The Role of ASK1 in Arsenic Trioxide-induced Cell Death

Stanley Kwan

Experimental Medicine

McGill University, Montreal

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ABSTRACT

Arsenic trioxide (ATO) is an effective treatment for acute promyelocytic leukemia (APL). While it is undergoing clinical trials for numerous malignancies including multiple myeloma, myelodysplastic syndrome, lymphoma and solid tumors, it has demonstrated only limited efficacy as a single agent. However, it may hold promise as part of a combination therapy. Thus investigation to elucidate the mechanisms of action underlying these clinical responses may lead to generation of rational combination therapies to increase its therapeutic spectrum. Previous work has described a pathway required for ATO-induced apoptosis in APL cells involving the generation of reactive oxygen species (ROS), and the subsequent induction of a specific mitogen-activated protein kinase (MAPK) cascade that includes both stress-activated protein kinase (SAPK)/ERK kinase 1 (SEK1) and c-Jun N-terminal kinases (JNK) activation. However, the link between ROS production and activation of SEK1 remains to be elucidated. Apoptosis signaling kinase 1 (ASK1) is a MAP3K upstream of SEK1 that has been implicated in the induction of stress-induced signaling.

Using murine embryonic fibroblasts (MEFs) derived from ASK1−/− mice; we provide evidence that ASK1 is a strong mediator for ATO-induced apoptosis and JNK activation. In an APL cell line, we show that ATO activates ASK1 in a dose- and time-dependent manner. However, knockdown of ASK1 in APL cells enhanced susceptibility to ATO, undergoing apoptosis and growth inhibition more than their wild type counterparts. The same impact was observed in the knockdown of SEK1, a direct downstream MAP2K of ASK1, and the knockdown of ASK1 in MCF-7 cells, a human breast cancer cell line. This led us to postulate that ASK1 had a pro-apoptotic function in non-transformed fibroblasts, but was pro-survival in malignant cells. Indeed, transformation of ASK1−/− MEFs restored their sensitivity to ATO-induced apoptosis and growth inhibition. Taken together, these results suggest that ASK1 can have both pro-apoptotic and anti-apoptotic roles depending on the transformation state of the cells.
One model of ASK1 regulation suggests that ASK1 is kept in an inactive form by reduced thioredoxin-1 (Trx1). During oxidative stress, Trx1 is oxidized and releases ASK1 for activation. Immunoprecipitation of ASK1 followed by immunoblotting for Trx1 in APL cells shows a strong basal association that is lost with ATO treatment. Furthermore, the activity of thioredoxin reductase 1 (TrxR1), an enzyme that converts oxidized Trx1 into reduced Trx1, is significantly decreased following ATO treatment. This suggests that ATO activates ASK1 signaling by ROS-mediated oxidation of Trx1 and by maintaining Trx1 in its oxidized state by decreasing TrxR1 activity. In addition, we show that inhibition of TrxR1 with the TrxR1 inhibitor Auranofin sensitizes APL cells to ATO-induced apoptosis. Overall, our results suggest that targeting Trx1 may enhance ATO-induced apoptosis in a novel combination therapy.
RÉSUMÉ

L’arsenic trioxyde (ATO) est un traitement efficace contre la leucémie promyélocitaine aigüe (APL). Alors qu’il est testé dans le cadre de plusieurs essais cliniques sur différents cancers comme le myélome multiple, le syndrome myélodysplasique, le lymphome et les tumeurs solides, il ne montre qu’une efficacité limitée en tant qu’agent unique. Quoiqu’il en soit, il pourrait être prometteur au sein d’une thérapie combinée. Ainsi, l’étude des mécanismes d’action responsables des bénéfices cliniques apportés par l’arsenic trioxide pourrait mener à la mise au point de nouvelles thérapies combinées afin d’élargir le spectre thérapeutique d’ATO. Des travaux antérieurs ont décri une voix de signalisation, requise pour l’induction de l’apoptose par l’arsenic dans des cellules d’APL, qui implique la génération d’espèces actives de l’oxygène (ROS), ainsi que l’induction subséquente d’une cascade de protéine kinases mitogène-activées (MAPK) spécifique qui inclut à la fois la protéine kinase stress-activée (SAPK)/ERK kinase 1 (SEK1) ainsi que l’activation de la kinase c-jun N-terminal (JNK). Néanmoins, le lien entre la production de ROS et l’activation de SEK1 reste à être élucidé. La kinase de signalisation de l’apoptose (ASK1) est une MAP3K en amont de SEK1 qui a été impliquée dans l’induction de la signalisation induite par le stress.

En utilisant des fibroblastes d’embryons de souris (MEFs) dérivées de souris ASK−/−, nous montrons qu’ASK1 est un médiateur fort de l’apoptose et de l’activation de JNK par ATO. Dans une lignée cellulaire APL, nous montrons qu’ATO active ASK1 en fonction du temps et de la dose. Par contre, une sous-régulation d’ASK1 dans des cellules APL augmente la susceptibilité envers ATO, ce qui se traduit par une diminution de la croissance et une augmentation de l’apoptose par rapport aux cellules APL sauvages. Un impact similaire est observé lors d’une sous-régulation de SEK1, une MAP2K directement en aval d’ASK1, ainsi que lors d’une sous-régulation d’ASK1 dans des cellules MCF-7, des cellules de cancer du sein humain. Cela nous a conduit à postuler qu’ASK1 a une fonction pro-apoptotique dans les fibroblastes non-transformés, mais anti-
apoaptotique dans des cellules malignes. En effet, la transformation des cellules MEFs ASK\(^{-/-}\) a restauré leur sensibilité envers l’arrêt de la croissance et l’apoptose induits par ATO, ce qui souligne les différences entre les cellules normales et les cellules transformées. Dans l’ensemble, ces résultats suggèrent qu’ASK1 a un rôle important dans la sensibilité à l’ATO qui dépend du contexte.

Un des schémas de régulation d’ASK1 suggère qu’ASK1 est séquestré sous une forme inactive par la thioredoxine-1 réduite (Trx1). Durant le stress oxydatif, Trx1 est oxydée et libère ASK1 afin qu’il puisse être activé. L’immuno-précipitation d’ASK1 suivie d’un immuno-marquage for Trx1 dans des cellules APL montre une association basale forte qui est perdue lors du traitement avec ATO. De plus, l’activité de la réductase de la thioredoxine (TrxR1), une enzyme qui convertit Trx1 oxydée en Trx1 réduite, est diminuée de façon significative après traitement avec ATO. Cela suggère qu’ATO active la signalisation d’ASK1 en oxydant Trx1, via les ROS, ainsi qu’en inhibant la réduction de Trx1 en diminuant l’activité de TrxR1. De plus, nous montrons que l’inhibiteur de TrxR1, Auranofin, sensibilise les cellules APL à l’apoptose induite par ATO. Cela suggère que la régulation d’ASK1 dépend du statut redox de Trx1. Dans l’ensemble, nos résultats suggèrent que le fait de cibler Trx1 pourrait augmenter la signalisation d’ASK1 et l’apoptose induite par ATO au sein d’une nouvelle thérapie combinée.
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CONTRIBUTIONS OF AUTHORS

The thesis is composed of 4 chapters prepared in accordance to the guidelines outlined by the department of Graduate and Postdoctoral Studies. The contribution of each author described below.

Chapter 2
The majority of the experiments were completed by Stanley Kwan. Myrian Colombo completed Figures 1B, 1C and 2.

Chapter 3
The majority of the experiments were completed by Stanley Kwan. Nicolas Garnier completed Figure 4.
ABBREVIATIONS

ASK1 = Apoptosis signal-regulating kinase 1
AML = Acute myelogenous leukemia
AMS = 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid
Ang II = Angiotensin II
APL = Acute promyelocytic leukemia
AQP = Aquaporin
ATO = Arsenic trioxide
ASCT = Autologous stem cell transplantation
ATRA = All-trans retinoic acid
ATF-2 = Activating transcription factor-2
ATM = Sodium aurothiomalate
BSO = Buthionine sulfoximine
CML = Chronic myeloid leukemia
CCC = C-terminal coiled-coil
CREB = cAMP response element-binding protein
DAR = S-dimethylarsino-glutathione
DTNB = 5,5’-di-thio-bis-(2-nitrobenzoic acid)
ERK1/2 = Extracellular signal-regulated kinase 1/2
FAB = French-American-British
FKHR = Forkhead transcription factor
GPx = Glutathione peroxidase
GSH = Glutathione (γ-glutamylcysteinylglycine)
HDCT = High-dose chemotherapy
IKK = IKβ kinase
JNK = c-jun N-terminal kinase
MAPK = Mitogen-activated protein kinase
MAPKK = MAPK kinase
MAPKKK = MAPK kinase kinase
MEF = Mouse embryonic fibroblast
MDS = Myelodysplastic syndrome
MM = Multiple myeloma
MRP1/ABCC1 = Multidrug resistance protein 1
NAC = N-acetyl-cysteine
NF-κB = Nuclear factor kappa B
NPM = Nucleophosmin
NuMA = Nuclear matrix
PLZF = Promyelocytic leukemia zinc finger
PML = Promyelocytic leukemia gene
PARP = poly (ADP-ribose) polymerase
PP5 = Protein phosphatase 5
PTPC = Permeability transition pore complex
RARα = Retinoic acid receptor acid receptor α
ROS = Reactive oxygen species
SAPK = Stress-activated protein kinase
SEK1 = Stress-enhanced kinase 1
SOD = Super-oxide dismutase
SRB = Sulforhodamine-B
TNF = Tumor necrosis factor
TRAFs = Tumor necrosis factor-α receptor-associated factors
Trx1 = Thioredoxin 1
TrxR1 = Thioredoxin reductase 1
CHAPTER 1: LITERATURE REVIEW

1. Introduction to Cancer

Cancer defines a broad range of diseases comprised of abnormal cells that divide in a dysregulated fashion and can spread to other tissues through the blood circulation and lymphatic system. It can be categorized broadly into carcinoma, sarcoma, leukemia, lymphoma, myeloma, and central nervous system diseases.\(^1\) The six classical hallmarks of cancer are resisting cell death, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality and inducing angiogenesis.\(^2\)

Cancer is a worldwide problem and is a leading cause of death. In Canada, over 500 Canadians are diagnosed with cancer every day while 200 die from it.\(^3\) The number of individuals diagnosed with cancer continues to rise, with prostate cancer being the most commonly diagnosed cancer in men and breast cancer being the most commonly diagnosed cancer in women. However, the leading cause of cancer death in both men and women is lung cancer.

While only a small proportion of cancers are attributed to genetic defects (5-10%), the majority are greatly influenced by environmental and lifestyle factors.\(^4\) These include and are not limited to age, cigarette smoking, alcohol, sun exposure, environmental pollutants, infections, stress, obesity, physical inactivity, hormones and diet.\(^4,5\) Age is the most important risk factor for developing cancer and will remain a significant risk factor due to the aging population.\(^5\)

1.1. Treatment modalities

Conventional treatment modalities include surgery, radiation, chemotherapy, hormone therapy, and biological therapy.\(^5,6\) These modalities are often used in combination depending on the type and location of the cancer, the extent of cancer spread, the patient’s age and general health, among other factors. In the
case of leukemia, choice of treatment depends mainly on the type of leukemia, age and the presence of leukemic cells in the cerebrospinal fluid and is limited by the unavailability of surgery. Patients with acute leukemia require immediate treatment with the goal of first achieving remission and then preventing relapse with consolidation therapy leading to a cure. On the other hand, patients with chronic leukemia are rarely cured and may require stem cell transplants.

2. Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a clinically distinct subtype of acute myelogenous leukemia (AML) characterized by the accumulation of promyelocytes due to a block in granulocytic differentiation. It is morphologically classified by the French-American-British (FAB) classification as AML-M3. Patients with the disease experience a high tendency for severe bleeding due to fibrinogenopenia and disseminated intravascular coagulation. Some features of APL are illustrated in figure 1.1.

**Figure 1.1:** (Adapted from Liu et al) Features of acute promyelocytic leukemia. (A) Severe bleeding and (B) accumulation of abnormal promyelocytes in the bone marrow of an APL patient.
2.1. Genetic Basis and Pathogenesis

Cytogenetically, APL is characterized most often by a reciprocal translocation between chromosomes 15 and 17 resulting in the fusion of the promyelocytic leukemia (PML) gene and the retinoic acid receptor α (RARα) gene. RARα is a ligand-dependent transcription factor stimulated by all-trans retinoic acid (ATRA). RARα signaling is normally required for the transcription of genes necessary for myeloid differentiation. However, in APL, the resulting aberrant PML/RARα oncoprotein binds to DNA with higher affinity and recruits corepressors and histone deacetylases to silence and block the transcription of target genes necessary in myeloid differentiation. While the majority of APL cases are attributed to the translocation t(15:17), a small subset of APL can be caused by other gene arrangement combinations involving the fusion of the RARα gene to other partners, including promyelocytic leukemia zinc finger (PLZF), nucleophosmin (NPM) and nuclear matrix (NuMA). In all scenarios, RARα signaling prevents normal myeloid development.

2.2. Treatment Options

Until the early 1980s, treatment of APL consisted of cytotoxic chemotherapy treatment, usually with anthracycline in combination with cytosine arabinoside, similar to the treatment of other AML subtypes. However, a study conducted by a Chinese group demonstrated that all-trans retinoic acid (ATRA), a derivative of vitamin A, was effective in APL patients; especially those refractory to conventional treatment. This finding changed the traditional paradigm for cancer treatment from strictly cytotoxic chemotherapy to induction of cellular differentiation as a feasible means of treatment. Since then, the efficacy of ATRA has been validated through numerous studies and it is now used as first line therapy to treat APL patients. Despite the successes of ATRA, relapse and acquired resistance is common in patients, necessitating other therapeutics. The void is filled by arsenic trioxide (ATO), a compound that has shown remarkable
efficacy in treatment of patients with APL, especially those who have relapsed and are refractory to ATRA treatment.\textsuperscript{20} The chemical structures of the ATRA and ATO are illustrated in Figure 1.2.

![Chemical structures of ATRA and Arsenic trioxide](image)

**Figure 1.2 (Adapted from Liu et al)\textsuperscript{19}** Chemical structure of ATRA (left) and arsenic trioxide (right)

3. Arsenic Trioxide as a Therapeutic Agent

3.1. Physical and Chemical properties of Arsenic and Arsenic Compounds

Arsenic is a group V metalloid possessing chemical properties intermediate between metals and non-metals.\textsuperscript{21} It is found naturally in more than 200 different mineral forms and is present in the earth’s crust with an abundance of 5 mg/kg.\textsuperscript{22} Each form has its unique physical, chemical, and toxicological properties.\textsuperscript{23} Anthropogenic sources further increase exposure to arsenic, raising concerns of pollution in soil and groundwater.\textsuperscript{22}

Arsenic can assume several oxidation states but there are two in particular that are biologically important and are associated with its cytotoxic potential: +3 and +5 with the +3 state being more potent.\textsuperscript{21} In addition, it also exists as inorganic and organic forms.\textsuperscript{21} The three major inorganic forms are arsenic trisulfide ($\text{As}_2\text{S}_3$), arsenic disulfide ($\text{As}_2\text{S}_2$) and arsenic trioxide ($\text{As}_2\text{O}_3$) also known as yellow, red and white arsenic respectively (Figure 1.3).\textsuperscript{24}
A) Arsenic Trisulfide
\((\text{As}_2\text{S}_3)\)
Yellow Arsenic
Orpiment\(^{25}\)

B) Arsenic Disulfide
\((\text{As}_2\text{S}_2)\)
Red Arsenic
Realgar\(^{26}\)

C) Arsenic Trioxide
\((\text{As}_2\text{O}_3)\)
White Arsenic
Arsenolite\(^{27}\)

Figure 1.3 Types of inorganic arsenic

3.2. Toxicity of Arsenic Compounds

3.2.1. Environmental Contamination

Arsenic is found naturally in the atmosphere, soils, rocks, and water and is circulated into the environment through natural means and anthropogenic activities.\(^{28}\) It poses a health risk predominantly when present in food and drinking water, where chronic exposure can lead to serious health problems in many parts of the world.\(^{28,29}\) In India and Bangladesh alone, there has been an estimated 60-100 million people who are at risk of disease from drinking arsenic-contaminated water.\(^{22}\) The accumulating reports of the toxicological effects of arsenic in drinking water prompted the World Health Organization to limit the acceptable concentration of arsenic in drinking water to 10 \(\mu g\) per liter as of 1993.\(^{28}\) However, many developing countries still lack the resources to adequately meet these safety guidelines.
3.2.2. Arsenic Metabolism

Arsenic can be absorbed into the body through various means but ingestion and gastrointestinal absorption of inorganic arsenic is the most common path. Ingested arsenic is rapidly absorbed into the blood from the gastrointestinal tract and then subsequently transported into the body cells through aquaporins (AQP).\textsuperscript{23,30} This occurs predominantly in the liver. However, different forms of arsenic distribute differently in the body and have varying toxicities.\textsuperscript{31} The liver is the primary site of arsenic metabolism where arsenic undergoes biotransformation, a series of biochemical reactions that convert a compound into a more water-soluble compound to facilitate excretion.\textsuperscript{32} This process includes many steps including reductions and oxidative methylations to form pentavalent organic metabolites.\textsuperscript{33} The intermediates in the process, particularly the methylated intermediates, are more toxic than inorganic arsenic.\textsuperscript{34} Arsenic is then excreted predominantly in the urine and feces.

3.2.3. Health Risks to Arsenic Exposure

Arsenic has been recognized for centuries as having significant acute toxicity and chronic toxicity following exposure. Acute exposure can cause death and has been used as an intentional poison throughout history as a murder weapon. Chronic toxicity is caused by long term exposure to low doses of arsenic and has stimulated more concern due to the increasing levels of environmental arsenic.\textsuperscript{35} Millions of people suffer from the effects of chronic arsenic poisoning which is now regarded as an international public health problem that needs to be addressed.\textsuperscript{35}

Arsenic toxicity depends on a variety of factors. Soluble inorganic arsenicals are generally more toxic than organic ones;\textsuperscript{35-37} however, there is evidence that methylated organic ATO metabolites are more potent growth inhibitors and apoptotic inducers than inorganic arsenicals.\textsuperscript{38} Our lab has already shown that S-
dimethylarsino-glutathione (DAR), an organic arsenical synthesized by conjugating dimethylarsenic to glutathione, is more potent than ATO in inducing oxidative stress and apoptosis.\textsuperscript{39} Furthermore, the trivalent form of arsenic is more toxic than the pentavalent form due to its ability to strongly bind thiol groups (\textendash\text{SH}) of proteins with a high cysteine content.\textsuperscript{37,40}

Acute exposure to large doses of arsenic can yield symptoms as early as 30 minutes starting with burning lips and dysphagia.\textsuperscript{35} This may be followed by violent vomiting and eventually hematemesis. Gastrointestinal symptoms are also readily present and may lead to electrolyte imbalance, hypertension and hypoxia. Multi-organ failure can ensue, causing death.\textsuperscript{35,36,41}

Chronic exposure to arsenic has been implicated in numerous cancerous conditions, including strong evidence for the development of lung,\textsuperscript{42} bladder\textsuperscript{43} and skin cancer.\textsuperscript{35,36} Epidemiologic studies are underway to provide evidence for the development of other cancers as well. Chronic arsenic exposure has also been linked to skin disease, diabetes mellitus, hypertension, cardiovascular disease, perturbed porphyrin metabolism and irreversible non-cirrhotic portal hypertension among others.\textsuperscript{35,36,41} The symptoms of chronic exposure differ among individuals, population groups and geographical areas.\textsuperscript{44}

3.3. Chronology of Arsenic as a Therapeutic Agent

While widely recognized for its role as a poison, arsenic paradoxically has an equally enriching history as a medicine and has been used for more than 2400 years in this capacity.\textsuperscript{45} The major milestones of arsenic usage are illustrated in Figure 1.4. Hippocrates was thought to have used arsenic-containing minerals realgar and orpiment pastes to treat ulcers. Dioscorides later recognized that ATO could serve as a therapeutic when given at low doses in the treatment of tuberculosis and emphysema. Concurrently, arsenic was used in traditional Asian folk medicines to treat ailments such as malaria and fever.
Arsenic gained popularity in Europe in the 17th century when William Withering advocated for arsenic-based therapies by justifying that medicines are simply poisons but used at low doses. Over a century later, Thomas Fowler created a therapeutic of potassium bicarbonate-based solution of arsenic that was used to treat a wide variety of ailments. Fowler’s solution gained the nickname “the mule” for its strength and efficacy. It was reported to have an anti-leukemic effect but was eventually replaced by radiation therapy. By 1910, Paul Ehrlich was using an organic-based arsenic therapeutic called salversan to treat tuberculosis and syphilis.

With the advent of other medicines including antibiotics, the use of arsenic declined. It made a resurgence through the help of a published report showing good responses in patients with chronic myeloid leukemia (CML) to ATO. However, these patients later developed chronic arsenic poisoning, exhibiting symptoms including skin pigmentation, skin keratosis, liver cirrhosis, polyneuritis and gastrointestinal problems.

The major breakthrough came for arsenic in the 1970s by studies conducted at the Harbin Medical University in China where they carefully monitored cancer patients treated with intravenous infusions of Ailing 1, a crude ATO solution. The treatment showed tremendous efficacy in APL patients and was carefully followed up by clinical trials in Shanghai Second Medical University, Europe and the United States. These studies confirmed ATO as an effective therapeutic in APL relapsed patients, with a disease that is refractory to ATRA treatment.
3.4. ATO in the Treatment of Acute Promyelocytic Leukemia

The successes of ATO in the clinical trials in China, Europe and the United States led to the approval of the drug for treatment of relapsed APL by the U.S. Food and Drug Administration (FDA) in September 2000 under the brand name, Trisenox™. However, it was not certain whether ATO would be equally effective in patients with newly diagnosed APL. A Chinese group from the Shanghai Institute of Hematology studied and compared the responses to ATO in relapsed and newly diagnosed APL patients and observed similar rates of remission in both groups. However, they showed that ATO induced significant
hepatic toxicity in the newly diagnosed APL patients and subsequently recommended ATO for use only in maintenance or consolidation therapy following remission by ATRA. Since then, ATO in newly diagnosed patients has been further studied in combination with standard ATRA/chemotherapy to reinforce the efficacy of the regimen.\textsuperscript{55,56} Ravandi et al. and Shen et al have shown that the combination of ATRA and ATO in newly diagnosed APL patients is an effective and safe substitute for conventional chemotherapy–containing regimens and either ATRA or ATO alone.\textsuperscript{57,58} Furthermore, ATO has been studied as a substitute for induction and consolidation regimen to minimize cytotoxicity.

3.4.1. Clinical Responses to ATO in Acute Promyelocytic Leukemia Patients

Lengfelder et al. recently consolidated the results from all the clinical studies of ATO in treating relapsed APL and newly diagnosed APL.\textsuperscript{56} Overall, 15 studies and 304 patients were included in the evaluation of ATO in patients with relapsed APL after first line ATRA and chemotherapy treatment. The responses to ATO treatment were significantly positive, with 86% of the patients achieving complete remission while only 7% showed an inadequate response and another 7% died from induction therapy. In comparison, a total of 314 of 362 (87%) of the patients from the studies using ATO in newly diagnosed APL patients achieved a complete response with comparable toxicities.

3.4.2. Adverse Effects of ATO treatment

While many anti-cancer drugs and chemotherapies elicit significant side effects, ATO has relatively few significant side effects. Nearly half the APL relapsed patients treated with ATO experienced some form of mild side effects including fatigue, fever, edema, nausea, anorexia, diarrhea, emesis, headache, insomnia,
cough, dyspnea, dermatitis, tachycardia, pain, hypomagnesemia and hyperglycemia.\textsuperscript{20,53,59}

Leukocytosis has been reported in 50\% of the patients, likely due to the differentiation of all the APL blasts at once as a direct effect on the leukemic cells, but could spontaneously resolve without chemotherapy.\textsuperscript{20,53,54} However, in 25-50\% of the cases, it can develop into retinoic acid syndrome with accompanying fever, skin rash, and edema.\textsuperscript{50} These symptoms are usually alleviated by administration of corticosteroids.

The most common serious symptoms occurring in more than 10\% of the patients include abdominal pain, epistaxis, dyspnea, hypoxia, bone pain, thrombocytopenia, neutropenia, hypokalemia and hyperglycemia.\textsuperscript{20,53,59} Other rare side effects include distal muscular dystrophy, cardiac dysrhythmias, prolonged QT intervals, and ventricular premature contractions.\textsuperscript{59,61-63}

3.5. Use of ATO in Other Malignancies

The relatively rare appearance of significant side effects compounded with its high efficacy in the treatment of relapsed APL patients stimulated interest to extend ATO into other cancer settings, particularly in hematological malignancies. Preclinical studies confirmed that ATO showed some efficacy against multiple malignant cell lines.\textsuperscript{64} Currently, there have been 96 completed or ongoing clinical trials with ATO as a single agent and in combinations, in a variety of malignancies including metastatic melanoma, acute myeloid leukemia, multiple myeloma, central nervous system tumors, myelodysplastic syndrome, hepatocellular carcinoma, chronic lymphocytic leukemia, colorectal cancer, breast cancer, and lymphoma among others.\textsuperscript{65} Lessons learned from these studies have shown that while ATO has limited efficacy as a single agent in settings outside of APL, it may hold promise when used as part of a combination therapy.
3.5.1. Multiple Myeloma

Multiple myeloma (MM) is an incurable hematological malignancy characterized by an accumulation of monoclonal plasma cells in the bone marrow.\textsuperscript{66,67} There are more than 15 000 newly diagnosed cases in the United States alone and has a median survival of 3-4 years.\textsuperscript{68}

Currently, patients with MM are treated with high-dose chemotherapy (HDCT), thalidomide, bortezomib, lenaldomide and autologous stem cell transplantation (ASCT) but patients eventually relapse and become refractory to conventional treatment.\textsuperscript{67,68}

When used as a monotherapy, numerous studies have shown that ATO can elicit a response in up to 33% of MM patients, where a response is defined as a greater than 25% reduction in serum M protein.\textsuperscript{69,70} The results in combination therapies with Bortezomib and ascorbic acid have been more encouraging; showing enhanced clinical activity against relapsed and refractory MM and is well tolerated.\textsuperscript{71,72}

3.5.2. Myelodysplastic Syndrome

Myelodysplastic syndrome (MDS) is a heterogeneous hematological malignancy characterized by a hyper-proliferative bone marrow, dysplasia of the cellular elements and ineffective hematopoiesis.\textsuperscript{73} Much of the morbidity and mortality is attributed to severe anemia, bleeding and infections.

The only curative method is high-dose chemotherapy with allogeneic transplantation. The disease still remains a challenge, particularly in elderly patients.\textsuperscript{74} Preclinical studies have shown that ATO can induce apoptosis in MDS cell lines at clinically achievable concentrations.\textsuperscript{74} Results from two phase II multicenter trials demonstrated that ATO treatment yielded an overall rate of
hematological improvement of 29% with one complete and one partial response. ATO is still undergoing evaluation for routine use in MDS patients.

3.5.3. Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm resulting from a balanced reciprocal translocation t(9;22)(q34;q11) in hematopoietic stem cells leading to the fusion of the c-abl oncogene and the break point cluster region. The resulting Bcr-Abl oncoprotein is a constitutively activated tyrosine kinase that promotes the unregulated growth and accumulation of myeloid cells in the bone marrow and in the blood circulation.

Many patients treated with conventional therapy with the tyrosine kinase inhibitor Imatinib have poor outcomes. This is highlighted by the 60% of patients who fail to achieve a complete response after 18 months on therapy. Resistance can develop due to the inability of Imatinib to completely inhibit Bcr-Abl and the overexpression of Bcr-Abl. ATO may play a role in down-regulating Bcr-Abl and is being investigated in combination with Imatinib to treat patients with Imatinib resistance. Thus far, no significant responses have been observed although the regimen is well tolerated.

3.5.4. Solid tumors

ATO has shown promising preclinical activity in solid tumor cancer cell lines by inhibiting proliferation, decreasing cell viability and inducing apoptosis. These cancer cell lines include prostate cancer, bladder cancer, ovarian cancer, colon cancer, gastric cancer, cervical cancer and esophageal cancer cell lines. This led to numerous clinical trials that are underway including in advanced metastatic melanoma, hepatocellular carcinoma, and advanced head and neck cancer, but has thus far only shown limited efficacy compared to in APL.
4. Mechanisms of ATO Signaling in APL

In recent years, the clinical successes of ATO in APL have stimulated investigation in understanding the mechanism of action of ATO. Initially, it was shown that arsenic-resistant APL cells underwent apoptosis in addition to being susceptible to ATO-induced partial differentiation, suggesting different mechanisms of ATO action. Currently, the working model suggests that ATO has dose dependent dual effects on APL cells, inducing apoptosis at higher concentrations (0.5 – 2 µM) and partial differentiation at lower concentrations (0.1 – 0.5 µM).

4.1. Induction of Partial Differentiation

Similarly to ATRA treatment, ATO is capable of inducing the degradation of the PML-RARα fusion protein to release the maturation block and promote differentiation in APL cells. In normal cells, the PML protein is found in macromolecular structures in the nucleus called nuclear bodies. In APL cells, the PML nuclear bodies exhibit a nuclear microspeckle pattern with a corresponding loss of PML function. PML is a tumor suppressor that antagonizes many processes for the initiation and development of malignancy, including growth arrest and apoptosis. In APL cells, the PML-RARα fusion oncoprotein blocks the expression of genes necessary for normal differentiation. Upon ATO treatment at low concentrations (0.1 – 0.5 µM), PML and PML-RARα are targeted to nuclear bodies, sumoylated, polyubiquitinated by the SUMO-dependent ubiquitin ligase RNF4, and then degraded by the proteasome.

The PML-RARα in APL cells recruits and constitutively binds co-repressors SMART/N-CoR and histone deacetylases that promotes the differentiation block through inhibiting the expression of genes necessary for normal myeloid differentiation. ATO can release the differentiation block by interfering with the
ability of the PML-RARα fusion protein to recruit the co-repressors thereby promoting the expression of genes necessary for myeloid differentiation.\textsuperscript{9,95}

However, the presence of the PML-RARα fusion protein does not contribute entirely to the exquisite sensitivity of APL cells to ATO. Work in our lab has shown that the sensitivity of APL cells to ATO is not due to the expression and regulation of PML-RARα.\textsuperscript{96,97}

4.2. Induction of Apoptosis

ATO in high concentrations (0.5 - 2 µM) has been shown to induce apoptosis in APL and non-APL hematological malignancy and solid tumor cell lines.\textsuperscript{88} Through numerous studies, many mechanisms are implicated in ATO-induced apoptosis including the generation of reactive oxygen species (ROS),\textsuperscript{98} decrease in the mitochondrial membrane potential $\Delta \Psi _m$,\textsuperscript{99} caspase cascade activation,\textsuperscript{100} down-regulation of Bcl-2 expression,\textsuperscript{101} cell cycle arrest,\textsuperscript{102} and the opening of the permeability transition pore complex (PTPC) in the mitochondrial membrane through direct interaction.\textsuperscript{103}

Apoptosis is an evolutionarily well-conserved regulated programmed cell death that occurs in different physiological and pathological situations.\textsuperscript{104} It is a necessary process to control cell number and tissue size as well as maintaining homeostasis by removing aberrant cells. It is characterized by specific morphological and biochemical features distinct from necrosis including cell shrinkage, nuclear DNA fragmentation and membrane blebbing.

The central executioners of apoptosis are a family of highly well-conserved intracellular cysteine proteases called caspases.\textsuperscript{104} They possess an active site that allows them to cleave substrates at specific sites. Initially expressed as a zymogen, they require cleavage at specific sites to become activated. Caspase activation occurs in a systematic manner where one caspase cleaves and activates
the next in the cascade. This allows for an efficient method to amplify and integrate pro-apoptotic signals.

4.2.1. Intrinsic Pathway

The mitochondria is regarded as the forum of death where pro-apoptotic proteins including cytochrome c are sequestered. Pro-apoptotic signals can converge on the mitochondria and induce the depolarization of the mitochondria membrane potential to open up the PTPC. The PTPC is a multi-protein complex that interacts with the apoptosis regulating protein Bcl-2/Bax family. ATO has been reported to decrease the mitochondrial membrane potential \( \Delta \Psi m \) and to directly bind to the thiol groups of the PTPC and cause the release of cytochrome c and other pro-apoptotic proteins into the cytoplasm. ATO further contributes to apoptosis by down-regulating the expression of Bcl-2, a known antagonist to the opening of the PTPC.

In the cytoplasm, cytochrome c binds and activates Apaf-1 which then activates procaspase-9 to begin the caspase cascade leading to cell death. At the bottom of the cascade, caspase 3 is activated and subsequently cleaves key substrates in the cell to result in the cellular and biochemical features of apoptosis. Among the cleavage events is the inactivation of poly (ADP-ribose) polymerase (PARP) which is thought to prevent the depletion of NAD and ATP, necessary components for the later events of apoptosis.

4.2.2. Extrinsic Pathway

The extrinsic death pathway employs the role of death receptors which are members of the tumor necrosis factor (TNF) receptor gene superfamily that transmit the death signal from the cell surface to intracellular signaling pathways involving first the activation of caspase 8. Kitamura et al. reported that ATO can contribute to inducing apoptosis through the activation of the extrinsic
pathway of apoptosis. In their study, they demonstrated that ATO activated caspase 8 and Bid in APL cells but not in ATO-resistant APL cells. Furthermore, inhibition of caspase 8 blocked the activation of caspase 3 and the depolarization of the mitochondrial membrane potential. They also showed that their ATO-resistant cells had up-regulated expression of glutathione (GSH) and pharmacologic inhibition of GSH in these cells restored sensitivity to ATO, caspase 8 activation, and depolarization of mitochondrial membrane potential. Overall, their results suggested that ATO activates extrinsic death pathway signaling in a ROS-dependent manner.

4.3. Reactive Oxygen Species Generation

The accumulation of ROS plays a significant role in ATO-induced cytotoxicity. ROS is important in many biochemical and physiological processes and its up-regulation has been associated with many cancers. It may also be responsible for the sensitivity to certain drugs and contribute to the regulation of cellular proliferation, DNA damage and genomic instability.

ROS are oxygen-containing chemical species called radicals that have the ability to exist independently. The extremely high chemical reactivity of these species is attributed to the possession of unpaired electrons in their outermost orbitals. Oxygen possesses two electrons on its outermost orbital, rendering it extremely susceptible to the formation of radicals. Examples of ROS includes the oxygen molecule, superoxide radicals, $O_2^*$ and the hydroxyl radical, $OH^*$.

In eukaryotic cells under physiological conditions, most of the ROS is generated from the mitochondrial electron transport chain where approximately 2% of the oxygen is converted into ROS. Other ROS generating sites in the cell includes the microsomal cytochrome P450, plasma membrane NAD/NADPH systems and peroxisomes. Their formation is catalyzed by oxidation/reduction
reactions by enzymes such as dehydrogenases, oxidases, dioxygenases and hydroxylases.

ROS can cause damage to all classes of macromolecules including DNA, RNA, proteins and lipid components leading to inhibition of cellular proliferation, apoptosis, or necrosis. The damage can occur through altering the redox potential of proteins, binding proteins to other macromolecules and altering the activity of transcription factors and many enzymes among other functions. Some of the enzymes altered include protein kinases and phosphatases. Due to the adverse effects of excess ROS accumulation, it has been under scrutiny for the cause of cellular aging, carcinogenesis, malignant transformation and many chronic diseases.

4.3.1. Mechanism of ATO-induced Reactive Oxygen Species Accumulation

The accumulation of ROS as a requirement for ATO-induced apoptosis is well documented from studies in our lab and others. While the exact mechanism remains to be elucidated, many targets have been implicated in this process. These targets include the activation of NADPH oxidase and NO synthase, perturbation of the mitochondrial electron transport chain and the inhibition of antioxidant enzymes including thioredoxin reductase and glutathione peroxidase.

4.3.2. Reactive Oxygen Species Signaling

ATO-induced ROS accumulation may regulate many different intracellular signaling pathways. Among these pathways include the stress-induced mitogen-activated protein kinase (MAPK) signaling cascades. Activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade mediates signals for growth and differentiation by activating downstream transcription
factors such as c-Myc and Elk1.\textsuperscript{120} ATO has also been shown to activate the SAPK/JNK which can enter into the nucleus and regulate the activation of activating transcription factor-2 (ATF-2) and c-Jun transcription factor to mediate apoptosis.\textsuperscript{122} Furthermore, the p38 MAPK has been reported to be activated by ATO and is involved in the regulation of transcription factors such as ATF-2 and cAMP response element-binding protein (CREB), leading to conflicting outcomes including cell proliferation and apoptosis.\textsuperscript{120} One report combining ATO and a p38-MAPK inhibitor show enhanced ATO-induced apoptosis and growth inhibition in APL and multiple myeloma cells.\textsuperscript{123,124}

4.3.3. Role of Antioxidants in Resistance to ATO-induced Cell Death

Given the damaging effects of excess ROS in cells, cells must rely on intracellular defense mechanisms to mitigate the threat. One mechanism of protection makes use of anti-oxidant enzymes including super-oxide dismutase (SOD), and catalase.\textsuperscript{125} While SOD metabolises superoxides, catalase metabolizes hydrogen peroxide to reduce ROS stress.\textsuperscript{126}

Cells also possess reductive-oxidative buffering systems to regulate the levels of ROS. The glutathione system involves the enzyme glutathione peroxidase (GPx) which uses reduced glutathione to catalyze the reduction of hydrogen peroxide.\textsuperscript{127} Published data from our lab has shown that pharmacologic depletion of glutathione in ATO-resistant APL cells re-sensitizes them to ATO.\textsuperscript{96} The unique sensitivity of APL cells to ATO can also be attributed to the lower level of basal cellular glutathione content and therefore less buffering against ROS.\textsuperscript{127} Furthermore, the decreased level of cellular glutathione may lead to arsenic accumulation because the arsenic cannot be glutathionylated and exported via the multidrug resistance protein 1 (MRP1/ABCC1) transporter.\textsuperscript{128} The thioredoxin (Trx) ROS buffering system involves the capacity of reduced Trx to become oxidized to help maintain proteins in their reduced state.\textsuperscript{129,130} Reduced Trx can be
restored by the enzyme thioredoxin reductase (TrxR). We have preliminary data demonstrating that inhibition of TrxR enhances ATO-induced cell death.

4.3.4. Role of Pro-oxidants in Potentiating ATO-induced Cell Death

Since ROS accumulation is an important mediator of ATO-induced apoptosis, manipulation of the redox environment in cells is an attractive strategy to enhance ATO sensitivity, particularly in non-APL settings with a high ROS buffering capacity. APL cells are uniquely sensitive to ATO in part due to their relatively lower GSH levels compared to other malignant cell lines. Indeed our lab has already shown that the pharmacologic depletion of GSH by buthionine sulfoximine (BSO) can synergize with ATO to induce apoptosis in a variety of malignant cell lines including ATO-resistant APL cells and diffuse large B-cell lymphoma. When ATO is combined with a pro-oxidant such as ascorbic acid, cytotoxicity is increased in chronic lymphocytic leukemia, multiple myeloma, and CML cell lines. The use of bryostatin 1, an analog of phorbol myristate acetate, has been shown to synergize with ATO to enhance ROS generation and cell killing of leukemic cells through the stimulation of NADPH oxidase activity. Our lab has also demonstrated that the widely known antioxidant trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a vitamin E analog, can synergize with ATO to increase intracellular oxidative stress and enhance cell death in APL, myeloma, and breast cancer cells.

4.4. NFκB Inhibition

ATO can also promote cell death by inhibition of nuclear factor kappa B (NF-κB) activation. NF-κB is a transcription factor that mediates the expression of pro-survival genes in APL cells and has an important role in many malignant diseases. Some of the target genes include those encoding cell adhesion molecules, pro-inflammatory cytokines, stress response enzymes, growth factors and anti-apoptotic enzymes. NF-κB is normally sequestered by Iκβa but can be
activated by the IKβ kinase (IKK) complex-mediated phosphorylation and
degradation of Iκβα in response to extracellular signals. ATO has been reported
to bind to the Cys-179 residue in the activation loop of the IKKα/β subunit of
IKK, thereby inhibiting it. This results in the inhibition of the NF-κB cell
survival signals and leads to enhanced cell death.

4.5. Activation of Mitogen-Activated Protein Kinases

ATO-induced ROS has been shown to regulate the evolutionarily well-conserved
mitogen-activated protein kinase (MAPK) signaling cascade which is involved in
many intracellular processes including gene expression, cell proliferation, cell
survival, apoptosis, differentiation and apoptosis. MAPK signaling cascades are
composed of different tiers which are activated sequentially through
phosphorylation. Typically, a MAPK kinase kinase (MAP3K) activates a MAPK
kinase (MAP2K) which then in turn activates a MAPK to propagate the
intracellular signal. Each tier is subject to its own regulation.

4.5.1. Role of JNK Activation in ATO-induced Apoptosis

While several different MAPK signaling cascades exist, the activation of the
SAPK/JNK pathway is most strongly implicated in ATO-induced apoptosis. This
pathway is normally activated in response to environmental stresses including UV
light, endotoxin, inflammation and tumor necrosis factor (TNF) to mediate pro-
apoptotic signals from the mitochondria. JNK is a MAPK that is activated upon
phosphorylation by upstream kinases MKK4 (SEK1) and MKK7 (SEK2). Upon activation, JNK phosphorylates and regulates transcription factors including
activating transcription factor-2 (ATF-2) and c-Jun.

Work done in our lab has demonstrated that APL cells exhibit significant ATO-
induced cell death that is preceded by ROS generation and JNK activation. However, ATO-resistant cell lines with up-regulated GSH content showed only
limited JNK activation at the same ATO concentrations. JNK activation and ATO-sensitivity could be restored in the ATO-resistant APL cell lines by pharmacologic depletion of GSH. Furthermore, pharmacologic inhibition of JNK reduced sensitivity to ATO in APL cells. These results suggest that ATO-induced ROS generation and JNK activation are required to induce apoptosis. Other groups have also shown that JNK activation is important in ATO-induced apoptosis in other leukemic and lymphoma cell lines including U937, Namalwa, Jurkat, and NKM-1.  

There has been conflicting reports on the role of p38 activation in ATO-induced apoptosis. In one instance, p38 was shown to have a negative regulatory role in ATO-induced apoptosis. Pharmacologic inhibition of p38 or its direct upstream activators Mkk3 and Mkk6 resulted in enhanced ATO-induced JNK activation and cell death in various leukemic cell lines. However, p38 activation has also been implicated in ATO-induced apoptosis. Kang and Lee reported that ATO induced ROS generation and apoptosis through the activation of p38 in HeLa cells. Furthermore, Iwarma et al. demonstrated that p38 activation played a pro-apoptotic role while JNK activation played an anti-apoptotic role in U937 cells treated with unconventionally high concentrations of ATO. The conflicting results may be attributed to the different cell types used and the concentrations of ATO administered.

4.5.2. SEK1 Activation

Further evidence suggest that ATO-induced apoptosis is dependent on JNK activation and signaling. Published data from our lab has shown that JNK activation is abrogated in stress-enhanced kinase 1 (SEK1) deficient mouse embryonic fibroblasts (MEF) compared to their wild-type counterparts with a corresponding reduced sensitivity to ATO-induced cell death. SEK1 is a MAP2K that directly phosphorylates and activates JNK in response to stress signals.
4.5.3. JNK Targets

JNK signaling has been reported to be able to induce the expression of genes for both cell survival and cell death, but only sustained JNK activation mediates pro-apoptotic signaling. Such is the case for treatment with ATO where JNK activation is sustained for at least 24 hours. Upon activation, JNK phosphorylates and activates a wide variety of proteins that mediate pro-apoptotic gene expression including c-jun, Elk-1 and ATF2. JNK activation may also induce apoptosis via a transcription-independent mechanism by activation of the intrinsic death pathway through the Bax subfamily of Bcl-2-related proteins and through the phosphorylation of 14-3-3 proteins to regulate nuclear targeting of c-Abl in apoptotic responses.

4.5.4. Signal Transduction from ATO-induced ROS Generation to JNK Activation

ATO is transported inside the cell through aquaporins. Once inside the cell, the exact mechanism of signal transduction from oxidative stress generation to JNK activation is not entirely clear. JNK activation has been reported to require small GTP-binding proteins Ras, Rac, Cdc42 and Rho however; some evidence suggests only Rac, Cdc42 and Rho are implicated in ATO-induced JNK activation.

4.5.5. AKT Pro-survival Targets

ATO-induced JNK activation and apoptosis is highly regulated in many components of the signaling pathway. One of the key regulators is AKT, a serine/threonine protein kinase that mediates cell survival signals through the phosphorylation and inactivation of pro-apoptotic proteins including BAD, caspase-9 and forkhead (FKHR) family of transcription factors. AKT has
been reported to suppress the p38 and JNK pathways through the inactivation of the SEK1\textsuperscript{161} and the upstream apoptosis signal-regulating kinase 1 (ASK1).\textsuperscript{162} Furthermore, it may also bind to the JNK pathway scaffold proteins JIP1\textsuperscript{163} and POSH\textsuperscript{164} to prevent the assembly of the JNK signaling complex. Our lab has shown that in response to ATO, AKT protein levels and activity are decreased, correlating with JNK activation and sensitivity to ATO.\textsuperscript{165}

Since AKT negatively regulates ATO-induced apoptotic pathways at many different points, inhibition of AKT may enhance ATO-induced apoptosis. In one report, pharmacologic inhibitors of AKT potentiated ATO-induced apoptosis in myeloid leukemic cells through glutathione depletion and enhanced ROS accumulation.\textsuperscript{166}

5. Role of ASK1 in ATO-Induced Apoptosis

Our lab has begun defining the pathway of ATO-induced apoptosis involving the MAPK cascade. We have shown that ROS accumulation is required and that JNK activation is necessary for maximum induction of ATO-induced apoptosis. Furthermore, genetic deficiency of the MAP2K, SEK1, resulted in abrogated ATO-induced JNK activation and apoptosis. Upstream of SEK1, there are several MAPKKK that are implicated in the induction of the SEK/JNK pathway. However, since stress-induced signaling is strongly implicated in ASK1 activation, we hypothesized that ASK1 mediates ATO-induced stress signaling and apoptosis.

ASK1 is a ubiquitously expressed MAPKKK that activates both JNK and p38 signaling pathways and is a strong mediator of apoptotic signals.\textsuperscript{167} It phosphorylates and activates downstream MAPKKs including SEK1 and SEK2 in response to cellular stress such as oxidative stress, endoplasmic reticulum stress, calcium overload and receptor mediated inflammatory signals.\textsuperscript{168-170} While recognized for its pro-apoptotic role, ASK1 may have other functions including
mediating differentiation and survival signals depending on the cell type and context.\textsuperscript{171-173}

5.1. Mechanism of ASK1 Activation

5.1.1. Role in Stress-Induced Cell Death

In response to various cytotoxic stresses, the MAPK cascade is activated to relay cell death signals terminating in p38 and JNK activation. However, there are more than 11 different mammalian MAPKKKs that have been implicated in the activation of JNK and/or p38 signaling.\textsuperscript{150} ASK1 has been shown to be important in this process where ASK1 deficient (ASK1\textsuperscript{-/-}) murine embryonic fibroblasts (MEFs) are resistant to ROS and TNF-induced apoptosis.\textsuperscript{174} TNF is a pro-inflammatory cytokine that regulates inflammation, proliferation and apoptosis in a ROS-dependent mechanism.

5.1.2. Activation of ASK1

ASK1 exerts its activity as part of a high molecular complex referred to as the ASK1 signalosome. The formation of the signalosome requires the homooligomerization of ASK1 through its C-terminal coiled-coil (CCC) domain conjugated to other proteins.\textsuperscript{175,176} In response to stress signals, the homooligomerized ASK1 becomes activated through the phosphorylation of threonine 838 within the activation segment in the kinase domain. Activated ASK1 can then phosphorylate and activate downstream MAPKK in the MAPK pathway.

5.2. Other roles of ASK1

While ASK1 is recognized for its pro-apoptotic role, several lines of evidence suggest that it may also have other functions in response to stress. ASK1 has been reported to mediate signals for differentiation and survival in the rat
pheochromocytoma cell line PC12\textsuperscript{172} and keratinocytes.\textsuperscript{177} It has also been implicated in the regulation of erythroid differentiation.\textsuperscript{178}

ASK1 may also contribute to diseases like cardiac hypertrophy through stress-induced signaling.\textsuperscript{179} There is evidence to suggest that ROS generation induced by angiotensin II (Ang II) plays an important role in the intracellular signaling response in cardiac hypertrophy.\textsuperscript{180} ASK1 may mediate G-protein-coupled receptor agonist-induced NFκB activation to induce cardiac hypertrophy.\textsuperscript{173}

Taken together, we can begin to paint a working model of ATO signaling in inducing apoptosis (Figure 1.5).
Figure 1.5 Proposed ATO-induced JNK signaling pathway
6. Combination therapies

While ATO has been demonstrated to be an effective therapy in APL, dose limiting toxicities limit its success in other settings when used as a single agent. However, it may hold promise when used in combination therapies therefore it is important to develop sensitization strategies to increase the cytotoxic effects of the drug. One strategy requires understanding the mechanisms of ATO-induced apoptosis to identify potential targets for combination therapies. This will allow the generation of rational combination therapies to increase its therapeutic spectrum and is the objective of this project.
CHAPTER 2: ROLE OF ASK1 IN ATO-INDUCED APOPTOSIS

Introduction

Arsenic trioxide (ATO) is an arsenic derivative that has important anti-tumor properties. It has been shown to be an effective treatment for acute promyelocytic leukemia (APL) especially in patients that have relapsed and have become refractory to all-trans retinoic acid (ATRA) treatment. ATO-induced anti-leukemic activity is a result of dose-dependent dual effects, comprising of partial differentiation through the degradation of PML/RARα at low concentrations (0.1 – 0.5 µM) and inducing apoptosis at high concentrations (0.5 – 2 µM).

The relatively rare appearance of significant side effects compounded with its high efficacy in the treatment of relapsed APL patients stimulated interest to extend ATO into other cancer settings, particularly in hematological malignancies. Currently, there have been 96 completed or ongoing clinical trials with ATO as a single agent and in combinations in a variety of malignancies including metastatic melanoma, acute myeloid leukemia, multiple myeloma, central nervous system tumors, myelodysplastic syndrome, hepatocellular carcinoma, chronic lymphocytic leukemia, colorectal cancer, breast cancer, and lymphoma among others. However, ATO demonstrated limited efficacy in non-APL settings due to dose-limiting toxicities. While ATO has limited efficacy as a single agent, it may hold promise when used as part of a combination therapy. Thus, it is important to understand the mechanism of ATO action to identify targets to develop rational combination therapies to increase its therapeutic spectrum. Indeed, using this strategy, we identified one potential combination therapy involving arsenic and the vitamin E-derivative trolox, which enhances tumor killing while protecting against toxicity.

Our laboratory and others have described a pathway required for ATO-induced apoptosis in APL cells. This pathway involves the generation of reactive oxygen species (ROS), and the subsequent induction of a specific mitogen-activated
protein kinase (MAPK) cascade that includes both stress-activated protein kinase (SAPK)/ERK kinase 1 (SEK1) and c-Jun N-terminal kinases (JNK) activation. Furthermore, ATO inhibits the pro-survival signals of AKT, a serine/threonine protein kinase, which negatively regulates the SEK-JNK pathway on several levels. However, little is known about what links ROS production and activation of the MAPK cascade.

ASK1 is a ubiquitously expressed MAP3K that activates both JNK and p38 signaling pathways and is a strong mediator of apoptotic signals. It phosphorylates and activates downstream MAPKKs including SEK1 and SEK2 in response to cellular stress such as oxidative stress, endoplasmic reticulum stress, calcium overload and receptor-mediated inflammatory signals. Cellular stress has been associated with many cancers and may be responsible for sensitivity to certain drugs and contribute to regulation of cellular proliferation, DNA damage and genomic instability. Thus, ASK1 plays a vital role in limiting the adverse consequences of redox imbalance.

Upstream of SEK1, there are several MAP3K that are implicated in the induction of the SEK/JNK pathway. However, since stress-induced signaling is strongly implicated in ASK1 activation, we hypothesized that ASK1 mediates ATO-induced stress signaling and apoptosis. Using MEF cells derived from ASK1 knockout mice, we provide evidence that ASK1 was necessary for ATO-induced apoptosis and JNK activation. We then investigated the role of ASK1 in NB4 cells and demonstrated a dose-dependent and time-dependent increase in ASK1 activation following treatment with ATO. However, knockdown of ASK1 in NB4 cells enhanced susceptibility to ATO. The same phenotype was observed in the knockdown of SEK1 in NB4 cells and the knockdown of ASK1 in MCF-7 breast cancer cells. To determine whether the opposite actions of ASK1 on ATO-induced apoptosis were an inherent difference between normal and malignant cells, we transformed ASK1 knockout MEFs with E1A and RAS oncogenes. Transformation significantly increased sensitivity to ATO-induced apoptosis and
growth inhibition, suggesting that ASK1 can have both pro-apoptotic and anti-apoptotic roles depending on transformation state of the cells.
Materials and Methods

Cell culture

All cells were grown in a humidified chamber at 37°C in a 5 % CO₂ environment. NB4 cells (kindly provided by Dr M Lanotte) and SEK1 knockdown NB4 cells were maintained in RPMI 1640 media (Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent) and penicillin/streptomycin (Wisent). SEK1 knockdown NB4 cells were also maintained in 1 μg/mL puromycin (GIBCO). MCF-7 (ATCC), 293T (ATCC), wild-type MEF, ASK1⁻/⁻ MEF cells (kindly provided by Dr H Ichijo) and E1A-Ras transformed ASK1⁻/⁻ MEF cells were cultured in Dulbecco’s modified eagle’s medium media (Wisent) supplemented with 10% FBS and penicillin/streptomycin. E1A-Ras transformed ASK1⁻/⁻ MEF cells were also supplemented with 2 μg/mL puromycin.

Trypan Blue Exclusion

NB4 and SEK1 knockdown NB4 cells were seeded at 1 x 10⁵ cells/mL in 24 well plates. Cells were treated with 0.5 μM, 1 μM, or 2 μM ATO for 2 days. MCF-7, wild-type MEF, ASK1⁻/⁻ MEF and E1A-Ras transformed ASK1⁻/⁻ MEF cells were seeded at 1.5 x 10⁴ cells/mL in 6 well plates. Cells were treated with 1 μM, 2.5 μM, 5 μM, and 10 μM ATO for 2 days. Viable cells were counted by trypan blue exclusion (Invitrogen) with a hemacytometer.

CellTiter-Glo Luminescent Cell Viability Assay

Wild-type MEF and ASK1⁻/⁻ MEF cells were seeded at 1000 cells / 100 μL and treated with various concentrations of ATO for up to 7 days. Cell viability of MEF cells were measured by luminescence detection using CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) as per manufacturer’s instructions.
Sulforhodamine B Colorimetric Assay

Cell viability of wild-type MEF, ASK1\(^{-/-}\) MEF, E1A-Ras transformed ASK1\(^{-/-}\) MEF and MCF-7 cells were measured by absorbance detection using Sulforhodamine B colorimetric assay. Briefly, cells growing in log phase were seeded at 500 cells/100 uL in a 96 well plate on day 0. After 24h hours, cells were treated with 1 μM, 2.5. μM, 5 μM and 10 μM for 3, 5, and 7 days. Cells were re-treated on day 4. After desired treatment time, the supernatant from the 96 well was removed and the cells were fixed with 10% TCA for 30 minutes at 4 °C. Following fixation, cells were washed 4 times using alternating dispensing and removal procedures with distilled water and air-dried overnight at room temperature. The 96 well plates were stained with 100 μL of 0.4% Sulforhodamine B for 30 min with gentle shaking. Following staining, plates were washed 4 times with 1% acetic acid and air-dried overnight. The following day, 100 μL of 10 mM Tris base solution was added to each well and absorbance was measured at 570 nm and 492 nm.

PI stain

NB4 and SEK1 knockdown NB4 cells were seeded at 1 x 10^5 cells/mL in 24 well plates. Cells were treated with 0.5 μM, 1 μM, or 2 μM ATO for 2 days. MCF-7, wild-type MEF, ASK1\(^{-/-}\) MEF and E1A-Ras transformed ASK1\(^{-/-}\) MEF cells were seeded at 1.5 x 10^4 cells/mL in 6 well plates and treated the following day with 1 μM, 2.5 μM, 5 μM or 10 μM ATO for up to 48 hours. Cells were collected and washed in PBS supplemented with 5% FBS and 0.01 M NaN\(_3\). They were then pelleted and resuspended in 0.2 mL hypotonic fluorochrome solution (50 mg/ml propidium iodide (Sigma), 0.1% sodium citrate, 0.1% Triton X-100). Fluorescence was detected by flow cytometry and analysis was performed using FloJo v7.6.2. Ten thousand events were recorded per sample and analyzed for cell cycle and apoptosis. Cells undergoing DNA fragmentation and apoptosis
were defined as events with fluorescence weaker than the G0-G1 cell cycle peak (Sub-G0).

**JNK Kinase Assay**

Wild-type MEF and ASK1-/- MEF cells were seeded at 1 x 10^8 cells on a 10 cm plate in 10 mL serum free media for 24 hours. They were then treated with various concentrations of ATO for 24 hours. Following treatment, cells were washed once with cold phosphate-buffered saline (PBS) and lysed in the presence of phosphatase and protease inhibitors. An anti-JNK1 antibody (Santa Cruz Biotechnology) and Protein A-Sepharose beads (Sigma) were used to precipitate JNK1 from 200 μg of cell lysates for each sample by nutation at 4 °C for 1 hour. Following multiple, successive washes with kinase buffer containing 3 M urea and then kinase buffer alone, immune complexes were incubated with glutathione-S-transferase (GST)-c-cjun and [γ^^32P]ATP for 30 minutes at 30 °C. GST-c-cjun was electrophoresed on an acrylamide gel and visualized by autoradiography. Western blotting was performed on samples prior to the immunoprecipitation to ensure that ATO treatment did not affect absolute JNK1 expression levels.

**Western Blot**

Cell extracts were prepared by washing 5 x 10^6 cells with cold PBS and resuspending cell pellets in 0.3 mL radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl (pH 8), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease/phosphatase inhibitors (Roche) at 4 °C. Extracts were sonicated using a Sonic Dismembrator (Model 300; Fisher Scientific) for 2 x 10 seconds at 15 % intensity and then centrifuged at 13 000 rpm in a microcentrifuge at 4 °C for 10 minutes. Supernatants were transferred to fresh tubes and protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad). To detect JNK1, E1A, SEK1 and GAPDH, 50 μg of protein was prepared. To detect
ASK1, phospho ASK1 T838, and α-actinin, 100 μg of protein was prepared. Volumes of samples were added to an equal volume of 2x sample buffer and run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and stained with 0.1 % Ponceau S in 5% acetic acid to ensure equal protein loading. Membranes were then blocked with 5% milk (JNK1, SEK1, E1A, GAPDH and α-actinin) or 5% bovine serum albumin (ASK1 and phospho ASK1 T838) in TBS containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. The membrane was then hybridized overnight at 4 °C with antibody against JNK1 (Santa Cruz, 1:500), SEK1 (Cell Signaling, 1:1000), E1A (Calbiochem, 1:100), GAPDH (Cell Signaling, 1:1000 dilution), Phospho ASK1 T838 (kind gift from Dr. H Ichijo, 1:2000 dilution),125 ASK1 (Cell Signaling, 1:1000 dilution) and α-actinin (Santa Cruz, 1:2000 dilution). Membranes were washed 4 x with in TBST for 10 minutes and then incubated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 1 hour at room temperature. Membranes were once again washed 4 x with TBST for 10 minutes each and the peroxidase activity was visualized by enhanced chemiluminescence (ECL; GE Healthcare Biosciences-Amersham) or chemiluminescent HRP substrate (Millipore Coorporation).

**shRNA Lentiviral Particle Transduction**

NB4 cells were seeded at 0.5 x 10^6 cells in 2 mL RPMI 1640 media supplemented with 10% fetal bovine serum and penicillin/streptomycin in a 12 well plate. MCF-7 cells were seeded at 5 x 10^4 cells/ mL in 2 mL Dulbecco’s modified eagle’s medium media supplemented with 10% FBS and penicillin/streptomycin. The next day, the culture media was replaced with the same media but supplemented with 5 μg/mL polybrene (Santa Cruz). To infect cells, 40 μl of shRNA lentiviral particles for ASK1 (Santa Cruz), SEK1 (Sigma) and their respective non-target controls were added dropwise to the culture and incubated overnight. The cell cultures were sequentially expanded in their respective fresh culture media without polybrene for 4 days. Following expansion, cell cultures
were selected in 1 ug/mL (NB4 cells) and 2 ug/mL (MCF-7 cells) puromycin-supplemented media for one week.

**Production of Viral Infecting Particles**

293T Phoenix cells were seeded at 5 x 10^6 cells in a 10 cm dish for 24 hours. Cells were washed and culture media was replaced with 3 mL chloroquine mix (12.5 μM chloroquine in 3 mL serum free media) and incubated at 37 °C for 5 minutes. After incubation, 1.5 mL of transfection mixture (20 μg of pLPC or pLPC-E1A-Ras vector in OPTI-MEM I (Invitrogen)) and 1.5 mL of lipofectamine mixture (80 μL Lipofectamine (Invitrogen) in OPTI-MEM I) were added to the cells. The plates were incubated for 5 hours at 37 °C followed by the addition of 4 mL of 10% culture media and further incubated overnight at 37 °C. The next day, the culture media was replaced with 4 mL of fresh media and incubated for 48 hours. The cell culture supernatant was collected and centrifuged at 1200 rpm for 5 minutes to pellet unwanted residual cells. The cell-free supernatant was collected for viral infection.

**Transformation of MEFs with E1A-Ras**

Wild-type and ASK1−/− MEF cells were seeded at 1.5 x 10^5 cells on a 10 cm plate and subjected to 2 rounds of viral infection. Each round consists of first replacing the culture media with viral supernatant mixture (2 mL viral supernatant + 3 mL of culture media supplemented with 2 μg/mL polybrene (Santa Cruz) and incubating overnight at 37 °C. After the second round of infection, the cells were incubated in culture media for 24 hours followed by selection in 2 μg/mL of puromycin for 1 week.
Statistical analysis

All statistical tests aiming at evaluating significance were determined by analysis of variance (one-way analysis of variance) followed by Newman–Keuls post-tests using Prism version 4.03 (GraphPad software, San Diego, CA, USA).
Results

**ASK1 is responsible for ATO-induced JNK activation and apoptosis in mouse embryonic fibroblasts**

We have previously shown activation of JNK to be necessary for maximum induction of apoptosis with ATO treatment. Pharmacologic inhibition of JNK in NB4 cells and genetic inhibition in mouse embryonic fibroblasts (MEFs) deficient in the direct upstream JNK activator SEK1 showed reduced sensitivity to ATO-induced apoptosis. Given that ASK1 is the direct upstream activator of SEK1, we first examined whether ASK1 was responsible for ATO-induced apoptosis by using MEFs derived from ASK1 knockout (ASK1−/−) mice. Wild-type and ASK1−/− MEFs were cultured with a range of concentrations of ATO for 1 week and cell viability (Figure 1A) was assessed using Titer-Glo assay. The number of metabolically active cells were measured and revealed that ATO-induced cytotoxicity was significantly reduced in ASK1−/− MEFs, with nearly 45% in reduction in cell viability with the 2.5 µM treatment. Viable cell numbers (Figure 1B) and cell death (Figure 1C) were assessed after a 48 hour treatment with a variety of ATO concentrations, demonstrating ASK1−/− MEFs to be more resistant to ATO-induced growth inhibition and apoptosis.

Given that JNK contributes to induction of apoptosis by ATO and the upstream ASK1 is also responsible for conferring sensitivity to ATO, we hypothesized that ASK1 plays a role in ATO-induced JNK activation. We expect ASK1−/− MEFs to have abrogated ATO-induced JNK activation. Indeed when we examined JNK activity in response to ATO treatment using a JNK kinase assay, we saw significant JNK activation in the wild-type MEFs in a dose dependent manner; a phenomenon not observed in ASK1−/− MEFs (Figure 2). In fact, JNK activation in the ASK1−/− MEFs was close to baseline untreated control, even following the 5 µM treatment, suggesting a predominant role for ASK1 in ATO-induced stress signaling in MEFs. The results were surprising considering that deletion of a single MAP3K, among many, elicited such a prominent effect in ATO signaling.
ASK1 is activated by ATO in APL Cells

Given that ASK1 is a MAP3K that plays a predominant role in ATO-induced cell death in MEFs, we wanted to expand our findings in the prototypical cancer setting for ATO and explore the role of ASK1 in the APL cell line, NB4. In order to examine the effect of ATO in ASK1 activation in NB4 cells, an antibody recognizing the activating phosphorylation site of ASK1 on threonine 838 was used. In figure 3A, we show a dose-dependent increase in ASK1 phosphorylation after a 24 hour treatment with a range of ATO concentrations. Next, we examined the time dependency of ASK1 phosphorylation. When NB4 cells were treated with 2 μM ATO for up to 24 hours, a time-dependent increase in ASK1 phosphorylation was observed as early as 6 hours and was sustained for at least 24 hours as illustrated in Figure 3B. These results indicate that ASK1 is activated by ATO in APL cells.

Knockdown of ASK1 enhances ATO-induced apoptosis in APL cells

To investigate whether ASK1 is necessary in ATO-induced apoptosis in APL cells, we knocked down ASK1 expression using shRNA lentiviral particles. Figure 4A confirms the protein expression level of ASK1 is significantly decreased one week post-puromycin selection. We compared control shRNA and ASK1 shRNA lentivirus-infected with wild-type NB4 cells for their sensitivity to 0.5- 2 μM ATO. Cell viability was assessed by trypan blue exclusion and cell death by propidium iodide staining. Unexpectedly, the knockdown of ASK1 significantly enhanced ATO-induced growth inhibition (Figure 4B) and apoptosis (Figure 4C) compared to control shRNA infected cells and their wild-type counterparts. These results suggest an anti-apoptotic and pro-survival role for ASK1 in APL cells.
**SEK1 knockdown APL cells exhibit enhanced sensitivity to ATO-induced apoptosis**

Previous published work in our lab implicated SEK1 as an important mediator of ATO-induced JNK activation and apoptosis. SEK1−/− MEFs exhibited enhanced resistance to ATO-induced apoptosis, similar to the phenotype observed in ASK1−/− MEFs. Given that knockdown of ASK1 in NB4 cells potentiated ATO-induced apoptosis in contrast to the effects seen in ASK1−/− MEFs, we wanted to determine the impact of ATO in SEK1 knockdowns in NB4 cells. The genetic approach of the knockdown made use of SEK1 shRNA lentiviral particles and subsequent selection in 1 µg/mL puromycin. The efficiency of the knockdown was confirmed by the disappearance of SEK1 expression as shown in Figure 5A. A growth assay revealed that SEK1 knockdown NB4 cells were much more sensitive to growth inhibition compared to their WT or control shRNA transfected counterparts (Figure 5B). PI staining also confirmed SEK1 knockdown NB4 cells to be significantly more sensitive to cell death following ATO treatment (Figure 5C). Taken together, the results support the pro-survival role of ASK1 signaling in APL cells in response to ATO.

**Knockdown of ASK1 enhances ATO-induced apoptosis in MCF-7 cells**

The unprecedented negative regulatory role of ASK1 in ATO-induced apoptosis in APL cells prompted us to further examine the role of ASK1 in another human cancer cell line to see if the results can be extended into another setting. Therefore, the MCF-7 human breast cancer cell line, an excellent candidate that readily undergoes genetic manipulation, was selected for comparison. ASK1 was knocked down in MCF-7 cells using shRNA lentiviral particle infection and ASK1 expression was monitored by immunoblot as shown in Figure 6A. We treated these cells with up to 10 µM ATO due to their reduced sensitivity to ATO compared to APL cells. After 7 days, cell viability was assessed by SRB assay, showing enhanced dose-dependent growth inhibition in the ASK1 knockdown
MCF-7 cells compared to the control shRNA transfected and wild-type counterparts as shown in Figure 6B. Correspondingly, the shASK1 MCF-7 cells also showed enhanced sensitivity to ATO-induced cell death following a 24 hour treatment (Figure 6C). Taken together, these results suggest that ASK1 may play an anti-apoptotic and protective role in malignant cell lines.

Transformation of wild-type and ASK1\(^{-/-}\) MEFs with E1A-Ras enhances sensitivity to ATO-induced apoptosis

We next tested whether transformation changed the function of ASK1 by transforming both ASK1\(^{+/+}\) and ASK1\(^{-/-}\) MEFs with E1A-Ras. Ras is a frequently mutated oncogene in cancers and cooperates with the adenoviral E1A oncoprotein to transform primary human cells.\(^{186}\) In transformed ASK1\(^{+/+}\) MEFs, expression of the oncogene was confirmed in an immunoblot for E1A as shown in Figure 7A. A growth assay and cell death assay revealed the E1A-Ras transformed ASK1\(^{+/+}\) MEFs to be significantly more sensitive to ATO-induced growth inhibition (Figure 7B) and apoptosis (Figure 7C). These results are consistent with previous reports that immortalized cells are more sensitive to ATO-induced cell death.\(^{187-189}\) Furthermore, we generated E1A-Ras transformed ASK1\(^{-/-}\) MEFs. The expression level of the E1A-Ras oncoprotein in the transformed cells was confirmed in Figure 8A by immunoblotting for E1A. When treated with ATO, the transformed ASK1\(^{-/-}\) cells are significantly more sensitive to ATO-induced growth inhibition (Figure 8B) and apoptosis (Figure 8C) compared to the parental and empty vector transfected ASK1\(^{-/-}\) MEFs, comparable to that of the ASK1\(^{+/+}\) MEFs. These results support the working hypothesis that ASK1 has contrasting roles in normal versus malignant cells.
Figure 1: Deficiency of ASK1 in MEF cells enhances resistance to ATO-Induced apoptosis. (A) MEFs from wild-type (WT) mice and ASK1 deficient (ASK1−/−) mice were cultured in various concentrations of ATO for 7 days. Cell viability was assessed by Titer-Glo Assay as per manufacturer’s instructions. WT and ASK1−/− MEFs were also treated with up to 5μM ATO for 48 hours and viable cell numbers (B) and cell death (C) were assessed by trypan blue exclusion and PI-staining, respectively. Each bar represents an average of three independent samples with standard deviation bars shown. Asterisks indicate significant differences between WT and ASK1−/− MEF treatment matched pairs (* p<0.05; ** p<0.01; *** p<0.001).
**Figure 2:** ASK1 mediates ATO-induced JNK activation in MEFs. WT and ASK1<sup>−/−</sup> MEFs were treated for 16 hours with different concentrations of ATO. Whole cell lysates were used in a JNK kinase assays (top panel) to measure JNK activity. JNK or β-actin immunoblots (middle and bottom panels respectively) serve as controls.
Figure 3: ATO activates ASK1 in APL cells. NB4 cells were cultured in media with various concentrations of ATO for 24 hours (A) or in 2 μM ATO for different durations (B) and immunoblotted for phospho-ASK1-T838, ASK1 and α-actinin.
Figure 4: Knockdown of ASK1 in APL cells enhances sensitivity to ATO. NB4 cells were infected with 200 000 infectious units of virus of ASK1 shRNA or control shRNA lentiviral particles. Following a 7 day selection in puromycin, whole cell lysates were prepared and immunoblotted for ASK1 and α-actinin (A). Transfected cells were also treated with various concentrations of ATO for 48 hours and cell viability was evaluated using trypan blue staining (B). Cell death was detected by PI Staining (C) following a 24 hour treatment with various concentrations of ATO. Each bar represents an average of three independent samples with standard deviation bars shown. Asterisks indicate significant differences between the control shRNA and ASK1 shRNA treatment matched pairs (* p<0.05; ** p<0.01; *** p<0.001).
Figure 5: Knockdown of SEK1 enhances ATO-induced apoptosis in APL cells. NB4 cells were infected with 200,000 infectious units of SEK1 shRNA or control shRNA lentiviral particles. Western blots detailing the expression levels of SEK1 (A) are shown. Transfected cells were also treated with a range of doses of ATO for 24 hours and cell viability (B) was evaluated using trypan blue exclusion while cell death (C) was assayed by PI-staining. Asterisks indicate significant differences between the control shRNA and SEK1 shRNA treatment matched pairs (* p<0.05; ** p<0.01; *** p<0.001).
Figure 6: Knockdown of ASK1 in human breast cancer cells increases sensitivity to ATO. MCF-7 cells were infected with 200 000 infectious units of virus of ASK1 shRNA or control shRNA lentiviral particles. Following a 7 day selection in puromycin, whole cell lysates were prepared and immunoblotted for ASK1 and α-actinin (A). Transfected cells were also treated for 7 days with a range of doses of ATO and cell density was measured using a Sulforhodamine-B (SRB) assay (B). Cell death (C) was assessed by PI-staining following treatment with ATO at the indicated concentrations for 2 days. Each bar represents an average of three independent samples with standard deviation bars shown. Asterisks indicate significant differences between the control shRNA and ASK1 shRNA treatment matched pairs (* p<0.05; ** p<0.01; *** p<0.001).
Figure 7: Transformation of WT MEFs with E1A-Ras increases sensitivity to ATO. WT MEFs were transfected with 20 μg of pLPC or pLPC-E1A-Ras. Following a 7 day selection with puromycin, whole cell lysates were prepared and immunoblotted for E1A and GAPDH (A). Transfected cells were also treated with various concentrations of ATO for 48 hours and growth inhibition (B) was assessed by counting live, trypan blue-negative cells and expressing it as a percentage of viable cells relative to the untreated control. Apoptosis was assessed by PI staining (C). Each bar represents an average of three independent samples with standard deviation bars shown. Asterisks indicate significant differences between EV and E1A-Ras transformed MEF treatment matched pairs (* p<0.05; ** p<0.01; *** p<0.001).
Figure 8: E1A-Ras transformed ASK1−/− MEFs are more susceptible to ATO-induced apoptosis than their parental counterparts. ASK1−/− MEFs were transfected with 20 μg of pLPC or pLPC-E1A-Ras. Whole cell lysates were prepared and immunoblotted for E1A and GAPDH 7 days after selection in 2 μg/mL puromycin (A). Transfected cells were evaluated for cell viability (B) using SRB assay after culturing the cells in the indicated concentrations of ATO for 7 days. Transfected cells were also treated with a range of concentrations of ATO for 48 hours and cell death (C) was assessed using PI-staining. Each bar represents an average of three independent samples with standard deviation bars shown. Asterisks indicate significant differences between the EV and E1A-Ras transformed MEF treatment matched pairs (* p<0.05; ** p<0.01; *** p<0.001).
Discussion

Our lab has linked ATO-induced ROS and JNK activation to apoptosis. We have also identified SEK1, a MAP2K directly upstream of JNK activation, to be necessary for maximum induction of JNK activation and apoptosis by ATO. Located directly upstream of SEK1, ASK1 is one of many MAP3K situated on the top of a 3 tier evolutionarily conserved signaling cascade, which senses various internal and external stresses to elicit a wide variety of cellular responses including cell death and proliferation. In this study, we sought to identify the role of ASK1 in ATO-induced apoptosis.

Using MEF cells derived from ASK1/− mice, we provide evidence that ASK1 mediates ATO-induced apoptosis and JNK activation. We then investigated the role of ASK1 in NB4 cells and demonstrated a dose-dependent and time-dependent increase in ASK1 activation following treatment with ATO. However, knockdown of ASK1 in NB4 cells enhanced susceptibility to ATO, undergoing apoptosis and growth inhibition more than their wild type counterparts. The same impact was observed in the knockdown of SEK1, a direct downstream MAP2K of ASK1, resulting in an increase in ATO sensitivity. Furthermore, the increased susceptibility to ATO was also observed in the human breast cancer cell line MCF-7 when we knocked down ASK1. This led us to postulate that ASK1 had a pro-apoptotic function in non-transformed fibroblasts, but was pro-survival in malignant cells. Indeed, transformation of WT and ASK1/− MEFs significantly increased their sensitivity to ATO-induced apoptosis and growth inhibition, highlighting differences of ASK1 in normal versus transformed cells. Taken together, these results suggest that the role of ASK1 depends on cellular context, having opposite roles in normal versus transformed cell types.

To determine whether ASK1 activation is necessary for ATO-induced apoptosis, our initial experiments examined the effects of ATO on cell growth and cell death in ASK1/− MEFs. These experiments revealed that ASK1 played a predominant role in ATO-induced apoptosis and JNK activation since ASK1/− MEFs were
significantly resistant to ATO-induced apoptosis accompanied by remarkable reduction of sustained JNK activation compared to wild-type controls. The complete abrogation of JNK activity in these cells for at least 24 hours suggests that ASK1 plays a pivotal role in ATO-induced stress signaling and apoptosis. The results are surprising, considering the numerous MAP3K found upstream of JNK activation in stress-induced signaling but are consistent with the documented role of ASK1.

Since ASK1 has been shown to be necessary in ATO-induced cell death in MEFs, we wanted to determine if this remained true in a cancer setting. In NB4 cells, the activation of ASK1 induced by ATO occurred in a dose-dependent and time-dependent manner, correlating with our published results on ROS accumulation, JNK activation, growth inhibition and cell death. In this regard, ATO appears to play a positive role in the induction of apoptosis. However, the present study also suggests that ASK1 may mediate pro-survival signals in APL cells. Our results from the ASK1 knockdown experiments in NB4 cells showed a markedly increased sensitivity to ATO-induced cell death and growth inhibition in the ASK1 knockdowns compared to their parental counterparts. This is consistent with results from another group, reporting a pro-survival role for ASK1.191 These results suggest that ASK1 may play a pro-survival role in transformed cells. To confirm these results, it will be necessary to validate the other signaling requirements in the ASK1 knockdown NB4 cells and evaluate JNK activity in response to ATO. It will also be interesting to evaluate p38 activation because several studies have implicated p38 as a negative regulator in ATO-induced apoptosis.123,147

We wanted to determine if the pro-survival role of ASK1 could be reproduced in a solid tumour-derived cell line, so we expanded our results to the human breast cancer cell line MCF-7, a relatively easy model to perform genetic manipulations. Consistent with the results in NB4 cells, ATO induced significantly more apoptosis and growth inhibition in a dose-dependent and time-dependent manner
when ASK1 was knocked down. Taken together, these results suggest that ASK1 mediates a pro-survival role, but requires further validation of its signaling requirements. While these results are surprising, stress-induced ASK1 activation has been reported to have other consequences. Expression of constitutively active ASK1 in the rat pheochromocytoma cell line PC12 was shown to induce neurite outgrowth, activate expression of mature neuron-specific proteins, and survive in serum-starved conditions, suggesting that ASK1 may mediate signals for differentiation and survival. ASK1 has also been implicated in G-protein-coupled receptor agonist-induced NF-κB activation in cardiomyocyte hypertrophy. These studies support the notion that ASK1 has a variety of functions depending on the biological context and is not limited only to a pro-apoptotic function.

It remains a possibility that the differences in the role of ASK1 in normal cells and the two different malignant cell lines are attributed to a normal versus malignant background. To test this hypothesis, ASK1−/− MEFs were transformed with E1A-Ras. Compared to their parental counterparts, the E1A-Ras transformed ASK1−/− MEFs were more sensitive to ATO-induced apoptosis and growth inhibition suggesting enhanced ATO cytotoxicity in the transformed cells. These results are comparable to the impact of transforming WT MEFs. The increased cytotoxicity of ATO in transformed cells probably reflect cellular changes associated with the transformed state, which can include shortening of the cell cycle, excision repair deficiency, or an increase in DNA replicon size and many biochemical changes. In addition, ASK1 signaling in response to ATO seems to play a pro-survival role in transformed cells. The current results demonstrating a difference in ATO sensitivity between normal and malignant cells as well as the role of ASK1 in these settings are important because they shed light on ATO as a directed drug targeting tumors and sparing normal cells.

The role of ASK1 in normal and malignant cells is not only attributed to ASK1 activation, but to ASK1 downstream signaling. Published work from our lab has
demonstrated that genetic knockout of SEK1, a direct downstream target of ASK1, conferred reduced sensitivity to ATO-induced apoptosis and JNK activation in MEFs. Here, we show that SEK1 knockdown in NB4 cells resulted in significantly enhanced sensitivity to ATO, mirroring the effects of the ASK1 knockdown NB4 cells. These results support the notion that ASK1 activation and ASK1 signaling are responsible for the differences in sensitivity to ATO in the normal and malignant setting. However, we cannot exclude the possibility of inherent differences between mouse and human cell lines or a knockdown versus a genetic knockout. To address the first possibility, it would be interesting to perform an ASK1 knockdown in a malignant mouse cell line and assess its sensitivity to ATO. The latter possibility can be evaluated by an ASK1 knockdown in wild-type MEF cells.

Given that ATO has been reported to activate p38 and JNK signaling cascade, one instance where ATO signaling leads to cell survival is due to the increased activation of p38 relative to JNK. Activation of p38 signaling cascade has been shown to negatively regulate the induction of apoptosis in leukemic cell lines, where pharmacologic inhibition of p38 potentiates ATO-induced apoptosis and growth inhibition, as well as JNK activation. It would be interesting to evaluate and compare p38 activation in our ASK1 knockdown NB4 cells and ASK1 deficient MEFs in response to ATO. If the activity of the p38 pathway accounts for ATO sensitivity between normal and malignant cells, we would expect greater relative p38 activation in the ASK1 deficient MEFs following ATO treatment. Another possibility for the enhanced sensitivity to ATO in normal versus malignant cells could be attributed to the differential expression of ASK2, an important component of the ASK1 signalosome. ASK1 functions as part of two different high-molecular mass complexes to exert its kinase activity: one with a core structure consisting of a heteromeric complex with ASK2 and one whose core structure is composed of a homo-oligomer of ASK1. Accumulating evidence implicates ASK2 as an important component of the ASK1 signalosome where ASK1 is required to stabilize ASK2 to allow
ASK2 to activate ASK1 by direct phosphorylation to accelerate the autophosphorylation of ASK1. The increased sensitivity to ATO in the ASK1 knockdown NB4 cells may be at least partly attributed to an increase in ASK2 expression as a consequence of the incomplete ASK1 depletion as a compensatory mechanism. This would imply an increase in the formation of the more efficient ASK1 heteromeric complex, resulting in increased ATO-induced ASK1 signaling and cell death.

Overall, our results suggest that ASK1 has an important context-dependent role in ATO sensitivity. In the context of malignant cells, ASK1 may play a pro-survival role. Conversely, ASK1 may mediate apoptotic signals in normal cells. These differences in malignant versus normal cells make ASK1 an interesting therapeutic target to enhance ATO sensitivity in malignant cells.
CHAPTER 3: REGULATION OF ASK1 IN ATO-INDUCED APOPTOSIS

Introduction

ASK1 is a strong mediator of pro-apoptotic signals depending on the cellular context therefore its activity is highly regulated in the cell. ASK1 has been shown to be regulated by numerous players and mechanisms.

To date, there are 2 different phosphatases that have been reported to inactivate ASK1 through the dephosphorylation of the activating phosphorylation site on ASK1 on threonine 838 (humans) and threonine 845 (mice). Protein phosphatase 5 (PP5) is a serine/threonine protein that binds to ASK1 and dephosphorylates ASK1 in response to ROS stress thus providing a negative feedback mechanism.\textsuperscript{195} In addition, the serine/threonine phosphatase, PP2C\textepsilon, negatively regulates ASK1 activation under basal non-stressed conditions to maintain ASK1 in its dephosphorylated state.\textsuperscript{196}

Another regulator of ASK1 is AKT, a serine/threonine protein kinase that mediates cell survival signals by the phosphorylation and inactivation of pro-apoptotic proteins including BAD\textsuperscript{158}, caspase-9\textsuperscript{159} and forkhead (FKHR) family of transcription factors.\textsuperscript{160} AKT has been reported to suppress ASK1 activity induced by ROS and overexpression of ASK1 in 293 cells by associating with ASK1 and phosphorylating its serine 83 residue.\textsuperscript{162}

Tumor necrosis factor-\alpha receptor-associated factors (TRAFs) play an essential role in ASK1 activity regulation. ASK1 has been shown to associate with many members of the TRAF family including TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 but only TRAF2 and TRAF6 play an important role in ASK1 activity, serving as members of the higher molecular mass complex ASK1 signalosome.\textsuperscript{197} Noguchi et al. showed that H\textsubscript{2}O\textsubscript{2}-induced ASK1 activation and cell death were markedly reduced in cells from Traf2\textsuperscript{-/-} and Traf6\textsuperscript{-/-} mice, consistent with the role of TRAF2 and TRAF6 in activating ASK1.\textsuperscript{198}
ASK1 is negatively regulated by 14-3-3 proteins, a highly conserved family of phospho-serine/phospho-threonine binding proteins with a multitude of functions, including roles in metabolism, protein trafficking, signal transduction, apoptosis and cell-cycle regulation.199 Under basal non-stressed conditions, 14-3-3 binds to ASK1 on the basally phosphorylated serine 967 residue on the C-terminal domain of ASK1 to inhibit it.200 Upon stress-induced signaling, ASK1 interacting protein I (AIP1), a Ras-GAP protein that forms a complex with ASK1, facilitates the dissociation of 14-3-3 from ASK1 by recruiting the phosphatase PP2A which dephosphorylates ASK1 at the serine 967 residue (Figure 3.1).201

The most well-characterized negative regulator of ASK1 is thioredoxin 1 (Trx1), a ubiquitously expressed protein that has a variety of biological functions including regulating cellular redox environment, cell proliferation and apoptosis.202 Trx1 contains two cysteine residues within the redox active center responsible for its reducing ability.203 Once oxidized, Trx1 can become reduced by the flavoprotein Trx reductase 1 (TrxR1).204 The interaction of Trx1 and ASK1 depends on the redox status of Trx1. Reduced Trx1 binds to ASK1 under basal non-stressed conditions at the N-terminal region to inhibit ASK1.168 ROS converts Trx1 to its oxidized form, releasing ASK1 from Trx1 inhibition (Figure 3.1). Given that ATO induces apoptosis through an ROS-dependent and ASK1-dependent mechanism in normal cells, we hypothesize that Trx1 plays an important in regulating sensitivity to ATO. Modulation of Trx1 redox state is therefore a potentially attractive strategy to enhance ATO-induced apoptosis. Recently, TrxR1 has been suggested as a new target for anticancer drug development.205

Here, we provide preliminary data on elucidating the role of Trx1 as a regulator of ASK1 in ATO-induced apoptosis. We provide evidence that ATO may activate ASK1 signaling by disrupting the interaction between ASK1 and Trx1.
Furthermore, inhibition of TrxR1, which prevents reduction of Trx1, may enhance ATO-induced apoptosis.

**Figure 3.1** Regulation of ASK1 by Trx and 14-3-3
Materials and Methods

Cell culture

All cells were grown in a humidified chamber at 37°C in a 5% CO₂ environment. NB4 cells (kindly provided by Dr M Lanotte) were maintained in RPMI 1640 media (Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent) and penicillin/streptomycin (Wisent).

Immunoprecipitation

NB4 cells were seeded at 2 x10^6 cells in a T25 flask and allowed to grow overnight. They were treated with various concentrations of ATO for 24 hours or in 2 μM ATO for up to 24 hours. Cells were lysed at 4 °C in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton x-100) supplemented with protease and phosphatase inhibitors (Roche). After determining the protein concentration by Bradford Assay, 750 μg lysates in 500 μL of lysis buffer were prepared and pre-cleared in protein A agarose beads (Sigma) for 1 hour at 4 °C under gentle nutation. An aliquot of 50 μL was taken as the input control for Western blotting to confirm an equal amount of ASK1 and Trx1. Lysates were nutated in 1 μg of ASK1 antibody (Santa Cruz) overnight at 4 °C. The cell lysates were then nutated with 40 μL of 50% beads for 6 hours at 4 °C to capture the immune complexes. The beads were washed extensively with lysis buffer and the protein was eluted in 55 μL of 2X SDS loading buffer. The proteins were resolved on a SDS-PAGE for subsequent Western blotting.

Thioredoxin Reductase Assay

NB4 cells were seeded at 2 x10^6 cells in a T25 flask and allowed to grow overnight. They were treated with various concentrations of ATO for 24 and whole cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl (pH 8), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS)
containing protease/phosphatase inhibitors (Roche) at 4 °C. The measurement of TrxR1 activity was detected using a DTNB reduction assay kit from Cayman Chemical Company following the manufacturer’s instructions. Briefly, 160 ug of cell lysate in 20 μL was mixed thoroughly with 20 μL NADPH, 20 μL (5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB), with or without 20 μL sodium aurothiomalate (ATM) and a variable volume of TrxR assay buffer (50 mM potassium phosphate, pH 7.0, containing 50 mM KCl, 1 mM EDTA, and 0.2 mg/mL BSA) to make it up to a volume of 200 μL in a 96 well plate. The reaction solutions using rat liver TrxR instead of sample cell lysate was set up as a positive control. The microtiter plate was shaken gently for 10 seconds and the absorbance was recorded once every minute at 405-414 nm using a plate reader. The reaction rate of each sample was expressed as a percentage of the untreated sample.

**PI stain**

NB4 were seeded at 1 x 10^5 cells/mL in 24 well plates. Cells were treated with 1 μM ATO in combination with 1 or 2.5 μM Auranofin for 24 hours. Cells were collected and washed in PBS supplemented with 5% FBS and 0.01 M NaN_3. They were then pelleted and resuspended in 0.2 mL hypotonic fluorochrome solution (50 mg/ml propidium iodide (Sigma), 0.1% sodium citrate, 0.1% Triton X-100). Fluorescence was detected by flow cytometry and analysis was performed using FloJo v7.6.2. Ten thousand events were recorded per sample and analyzed for cell cycle and apoptosis. Cells undergoing DNA fragmentation and apoptosis were defined as events with fluorescence weaker than the G_0-G_1 cell cycle peak (Sub-G_0).

**Western Blot**

Cell extracts were prepared by washing 5 x 10^6 cells with cold PBS and resuspending cell pellets in 0.3 mL radioimmunoprecipitation assay buffer (50
mmol/L Tris-HCl (pH 8), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease/phosphatase inhibitors (Roche) at 4 °C. Extracts were sonicated using a Sonic Dismembrator (Model 300; Fisher Scientific) for 2 x 10 seconds at 15 % intensity and then centrifuged at 13 000 rpm in a microcentrifuge at 4 °C for 10 minutes. Supernatants were transferred to fresh tubes and protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad). To detect phospho-14-3-3 serine 58 and 14-3-3, 50 μg of protein was prepared. Volumes of samples were added to an equal volume of 2x sample buffer and run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and stained with 0.1 % Ponceau S in 5 % acetic acid to ensure equal protein loading. Membranes were then blocked with 5% milk in TBS containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. The membrane was then hybridized overnight at 4 °C with antibody against phospho-14-3-3 serine 58 (Abcam, 1:1000), 14-3-3 (Abcam, 1:1000), JNK (Santa Cruz, 1:500) and Trx1 (Santa Cruz, 1:500). Membranes were washed 4 x with TBST for 10 minutes each wash and then incubated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 1 hour at room temperature. Membranes were once again washed 4 x with TBST for 10 minutes each and the peroxidase activity was visualized by enhanced chemiluminescence (ECL; GE Healthcare Biosciences-Amersham).

**Statistical analysis**

All statistical tests aiming at evaluating significance were determined by analysis of variance (one-way analysis of variance) followed by Newman–Keuls post-tests using Prism version 4.03 (GraphPad software, San Diego, CA, USA).
Results

**ATO disrupts the interaction between ASK1 and Trx1**

One model of ASK1 regulation stipulates that ASK1 is held in an inactive form by reduced Trx1, but becomes liberated when Trx1 is oxidized. To determine if ATO affects the interaction between ASK1 and Trx1, NB4 cells were treated with various concentrations of ATO for 24 hours (Figure 1A) or in 2 μM ATO for up to 24 hours (Figure 1B). The cell lysates were immunoprecipitated for ASK1 and immunoblotted for Trx1. The results indicate a strong basal association between ASK1 and Trx1 that is lost with as little as 0.5 μM ATO in 24 hours or 2 μM ATO in 1 hour. The results suggest the ATO disrupts the interaction between ASK1 and Trx1.

**Inhibition of thioredoxin reductase activity by ATO**

Thioredoxin reductase converts oxidized thioredoxin back into reduced thioredoxin. We wanted to determine whether ATO can inhibit TrxR1 activity, resulting in oxidized Trx1. NB4 cells were cultured in various concentrations of ATO for 24 hours and TrxR1 activity was assessed by a DTNB reduction assay from Cayman Chemical Company. TrxR1 activity was significantly reduced with as little as 0.5 μM ATO. When the ATO concentration was greater than 1 μM ATO, TrxR1 activity was completely inhibited. These results suggest that ATO may mediate ASK1 signaling partly through preventing the restoration of reduced thioredoxin.

**Inhibition of TrxR1 enhances ATO-induced apoptosis**

Given the role of reduced thioredoxin and its restoration by TrxR1 to inhibit ASK1 signaling, we hypothesized that inhibition of TrxR1 activity may enhance ATO-induced apoptosis. To test this, we treated NB4 cells with both ATO and an Auranofin, a TrxR1 inhibitor. We assessed cell death in response to the
combination treatment and observed significantly enhanced cell death with combination treatment compared to ATO alone. These results are promising, indicating a potential combination therapy with ATO and thioredoxin reductase inhibitors.

**ATO induces serine 58 phosphorylation of 14-3-3 proteins**

Independent of the Trx1 system of ASK1 regulation, 14-3-3 may also sequester ASK1 by direct binding as a dimer. Since the serine 58 phosphorylation on 14-3-3 is required for dimerization and client protein binding, we wanted to determine if ATO signaling phosphorylates 14-3-3 at serine 58 thereby preventing dimerization and interaction with ASK1. Indeed, when we evaluated phospho-14-3-3 serine 58 protein levels with immunoblotting in NB4 cells treated with ATO, we observed a time-dependent increase in serine 58 phosphorylation. This is consistent with the 14-3-3 model of ASK1 regulation.
Figure 1: ATO disrupts interaction between ASK1 and Trx1. NB4 cells were cultured in various concentrations of ATO for 24 hours (A) or in 2 μM ATO for different durations (B). The cell lysates were immunoprecipitated for ASK1 and the complexes were immunoblotted for Trx1.
Figure 2: ATO decreases thioredoxin reductase activity. NB4 cells were treated with 0.5, 1 and 2 μM ATO for 24 hours. Thioredoxin reductase activity was assessed using the Thioredoxin Reductase Assay Kit (a DTNB reduction assay) from Cayman Chemical Company. Asterisks indicate significant differences between the untreated control and the indicated treatment (* p<0.05; ** p<0.01; *** p<0.001).
Figure 3: A thioredoxin reductase inhibitor enhances ATO-induced apoptosis. NB4 cells were treated with 1 μM ATO and various concentrations of Auranofin, a thioredoxin reductase inhibitor. Apoptosis was assessed by PI staining. Asterisks indicate significant differences between the ATO treated and the combination treatment (* p<0.05; ** p<0.01; *** p<0.001).
Figure 4: ATO induces serine 58 phosphorylation of 14-3-3. NB4 cells were treated with 1 μM ATO for up to 6 hours and immunoblotted for phospho-14-3-3-S58 and 14-3-3.
Discussion

The role of ASK1 in ATO-induced apoptosis depends on cellular context. It is capable of playing both a pro-apoptotic and pro-survival role; therefore its regulation can determine the sensitivity to ATO. However, the molecular mechanism of ASK1 regulation in response to ATO is not well known.

Here we explored the role of Trx1 in the regulation of ASK1 in ATO-induced apoptosis. Reduced Trx1 has been reported to bind and sequester ASK1, while oxidized Trx1 dissociates from ASK1. Since ATO induces apoptosis through strong oxidative stress signals, the oxidation of Trx1 may be induced by ATO which leads to ASK1 activation and ASK1-mediated cell death. Our preliminary results indicate that ATO disrupts the interaction between ASK1 and Trx1 and inhibits the activity of thioredoxin reductase 1, the enzyme that converts oxidized Trx1 back into reduced Trx1. We further show that a thioredoxin reductase inhibitor may enhance ATO-induced cytotoxicity. Independent from the Trx1 regulation of ASK1, we also provide preliminary evidence that ATO induces the phosphorylation of 14-3-3 at serine 58, thereby preventing the dimerization of 14-3-3 and the sequestration of ASK1.

Consistent with the working hypothesis, ATO treatment in NB4 cells disrupted the interaction between ASK1 and Trx1 in a sustained manner, with as little as 0.5 μM, a concentration that does not induce apoptosis. Temporally, the ASK1-Trx1 interaction disruption preceded ATO-induced cell death and correlates with ATO-induced ROS generation. Presumably, reduced Trx1 is oxidized to release ASK1 thus we expect the ratio of oxidized to reduced Trx1 to increase. To test this hypothesis, it would be interesting to evaluate the change in redox state induced by ATO. Whole cell lysates treated with ATO can be alkylated with 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid (AMS) and run on a non-reducing Western blot to differentiate between the reduced (alkylated) and non-reduced (non-alkylated) proteins. We also expect that co-treatment with ATO and
N-acetyl-cysteine (NAC) at concentrations that decrease ATO-signaling will restore the oxidized Trx1:reduced Trx1 ratio.

The sustained loss in interaction between ASK1 and Trx1 in response to ATO could be partly attributed to the ATO-induced decrease in TrxR1 activity. While 0.5 μM ATO significantly decreased TrxR1 activity, a complete loss of TrxR1 activity was observed with the 1 μM treatment, the minimum dose needed to induce apoptosis in NB4 cells. To confirm the role of TrxR1, it would be interesting to overexpress TrxR1 and assess the impact on ATO-induced JNK activation and ASK1 activation.

We have begun to identify inhibitors of Trx1 and TrxR1 to maintain Trx1 in its oxidized state and unable to bind ASK1 thus synergizing with ATO cytotoxicity. One such compound is Auranofin, a potent thioredoxin reductase inhibitor. Preclinical studies have already implicated Auranofin in inducing apoptosis in a variety of cancer lines including cisplatin-resistant human ovarian cancer cells and adriamycin-resistant human K562 chronic myeloid leukemia cells through the inhibition of thioredoxin reductase. Our data suggests that Auranofin enhances ATO-induced apoptosis in NB4 cells and that targeting TrxR1 is a potential basis for cancer therapy by ATO. However, Auranofin may not mediate its pro-apoptotic effects through ASK1 regulation. We have previously shown that ASK1 play opposite roles in transformed versus normal cells. While it is activated in NB4 cells in response to ATO, it may mediate pro-survival signals (Chapter 2). Since inhibition of TrxR1 with Auranofin results in more oxidized Trx1 and ASK1 activation, we would expect increased cell survival in NB4 cells instead of the observed increase in cell death. Thus, inhibition of TrxR1 may induce cell death through an ASK1-independent mechanism. It is possible that Auranofin may enhance ATO-induced cytotoxicity independent of Trx1 regulation since it has other targets.
Independent of the role we find for Trx1 in ASK1 regulation, other proteins may also regulate ASK1 activation in response to ATO although their contribution and exact mechanism remains to be elucidated. These proteins include 14-3-3 proteins. Our data shows that serine 58 of 14-3-3 becomes phosphorylated in response to ATO, suggesting that 14-3-3 dimerization and protein binding is inhibited. While the release of ASK1 is one possibility of integrating apoptotic signals, 14-3-3 is also implicated in promoting survival by sequestering pro-apoptotic proteins upon phosphorylation by AKT.\(^{210}\) ATO-induced phosphorylation of the serine 58 residue of 14-3-3 may also prevent 14-3-3 from sequestering pro-apoptotic proteins, therefore leading to the release of pro-apoptotic proteins and cell death.

In summary, we provide preliminary data that Trx1 plays an important role in the regulation of ASK1 and that targeting TrxR1 is a potential basis for cancer therapy by ATO. The use of ATO and Auranofin is a potential rational combination therapy that increases the cytotoxicity of ATO and may increase its therapeutic spectrum.
CHAPTER 4: CONCLUSION

ATO has been demonstrated to possess important anti-tumour properties and is an effective treatment for APL. It has become increasingly clear through numerous clinical studies that elucidating the mechanism of ATO-induced apoptosis will allow for the identification of targets to generate rational combination therapies, enhance the cytotoxicity of ATO, and increase its therapeutic spectrum. Our lab has already elucidated a potential pathway involving ROS generation and JNK activation. Our findings in Chapter 2 aimed to characterize the role of ASK1, a well-documented mediator of stress-induced apoptosis, in our proposed pathway of ATO-induced cell death. Interestingly, we found opposite roles of ASK1 in normal versus transformed cells. In normal un-transformed cells, ASK1 plays a pro-apoptotic role. Transformation significantly increased sensitivity to ATO-induced apoptosis and growth inhibition, suggesting that ASK1 can have both pro-apoptotic and anti-apoptotic roles depending on the transformation state of the cells.

The importance of ASK1 in sensitivity to ATO led us to investigate the regulation of ASK1. We focused on the role of Trx1 since it is the most well characterized regulator of ASK1. Our preliminary data shows that ATO disrupts the interaction between ASK1 and Trx1. Furthermore, it decreases the activity of TrxR1, presumably leading to more oxidized Trx and enhanced ASK1 signaling. Interestingly, co-treatment of NB4 cells with ATO and Auranofin, an inhibitor of TrxR1, resulted in enhanced cell death. However, inhibition of TrxR1 may increase cell death in an ASK1-independent mechanism since ASK1 was shown to play a pro-survival role in NB4 cells. While the mechanism of action remains to be elucidated, targeting TrxR1 is a potential basis for cancer therapy by ATO.
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