effect of ROS and to maintain redox balance in a dynamic state. This situation would render them highly vulnerable to further oxidative insults by exogenous agents. Inhibition of the antioxidant system in cancer cells would lead to a severe accumulation of ROS resulting in cell death. On the other hand, normal cells may better tolerate treatment with ROS-inducing agents due to their low basal ROS output and normal metabolic regulation. Then, the differences in redox states between normal and cancer cells may provide a biological basis for selective killing of malignant cells, using agents that cause further ROS stress.

Employing an isogenic model of malignant transformation, here we demonstrated that oncogenically transformed cells are more sensitive to ROS-mediated cell death, and that As_2O_3 and trolox can be a selective combination therapy based on this biochemical platform. Several lines of evidence support this conclusion. (1) Cells transformed with E6/E7 exhibited a significant increase of $O_2\cdot^-$ compared to their parental cells. (2) This intrinsic oxidative stress rendered them highly dependent on the total antioxidant capacity to maintain redox balance. (3) The severe accumulation of $O_2\cdot^-$ and depletion of cellular buffering capacity in transformed cells caused increased HO-1 activation and massive cell death. In contrast, As_2O_3 and trolox caused only a modest increase of $O_2\cdot^-$ insufficient to cause significant cell death in non-transformed cells. Based on these findings, it is possible that the therapeutic selectivity of As_2O_3 and trolox is dependent on the biological difference in redox regulation between the oncogenically transformed cells and normal cells.

Growing evidence suggests that cancer cells produce high levels of ROS and are constantly under oxidative stress[1,19-21]. Elevated rates of ROS generation have been detected in seven human cancer cell lines (up to 0.5 nmol/10^4 cells per hour)[20]. In separate studies, quantitation of $O_2\cdot^-$ in primary blood samples from 30 patients with different types of leukemia demonstrated that the generation of $O_2\cdot^-$ was significantly increased compared to normal controls[22]. Furthermore, elevated oxidative modifications in DNA, proteins, and lipids have been detected in various primary cancer tissues including renal cell carcinoma, mammary invasive ductal carcinoma, and colorectal adenocarcinomas[22-27], further suggesting that cancer cells are inherently under oxidative stress. The mechanism responsible for increased ROS production in cancer cells is not clear at the present time. Active metabolism and the deregulation of cell growth likely contribute to the overproduction of ROS in malignant cells; it is known that cancer cells have increased glycolytic activity (Warburg effect) and a lower mitochondrial respiratory efficiency which may result in more free radical production due to a ‘‘leakage’’ of electrons from the respiratory chain[28,29].

As_2O_3 and trolox have been shown to cause various degrees of ROS increase and cytotoxicity in different cell lines. A moderate augmentation was observed in breast cancer cells and higher levels and toxic effects were detected in leukemic cells[8]. This variation may reflect diverse ROS basal levels in different cell lines. In the present study, the degree of ROS accumulation and cell death induced by the combination of As_2O_3 and trolox were dependent on endogenous ROS generation. The high basal ROS in E6/E7-transformed cells seem responsible for their high sensitivity to As_2O_3 and trolox combination. However, we have tested a variety of non-malignant cell lines and the addition of trolox has always shown to decrease As_2O_3-mediated toxicity. In addition, in tumor-bearing BDF_1 mice, treatment with 7.5mg/kg As_2O_3 and 20mg/kg trolox markedly reduced As_2O_3 induced liver toxicity as evidenced by a decrease in As_2O_3-induced hepatomegaly, markers of oxidative stress, and hepatocellular damage[30]. It is still intriguing that in non-transformed cells trolox not only abrogated As_2O_3-mediated toxicity, but also significantly decreased As_2O_3-mediated $O_2\cdot^-$ generation and protected cells from As_2O_3-mediated toxicity. We postulate that due to the higher antioxidant capacity and lower basal ROS levels in these cells, the bioavailabity of trolox as an

antioxidant promotes a depletion of As_2O_3-mediated ROS generated and thus, protects cells from the consequent oxidative damage that could have been promoted (Figure 4.5). We have previously demonstrated using electronic paramagnetic resonance that a single-electron transfer occurs when trolox is added to a reaction mixture containing As_2O_3[8]. As a consequence, trolox phenoxyl radicals are formed in aqueous solution. Therefore, it will be important to determine whether this radical is generated in MEF-E6/E7 and not in MEFs cells and therefore will contribute to the MEF-E6/E7 intracellular ROS pool increasing it to a threshold that will triggers cell death.

In conclusion, our study suggests that the intrinsic oxidative stress in cancer cells associated with oncogenic transformation provides a basis for developing strategies to preferentially kill cancer cells through an ROS-mediated mechanism, and combination therapies such as As_2O_3 and trolox can be used to achieve such activity *in vitro* and *in vivo*. Importantly, cancer cells in advanced disease stage usually exhibit genetic instability and show significant increase in ROS generation due in part to the ‘‘vicious cycle’’ in which ROS induce mutations leading to further metabolic malfunction and more ROS generation[3]. Such highly malignant cells are often resistant to conventional anticancer drugs. However, because these cells are under intrinsic ROS stress, using As_2O_3 and trolox to preferentially kill such malignant cells, warrants further testing in preclinical and clinical settings.

4.7 Acknowledgements

We thank Sophie Marcoux and Felicia Villasenor for technical help and Dr. Ala-Eddin Al Moustafa for helpful discussions.

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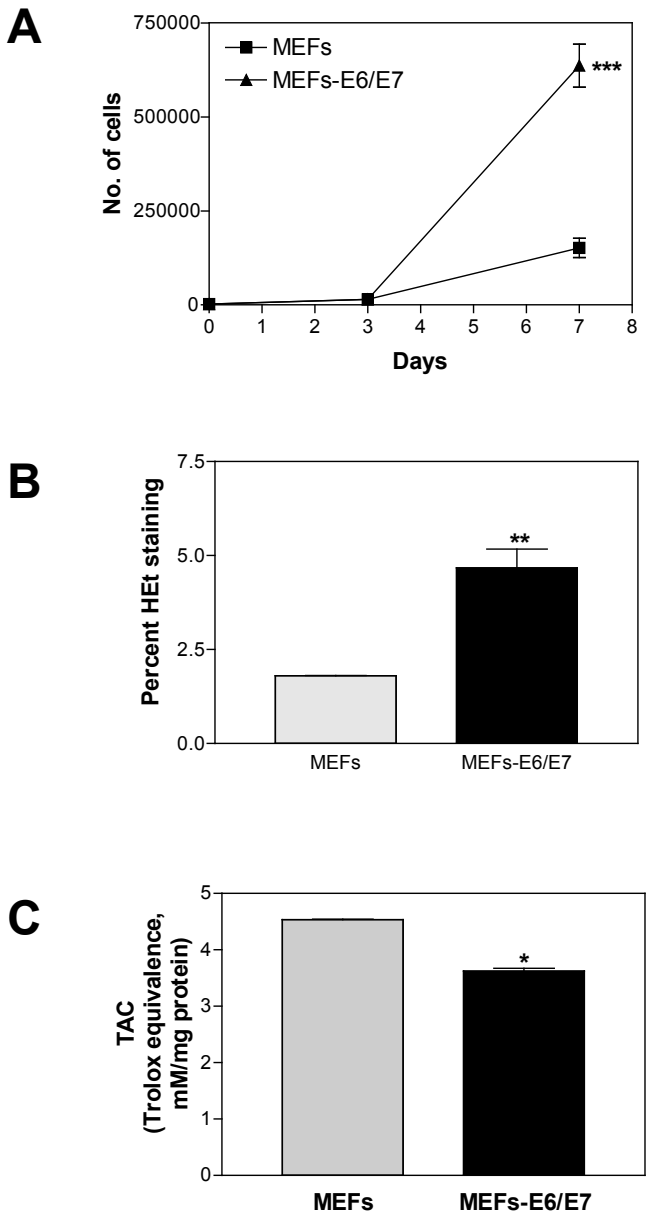

Figure 4.1: Oncogenic transformation by E6/E7 promotes cellular proliferation, increased ROS generation and decreased total antioxidant capacity.

(A) MEFs and MEFs-E6/E7 were seeded and cell viability was evaluated using trypan blue exclusion after 1, 3 and 6 days. (B) Cells were stained with HEt for detection of basal $O_2^{\cdot -}$ levels using flow cytometry. (C) Cellular total antioxidant capacity was measured using an Anti-Oxidant Assay Kit from Cayman Chemical Company. Experiments were performed in triplicates (n=3) and repeated three times. Asterisks indicate significant differences from MEFs cells (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

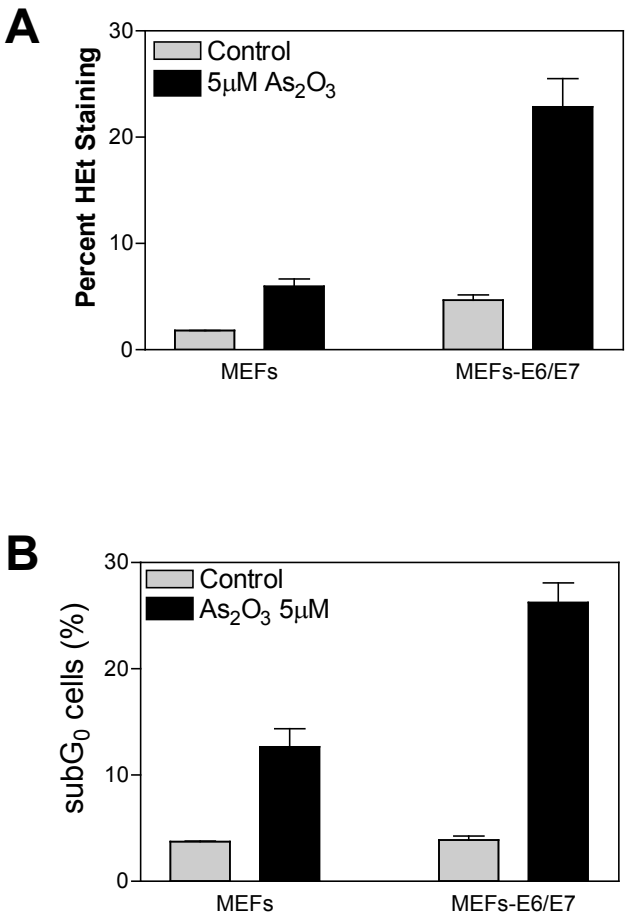

Figure 4.2: Oncogenically transformed cells are more sensitive to As_2O_3.

(A) MEFs and MEFs-E6/E7 were seeded and treated with 5μM As_2O_3. Apoptosis was detected by PI-staining. Quantitation of PI-positive cells in a hypotonic fluorochrome solution was performed after 48 hours of treatment. (B) Cells were treated with 5μM As_2O_3 and stained with HEt for detection of $O_2^{\cdot -}$ levels through flow cytometry. Experiments were performed in triplicates (n=3) and repeated three times with similar outcomes. Asterisks indicate significant differences from MEFs cells (*** $p<0.001$).

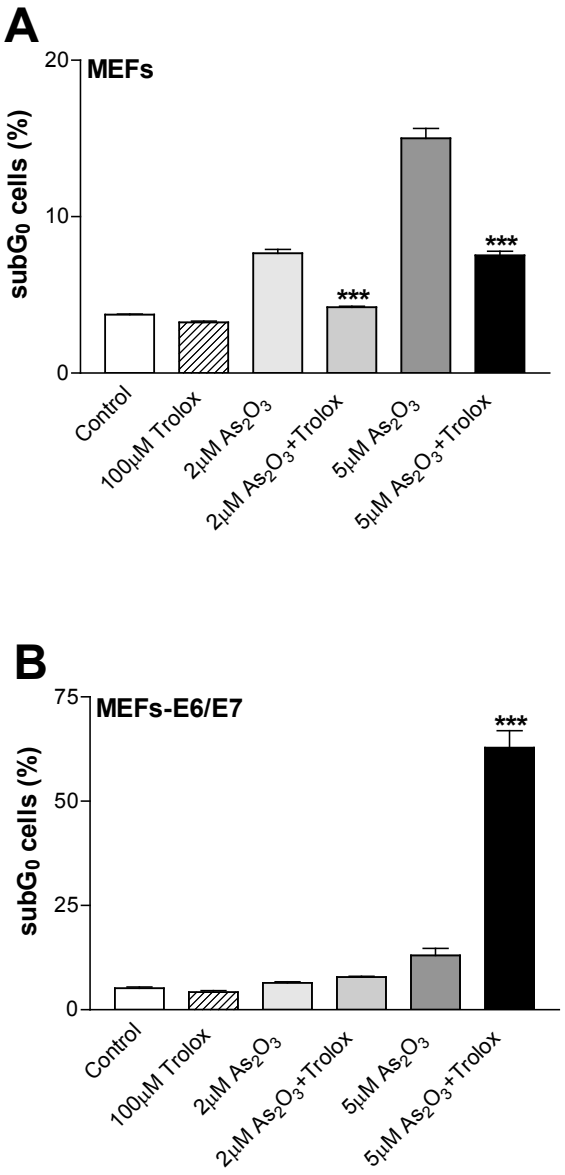

Figure 4.3: Trolox differently modulates As_2O_3–mediated toxicity in MEFs compared to MEFs-E6/E7.

(A) MEFs and (B) MEFs-E6/E7 were seeded and treated with 2 and 5μM As_2O_3. Apoptosis was detected by PI-staining. Quantitation of PI-positive cells in a hypotonic fluorochrome solution was performed after 48 hours of treatment. Experiments were performed in triplicates (n=3) and repeated three times with similar outcomes. Asterisks indicate significant differences from As_2O_3-treated cells (*** $p<0.001$).

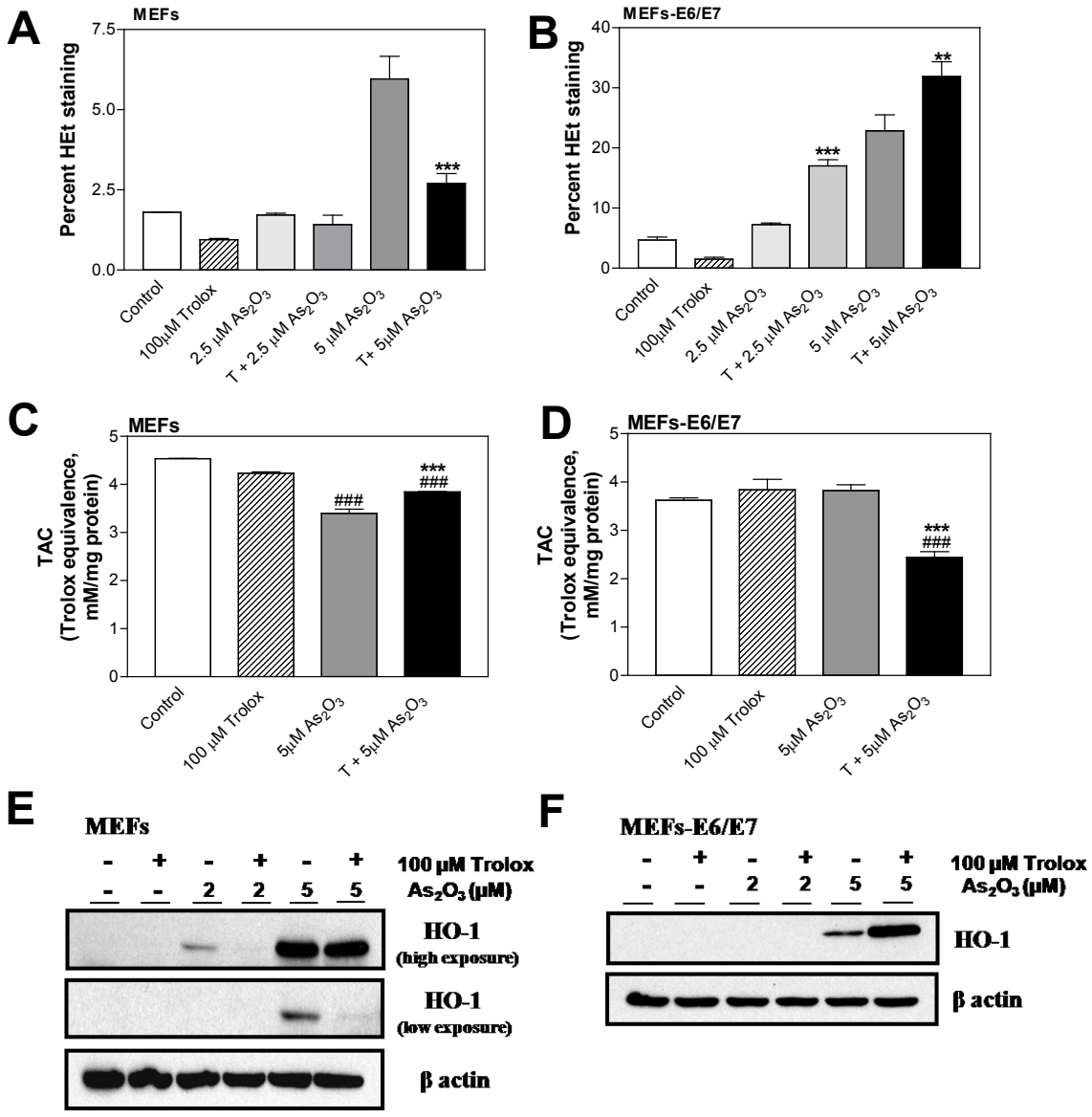

Figure 4.4: The modulation of As_2O_3–mediated toxicity involves changes in cellular oxidative stress. (A) Cells were seeded and treated with 2 and 5μM As_2O_3 and 100μM trolox for 48 hours. Cells were stained with HEt for detection of $O_2^{\cdot -}$ levels through flow cytometry. (B) Cellular total antioxidant capacity was measured using an Anti-Oxidant Assay Kit from Cayman Chemical Company. (C) Western blotting was performed to detect HO-1 protein levels after 24 hours of treatment. β-actin was used as a loading control. Experiments were performed in triplicates (n=3) and repeated three times with similar outcomes. Asterisks indicate significant differences from As_2O_3-treated cells (** $p<0.01$; *** $p<0.001$) or control cells ($^{###}$ $p<0.001$).

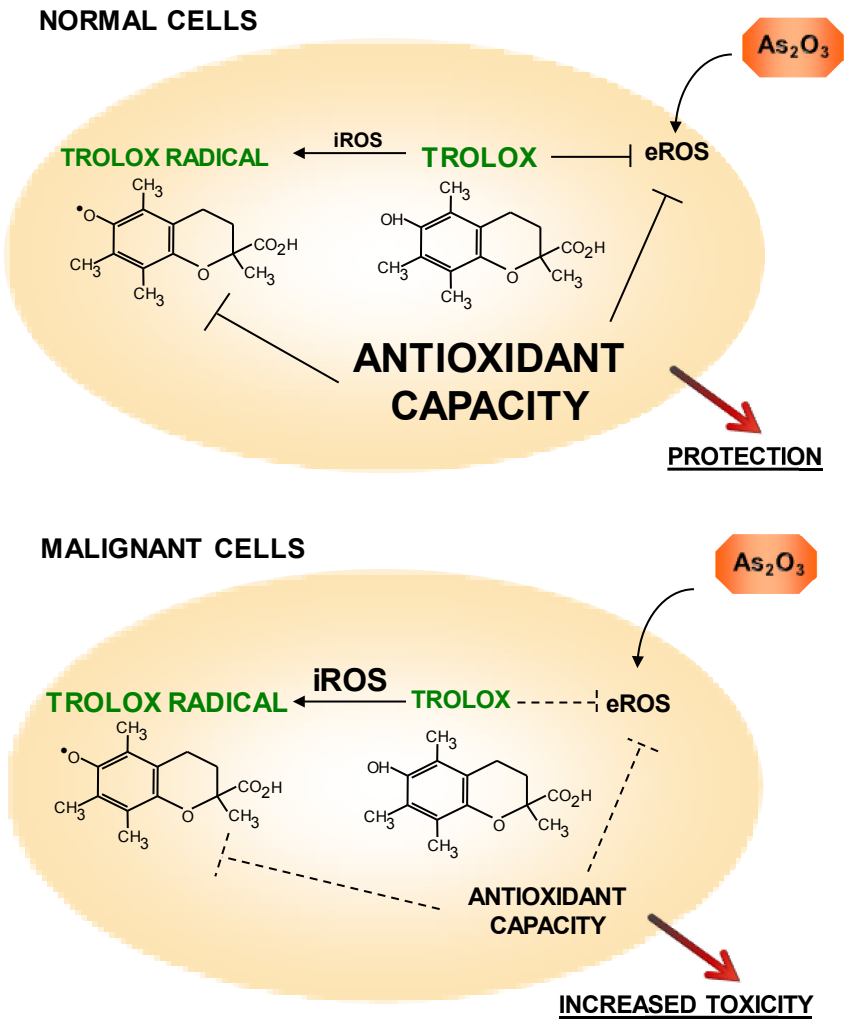

Figure 4.5: Intrinsic oxidative stress renders the malignant cells more sensitive to ROS-inducing combination therapies.

Cancer cells, compared to normal cells, are under increased oxidative stress (high iROS levels) associated with oncogenic transformation probably due to a decrease in antioxidant defenses which render them vulnerable to ROS-inducing chemotherapeutic drugs. Based on this biochemical characteristic, the combination of As_2O_3 and trolox may selectively kills cancer cells while protecting normal cells.

4.8 References

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CHAPTER 5

General Discussion

Arsenic trioxide has considerable efficacy in the treatment of acute promyelocytic leukemia, inducing high a rate of complete remissions (87%) in patients with the disease. Although initial studies focused on As_2O_3-mediated partial differentiation of malignant promyelocytes through PML-RARα degradation, subsequent investigations have revealed that As_2O_3 acts on numerous intracellular targets. As_2O_3 largely affects signal transduction pathways and induces apoptosis through the accumulation of free radicals and consequent induction of oxidative stress. Indeed, intracellular oxidative status has been shown to be important for As_2O_3 sensitivity.

Recent studies have shown that hematologic cancers other than APL and solid tumors derived from several tissue types may be responsive to As_2O_3. However, these cancer cells have higher redox buffering capacity which make them more competent to scavenge ROS generated by As_2O_3 abolishing its apoptosis-inducing ability. Therefore, therapeutic index is a key issue that limits the use of As_2O_3 and does not allow its potential in cancer therapy to be fully realized. Another issue that needs to be considered is selectivity, as As_2O_3 have been also shown to induce normal cell death and toxic side effects. Therefore, the manipulation of the biochemical environment, specifically in malignant cells, might allow for the design of strategies to increase the therapeutic effects of As_2O_3 using clinically achievable doses while reducing its toxicity. While the challenge of attaining specificity relies on gaining insights into the biological differences between normal and cancer cells, it is also of relevance to explore the use of drugs that would be expected to synergize with As_2O_3 in malignant cells, based mainly on their molecular mechanisms.

Herein, it has been shown that trolox can enhance As_2O_3-mediated cytotoxicity in NB4, AsR2, IM9, P388 and in a variety of breast cancer cells lines. We found an increase in intracellular oxidative stress when trolox and As_2O_3 were combined, leading to caspase 3 activation, PARP cleavage, mitochondrial membrane depolarization, cytochrome c release, and apoptosis. Importantly the concentrations used to attain this toxicity were

within the clinically achievable doses, thereby indicating that As_2O_3-related toxic effects would be limited.

Although the mechanism by which trolox enhances As_2O_3-mediated apoptosis in malignant cells remains unknown, we provide evidence that trolox potentiates As_2O_3-induced oxidative stress as indicated by an increase in HO-1 protein levels, JNK activation as well as increased cellular oxidative damage. We also demonstrated, using electronic paramagnetic resonance, that As_2O_3 may bio-activate trolox to a potentially tumoricidal phenoxyl radical (trolox$^{\bullet}$) in solution. These observations suggest that As_2O_3 not only generates ROS, but also induces the formation of trolox$^{\bullet}$, which contributes to the intracellular pool of ROS and consequently enhances cellular oxidative stress. We provided evidence that trolox$^{\bullet}$ plays a role in the augmentation of As_2O_3-induced apoptosis, as the combination of trolox with other chemotherapeutic drugs that also enhance ROS (e.g. doxorubicin, etoposide, AraC) were not synergistic and trolox$^{\bullet}$ was not generated (Figure 2.2 E). Intriguingly, an increase in toxicity when H_2O_2 and trolox were combined was not observed. In fact, trolox protected malignant cells from H_2O_2-mediated toxicity (Figure 5.1). Unfortunately, we did not assess whether addition of trolox to reaction mixtures containing H_2O_2 resulted in the formation of trolox$^{\bullet}$. If this were the case, the effects of trolox on As_2O_3-mediated toxicity would be a result of not only an increase in ROS but also of the presence of trolox$^{\bullet}$, and this hypothesis would be supported by results from Figure 2.6. It is possible that plasma membrane CoQ reductase (PMQR), the enzyme responsible for reducing trolox$^{\bullet}$ to trolox, may be affected by As_2O_3, therefore it will be important to determine the potential role of PMQR in the generation of trolox$^{\bullet}$. It will be also crucial, to elucidate the rates and mechanisms for the reactions between trolox and As_2O_3 and more imperative to evaluate whether trolox$^{\bullet}$ is generated in cells, and specifically in which of the cellular organelles trolox$^{\bullet}$ could be detected.

The possibility that the mitochondria may play an important role in the generation of ROS when NB4 cells are treated with the combination of As_2O_3 and trolox arose from experiments using MitoQ, a mitochondria-targeted antioxidant. MitoQ decreased substantially ROS and apoptotic cell death caused by the combination of As_2O_3 and

trolox. In addition, we examined the response to As_2O_3 and trolox of the human myeloid PLB985 cell line lacking gp91, the catalytic unit of NOX, and demonstrated that NOX activation is not necessary to elicit an apoptotic response. In NB4 cells, NOX inhibitors did not protect against As_2O_3-induced apoptosis (Figure 5.2). Based on this data, it will be important to determine whether the generation of trolox$^{\bullet}$ occurs in the mitochondria and the extent to which MitoQ could decrease trolox$^{\bullet}$ levels.

Other potential mechanism by which trolox potentiates As_2O_3-induced cell death relies on the modulation of arsenic intracellular influx and/or efflux. It has been demonstrated that vitamin E may modulate tumor cell permeability by altering levels of lipid peroxidation in surface membranes of malignant cells, raising the possibility that trolox could augment As_2O_3 cellular uptake or efflux[366]. We measured the intracellular levels of inorganic arsenic using atomic absorption spectroscopy and observed that NB4 cells accumulate substantially more inorganic arsenic when treated with the combination of As_2O_3 and trolox than when treated with As_2O_3 alone (Figure 5.3), suggesting that when trolox is present, As_2O_3 is either more efficiently absorbed or less efficiently effluxed.

In vitro experiments (Chapter 2 and 3) demonstrated that trolox could protect a variety of non-malignant cells from As_2O_3-mediated cytotoxicity and it was predicted that trolox would diminish or overcome the adverse effects associated with As_2O_3 monotherapy *in vivo*. Experiments performed in BDF_1 mice showed that indeed, trolox substantially decreased As_2O_3-associated hepatocellular damage, not only in non-tumor-bearing animals, but also in tumor-bearing animals with altered homeostasis. Based on our *in vitro* results in P388 cells, and the favorable *in vivo* toxicity profile, we chose the peritoneal P388 murine lymphoma model to assess antitumor efficacy of As_2O_3 and trolox *in vivo*. We observed that this combination increased the survival time and limited the metastatic spread in this model and propose that the combined use of As_2O_3 and trolox may increase the therapeutic index of As_2O_3 and possibly decrease the development of secondary tumors in patients with advanced lymphoma and other hematological malignancies. Although these results were dramatic and encouraging, we could not guarantee that the same efficacy would be obtained in the treatment of solid tumors. In the ascites model of murine lymphoma, both the tumor cells and their

treatment were administered directly into the intraperitoneal cavity and thus, As_2O_3 and trolox absorption through the gastrointestinal tract and metabolism was not considered. The effects of this combination in solid tumors will be related to the resulting As_2O_3 and trolox blood concentrations, which are affected by the absorption, distribution, metabolism and/or excretion of both drugs. Appropriately designed pharmacokinetic and pharmacodynamic studies should be performed to identify possible interaction of the two drugs, effective therapeutic dose, frequency of treatment and, optimal route of administration. As described in Chapter 1, As_2O_3 has a long history as a therapeutic drug, conversely, there are no reports on the administration of trolox in humans. Phase I studies should also be designed to define the route of administration and establish the highest dose of trolox that can be given safely with the fewest serious side effects (maximum tolerated dose). As shown in Figure 5.4, transitions from *in vitro* to preclinical and then to clinical testing for tumor modulation remain difficult and may be followed over long periods of time. Therefore, careful attention should be taken to design (applying correct models to the question), conduct (using multiple models) and interpretation (recognizing limitations and applying stringent criteria to outcomes) of efficacy studies for tumor modulation.

Clinical application of anticancer agents has been often hampered by toxicity against normal cells, and thus cancer-specific action is still one of the major challenges to be addressed. Experimentally, the selective cell death is partly achieved through various approaches. Clinically, however, poor selectivity of anticancer drugs can cause damage to normal cells, resulting in severe side effects, and thus, limit their clinical efficacy. Considering the opposite effects of the As_2O_3 and trolox combination, i.e., a cooperative action of As_2O_3 and trolox against lymphoma growth and metastases with the concomitant protection by trolox of normal cells *in vivo*, we propose that this combination would be used to treat advanced cancers with fewer side effects.

Our *in vivo* data suggest that trolox may behave as a prooxidant in cancer cells exposed to As_2O_3, while having antioxidant properties in normal cells. Using isogenic models of malignant transformation (Chapter 4), we show that the differences in cellular redox status modulate trolox properties. We demonstrated that oncogenic transformation leads to significantly higher basal levels of intracellular $O_2^{\cdot-}$ and lower total antioxidant

capacity. This intrinsic redox unbalance renders transformed cells more dependent on antioxidant defenses for survival and contributes to the increased sensitivity of these cells to As_2O_3 and trolox -induced apoptosis. It will be the goal of future research to utilize our understanding of the modulation of trolox redox properties to increase As_2O_3 antitumor capacity while limiting its toxicity in preclinical and clinical settings.

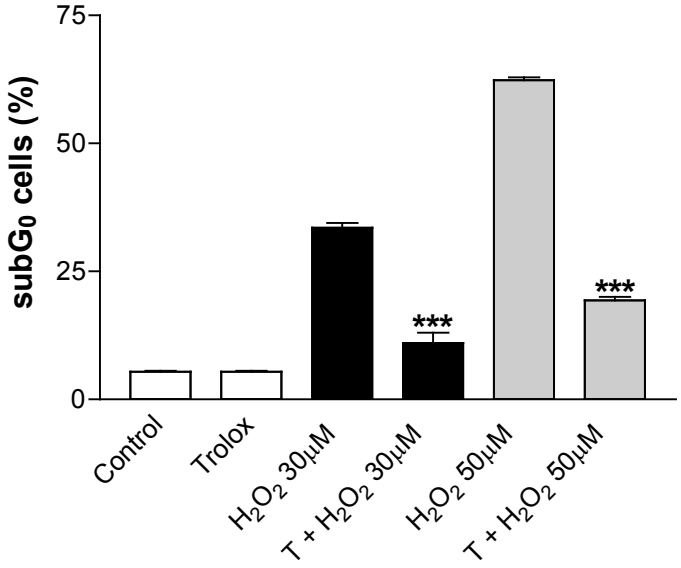


Figure 5.1: Trolox protects NB4 cells from H_2O_2-mediated apoptosis.

NB4 cells were treated with H_2O_2 alone or in combination with 100μM trolox for 48 hours. Apoptosis was detected by propidium iodide and flow cytometry. n=3 Asterisks indicate significant differences from H_2O_2-treated cells (***$p<0.001$).

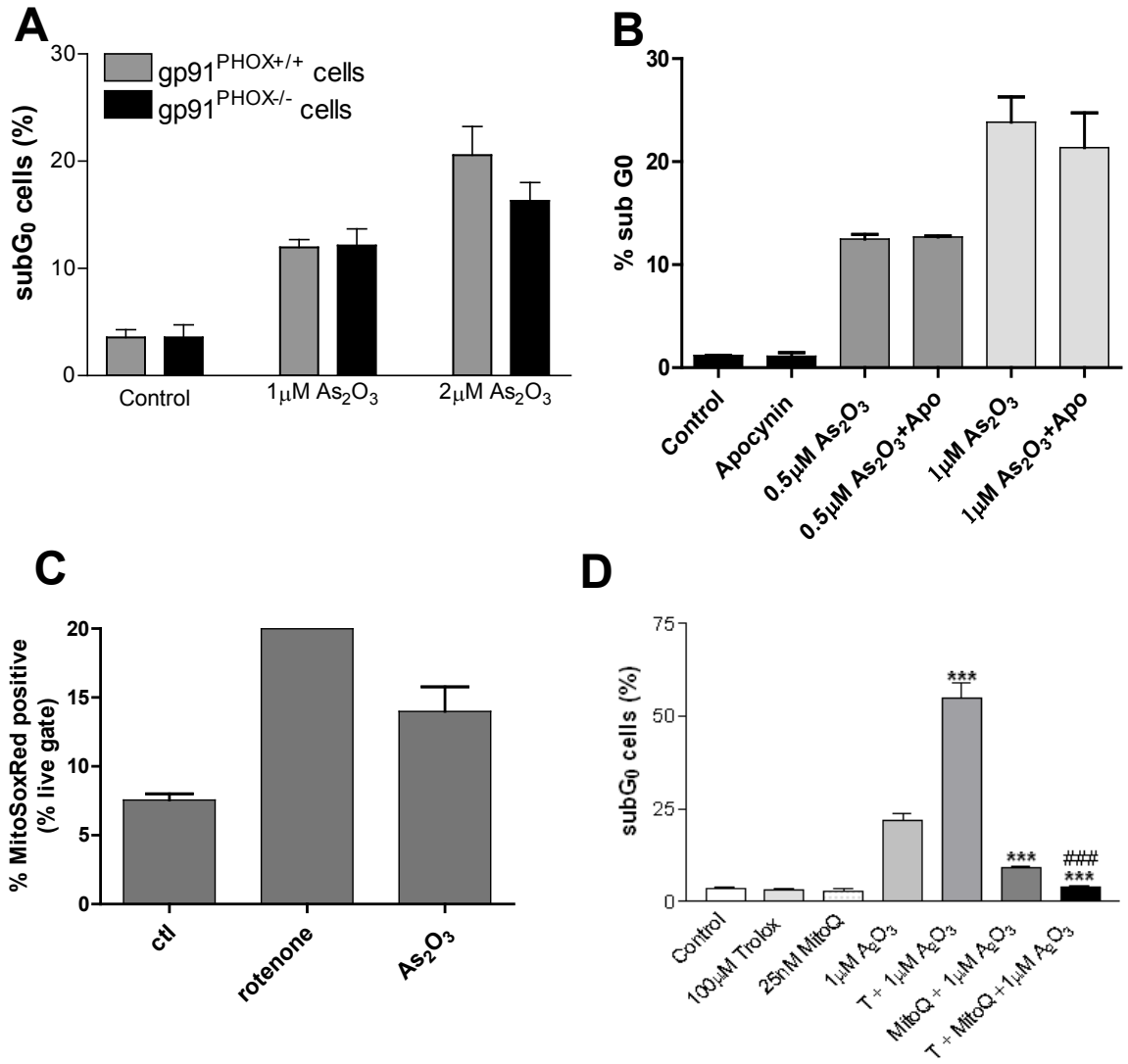


Figure 5.2: Mitochondrial antioxidants, but not NOX inhibitors protect against As_2O_3-induced apoptosis.

A) PLB985 $gp91^{+/+}$ and $gp91^{-/-}$ were seeded and treated with As_2O_3 or/and trolox for 48 hours. Apoptosis was detected by propidium iodide and flow cytometry. n=3 Asterisks indicate significant differences from As_2O_3-treated cells (**p<0.01; ***p<0.001). (B,C) NB4 cells were pretreated with B) the NOX inhibitor apocynin (60 μM) or C) the mitochondrial antioxidant MitoQ for 48 hours and then treated with As_2O_3 alone or in combination with trolox. Apoptosis was detected by propidium iodide and flow cytometry. n=3 Asterisks indicate significant differences from As_2O_3-treated cells (**p<0.01; ***p<0.001). D) NB4 cells were treated for 6 hours with 1.5 μM rotenone or 1 μM As_2O_3 and stained with MitoSoxRed. Staining was detected by flow cytometry and is expressed as percent staining above autofluorescence.

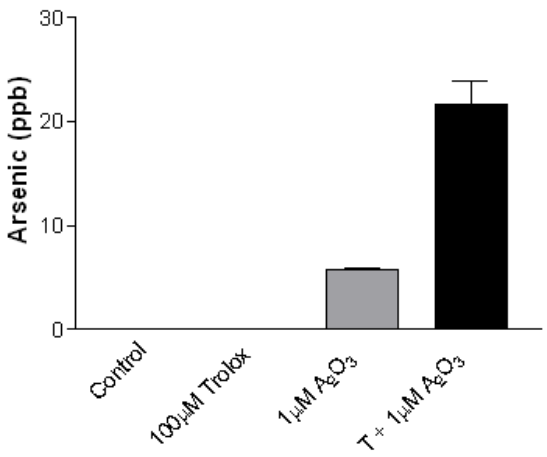
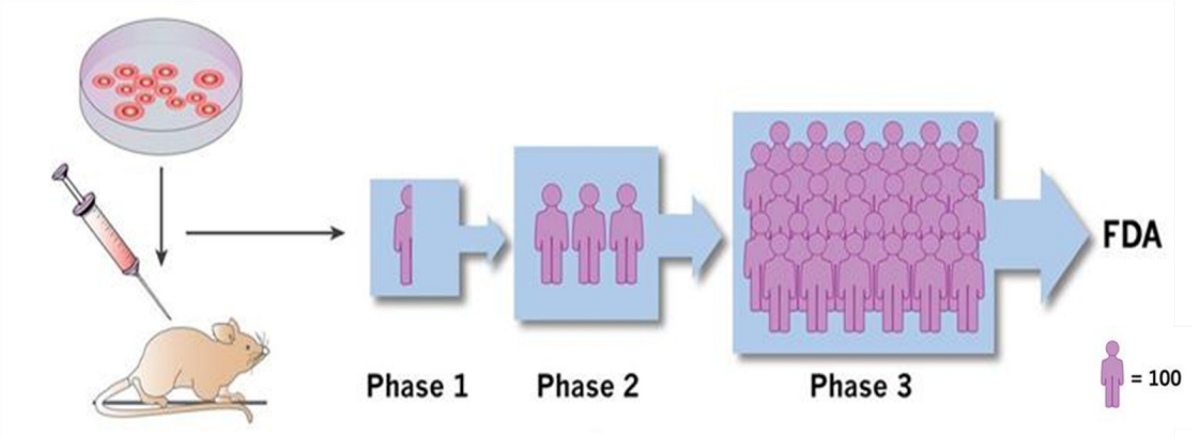


Figure 5.3: Trolox increases intracellular arsenic accumulation in NB4 cells.

NB4 cells were seeded and treated for 24 hours. Analyses of arsenic levels were performed by flow injection hydride analysis followed by atomic absorption spectroscopy. Data are presented as parts per billion (ppb) detected by sample. Asterisks indicate significant differences from As_2O_3-treated cells (*** $p<0.001$).



	Phase of Clinical Trial		
Characteristic	**Phase I (Dose Finding)**	**Phase II (Activity/Efficacy)**	**Phase III (Comparative)**
Primary Goals	• Determine MTD • Define DLT • Elucidate parameters of toxicity	• Determine activity/efficacy in defined populations • Inform the decision to move to a Phase III trial	• Compare a new drug or combination to therapy currently regarded as standard of care
Secondary Goals	• PK/PD issues • Scheduling issues • Target modulation effects • Preliminary efficacy data	• Estimate therapeutic index • Expand toxicity data • Evaluate additional dosing groups • Expand target modulation data • Quality of life measures	• Quality of life comparisons • Comparative costs

MTD, maximally tolerated dose; DLT, dose limiting toxicity; PK/PD, pharmacokinetic/pharmacodynamic.

Figure 5.4: Clinical trial phases in oncologic drug development

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The work presented in this thesis has addressed the effects of As_2O_3 and trolox in cancer and non-tumorigenic cells. *In vitro* as well as *in vivo* studies were conducted to identify possible mechanism of action. These studies have been published in peer-reviewed journals and have been presented in several international meetings. The main contribution of this work to original knowledge is as follows:

1. The i*n vitro* studies showing that trolox increases As_2O_3-mediated cytotoxicity in different malignant cell lines.

2. The identification, using electronic paramagnetic resonance, of trolox phenoxyl radical in As_2O_3-containing solutions.

3. The finding that the combination of As_2O_3 and trolox shows potential specificity for tumor cells.

4. The *in vivo* studies which evidence protective effects of trolox on As_2O_3-induced toxicity.

5. The *in vivo* studies in tumor-bearing mice, showing an increase in life span and inhibition of metastatic spread after As_2O_3 and trolox treatment.

6. The finding that the increase in ROS characteristic of oncogenically transformed cells can be exploited to selectively kill cancer cells using the combination of As_2O_3 and trolox.

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