University of Alberta

An investigation of aminoglutethimide cytotoxicity and its role in activation of cell death signaling pathways

by

Argishti Baghdasarian

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

PHARMACEUTICAL SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

Fall 2013
Edmonton, Alberta

ABSTRACT

Aminoglutethimide (AG), a drug used for the treatment of breast and ovarian cancers, is known to cause toxicities such as agranulocytosis. To investigate its toxicity mechanisms, we have used quantitative proteomic analysis to gain insight into the proteome of Human Leukemia 60 (HL-60) treated with AG. We identified 43 proteins that were changed significantly upon AG treatment among which 18 (42%) and 25 (58%) were up and down-regulated, respectively. The quantitative proteomics data showed that AG treatment led to the down-regulation of critical anti-apoptotic proteins responsible for inhibiting the release of pro-apoptotic factors from the mitochondria as well as cytoskeletal proteins such as nuclear lamina. This overall pro-apoptotic response was confirmed with flow cytometry which demonstrated apoptosis to be the main factor of cell death. This response correlated with the intensity of AG-induced protein radical formation in HL-60 cells, which may have an important role in cell death signaling mechanisms.

ACKNOWLEDGMENT:

My deepest appreciation goes to my supervisor, Dr. Arno G. Siraki for his continuous support and encouragement throughout the project. His willingness to grant me the freedom to learn and apply new tools to explore pharmaceutical sciences made the completion of this project possible.

I would also like to thank my supervisory committee members Dr. Richard Fahlman and Dr. John Seubert for their support and advice in the project.

Many thanks to my colleagues: Dr. Karim Michail, for his continuous support and encouraging words from the early stage of the project; Mr. Saifur Md. Khan, for his extensive collaboration and team effort with applying proteomic tools to my research; and Mr. Naif Aljuhani, for his support and encouragement.

I would like to extend my gratitude again to Dr. Arno G. Siraki, the Faculty of Pharmacy and Pharmaceutical Sciences, the University of Alberta, the Queen Elizabeth II Graduate Scholarship, and the Pfizer Canada Inc. Pharmacy Research Award for their financial support.

CONTENTS

LIST OF TABLES:

LIST OF FIGURES:

LIST OF ABBREVIATIONS:

ABAH	4-Aminobenzoic hydrazide
ACP-1	Acid phosphatase 1
ACTH	Adrenocorticotropic hormone
AG	Aminoglutethimide
ANP32B	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B
Apaf-1	Apoptotic protease activating factor-l
cAMP	Cyclic AMP
Bax	Bcl-2-associated X
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CARD	Caspase-recruitment domain
Caspases	Cysteine-dependent aspartate-directed proteases
CREB	cAMP response element-binding protein
CS	Cushing's syndrome
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DMPO	5,5-dimethyl-1-pyrroline N-oxide
ELISA	Enzyme-linked immunosorbent assay
FasL	Fas ligand
FasR	Fas receptor
FITC	Fluorescein isothiocyanate

FDA	Food and drug administration
GSN	Gelsolin
G/GO	Glucose/Glucose oxidase
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HL-60	Human promyelocytic leukemia cells
IDR	Idiosyncratic drug reactions
IL	Interleukin
LH	Luteotropic hormone
MD	Menadione
MS	Mass spectroscopy
MPO	Myeloperoxidase
NCCD	Nomenclature Committee on Cell Death
$O_2^{-\bullet}$	Superoxide anion radical
ODC	Ornithine decarboxylase
$P450_{11B1}$	11-beta hydroxylase
$P450_{21}$	21-alpha hydroxylase
$P450_{scc}$	Side Chain Cleavage Enzyme/20, 22-desmolase
PI	Propidium iodide
PIDDosome	Protein with a death domain
PP1CA	Protein phosphatase 1
PS	Phosphatidylserine
PKA	Protein Kinase A

ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSB	Sjogren syndrome antigen B
T_4	Thyroxine
TCEP	Tris-(2-Carboxyethyl)phosphine
TNF	Tumor necrosis factor
TPT1	Transitionally controlled tumor protein 1
UBE2K	Ubiquitin-conjugating enzyme E2K
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1

Introduction

1.1 Aminoglutethimide

1.1.1 Chemical properties

Aminoglutethimide (AG) or (*RS*)-3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione is a drug marketed as Cytadren® by Novartis pharmaceuticals. AG belongs to a family of aniline based aromatic amine compounds. It has a molecular formula of $C_{13}H_{16}N_2O_2$ and weight of 232.28 g/mol. It has a fine, white or creamy white crystalline powder texture. It also has a melting point of 150-159 ^{o}C and is freely soluble in most organic solvents.[1]

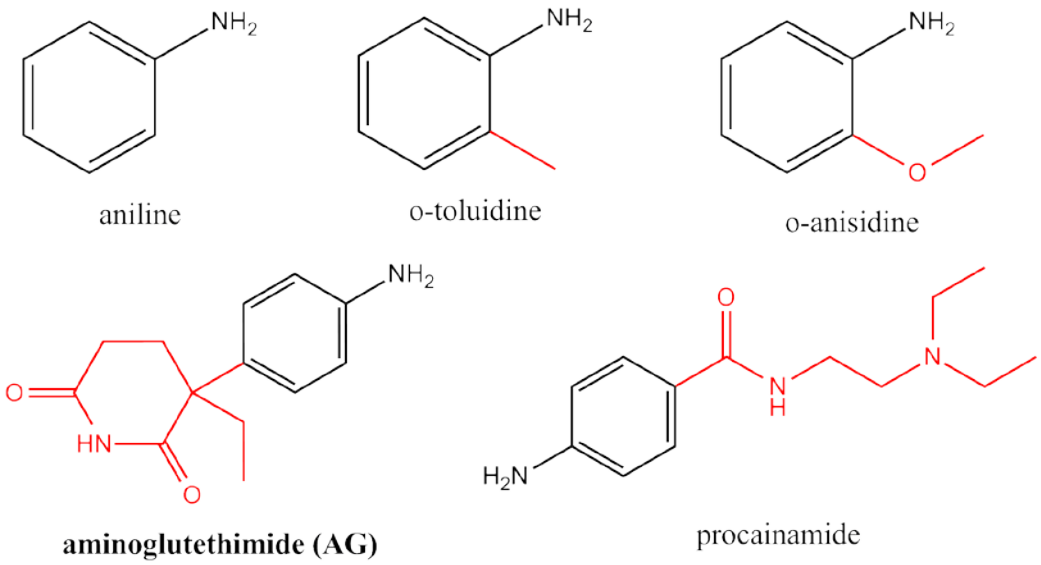


Figure 1.1 Structures of aniline based aromatic amine compounds

1.1.2 Pharmacokinetics & metabolism

1.1.2.1 Absorption

AG is rapidly and completely absorbed after oral administration. In one study, it was reported that six healthy male volunteers having ingested 250 mg tablets of AG experienced a maximum average plasma concentration of 5.9 μg/mL after 1.5 h duration.[2] In another pharmacokinetic study, radio-labelled AG was administered to 17 patients and confirmed that absorption of AG after oral intake was almost complete given its recovery in the urine.[3] The mechanism of AG absorption through the cell membrane has not been studied.

1.1.2.2 Distribution

AG distributes between the plasma and cells of human blood. The concentration in cells was recorded to be 1.4-1.7 times greater than that of the plasma. The binding of AG to the plasma proteins of human blood, measured by equilibrium dialysis ranged from 5 to 10 pg/mL.[4] It was shown that AG is also capable of crossing through the placenta.[5]

1.1.2.3 Metabolism

The enzymes responsible for AG metabolism are suggested to occur largely by the hepatic cytochrome P450 enzymes. For example, AG has been shown to induce the expression of CYP2B1 of rat liver which provides indirect evidence for its metabolism.[6] Nevertheless, there have been extensive studies

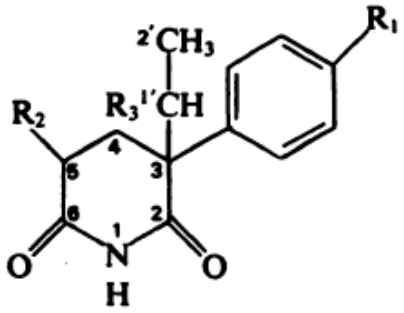
identifying the various metabolites of AG *in vivo*. The first and the major metabolite of AG was N-acetylaminoglutethimide in rats, guinea-pigs, and rabbits (with the exception of humans and dogs where the drug is mainly unchanged).[5, 7-9] Formyl-aminoglutethimide and p-nitroglutethimide were also reported later by Baker *et al.* (1981).[10] Jarman *et al.* (1983) reported hydroxylaminoglutethimide (3-ethyl-3-(4-hydroxylaminophenyl)-2,6-piperidinedione) as a novel metabolite of AG in the urine of patients treated chronically. It was shown that the formation of hydroxylaminoglutethimide was at the expense of N-acetylaminoglutethimide.[8, 11] Foster *et al.* (1984) reported 4 minor urinary metabolites of AG in humans (*p*-amino-5-hydroxyglutethimide, *p*-acetyl-amino-5-hydroxyglutethimide, *p*-amino-2'hydroxyglutethimide, and *p*-amino-1'-hydroxyglutethimide).[12] Dayrmple *et al.* (1988) reported 3-(4-acetamidophenyl)-3-(2-carboxamidoethyl)tetrahydrofuran-2-one, a novel metabolite not previously found in human urine.[9] The name and structures of urinary metabolites of AG are shown in Table 1.1.

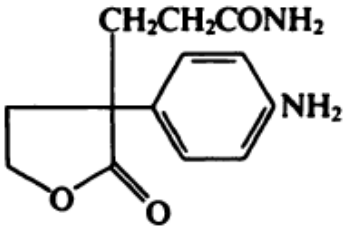
The half-life of AG is estimated to be 12.5 h and is shown to be reduced to 7 h after prolonged (32 weeks) treatment due probably to induction of hepatic enzymes that accelerate its metabolism.[5] During the first 48 h after the intake of AG, 34% to 54% of the oral dose is excreted in the urine unchanged and an additional fraction as the N-acetyl derivative.[2] A study showed 89% excretion of a single dose ^{14}C labelled AG administered to rats, guinea-pigs, rabbits and humans from urine and feces (51% in dogs) within 72 h.[9] A study exclusively done on rats

showed complete elimination within 48 h in urine and feces with doses of 5 and 50 mg/kg.[13]

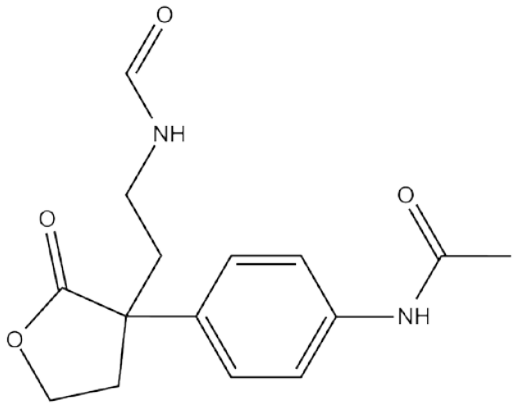
TABLE 1

Structures of aminoglutethimide and urinary metabolites in humans identified previously (reference given) and during the present study





Compound	R_1	R_2	R_3
Aminoglutethimide	NH_2	H	H
Acetylaminoglutethimide	$NHCOCH_3$	H	H
p-Nitroglutethimide	NO_2	H	H
Formylaminoglutethimide	NHCHO	H	H
Hydroxylaminoglutethimide	NHOH	H	H
p-Amino-5-hydroxyglutethimide	NH_2	OH	H
p-Acetylamino-5-hydroxyglutethimide	$NHCOCH_3$	OH	H
Lactone from *p*-amino-2′-hydroxyglutethimide	Structure shown above		
p-Amino-1′-hydroxyglutethimide	NH_2	H	OH



3-(4-acetamidophenyl)-3-(2-carboxamidoethyl)tetrahydrofuran-2-one

Table 1.1 Structures of urinary metabolites of AG from several studies

AG, Acetyl-AG, p-Nitroglutethimide, formyl-AG, hydroxyl-AG, p-amino-5-hydroxyglutethimide, lactone from p-amino-2'-hydroxyglutethimide, p-amino-1'-hydroxyglutethimide, and 3-(4-acetamidophenyl)-3-(2-carboxamidoethyl)tetrahydrofuran-2-one. Table adapted from Foster *et al.* (1984) with permission. (Copyright 1984 Drug Metabolism and Disposition).[12]

1.1.2.3.1 MPO and its mechanistic role in AG metabolism

A more recent study by Siraki *et al.* (2007) has shown in human promyelocytic leukemia (HL-60) cells that AG is capable of utilizing myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) to generate protein radicals (mainly MPO radicals) using polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot immuno-spin trapping (discussed further in chapter 3.3.2) (Figure 1.2). The presence of H_2O_2 was also shown to be necessary for the production of protein radicals.[14]

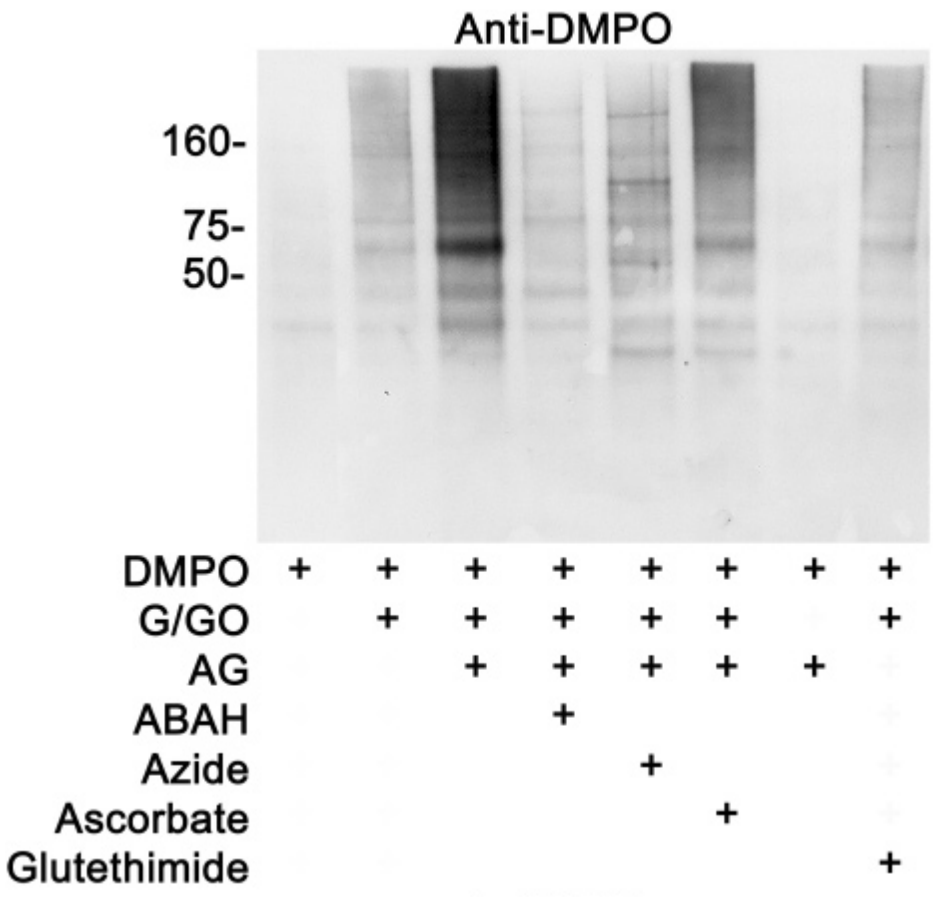


Figure 1.2 Western blot of HL-60 cell cytosol after incubation with AG and inhibitors. This Western blot demonstrates the detection of protein-DMPO in lane 3 which contains AG, H_2O_2, and DMPO (to trap protein radicals). Lane 4 containing the MPO inhibitor, 4-aminobenzoic acid hydrazide (ABAH), attenuated the detection of protein radicals suggesting that MPO metabolism of AG is necessary for protein radical formation. Adapted from Siraki *et al.* (2007) with permission. (Copyright 2007 American Chemical Society).[14]

MPO is a member of the heme peroxidase-cyclooxygenase superfamily that is found in abundant quantities in neutrophils. MPO is involved in the innate

immune defensive system by forming microbicidal oxidants (e.g. hypochlorous acid, HOCl) and radical metabolites from xenobiotics and drugs ($AH^{\bullet}$) during the respiratory burst of neutrophils (which occurs during an immune triggered by pathogens).[15] MPO is involved in two main reactions, halogenation and peroxidative reactions. The reaction begins when the resting iron (III) form of MPO is oxidized by H_2O_2 to form compound I, an iron (V) species (equation 1).

$$MPO^{3+} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

There are two known fates for compound I: it can undergo two electron reduction with a halide (i.e., Cl^-) to form hypochlorous acids (halogenation reaction – equation 2) or go through two successive one electron reduction steps to become compounds II and subsequently convert a substrate AH_2 (e.g. AG) to a radical ($AH^{\bullet}$) (peroxidative reaction – equation 3 and 4).[16]

$$\text{Compound I} + X^- + H^+ \rightarrow MPO^{3+} + HOX \quad (2)$$

$$\text{Compound I} + AH_2 \rightarrow AH^{\bullet} + \text{Compound II} \quad (3)$$

$$\text{Compound II} + AH_2 \rightarrow AH^{\bullet} + MPO^{3+} \quad (4)$$

The iron (III) form of MPO can also reversibly undergo one electron reduction with superoxide radical ($O_2^{\bullet -}$) to form compound III, a catalytically inactive enzyme state (equation 5).

$$MPO^{3+} + O_2^{\bullet -} \rightleftharpoons \text{Compound III} \quad (5)$$

As seen in equation 1, MPO consumes H_2O_2 and the rate of H_2O_2 utilization can be monitored by an H_2O_2 electrode.[17]

1.1.3 Pharmacology

1.1.3.1 Therapeutic uses and mechanism of action

1.1.3.1.1 Anticonvulsant activity

Originally, the study by Gross *et al.* (1956) enabled the marketing of AG as an anticonvulsant in the United States by May 1960.[18] However, due to some side-effects such as rashes, dizziness, drowsiness, behavioural changes, ataxia, headache, leukopenia, respiratory depression, etc. the American Medical Association's Council in 1962 concluded that due to its limited efficacy and high frequency of deleterious reactions (50% of patients treated), AG should be used as a supplement to other anticonvulsants of which the patients seem to not respond to.[19]

1.1.3.1.2 Corticosteroid biosynthesis inhibitor

1.1.3.1.2.1 Biochemical changes

The earliest study dates back to 1960's where the adrenal insufficiency was seen from children treated with AG. The supply of adrenocorticotropic hormone (ACTH) did not result in an increase in hydroxycorticoids even after the withdrawal of the drug. The unresponsive nature to ACTH supplements was also observed with AG treated puppies.[20, 21] Rats even experienced drug induced adrenal hypertrophy.[22]

1.1.3.1.2.2 Histological changes

Post-mortem studies of patients receiving AG revealed significant histological changes such as cellular hypertrophy, cytoplasmic vacuolation and excessive accumulation of lipid in the cortex of the glands.[20] Similar findings included increase in size and weight of the adrenal glands, an increased size of the zona fasciculata (middle zone of the adrenal cortex).[23]

1.1.3.1.2.3 Adrenocortical inhibition

1.1.3.1.2.3.1 Cytochrome P450 inhibition

Five known enzymes involved in steroidogenesis are known to be inhibited by AG and are shown in Figure 1.3: CYP11A1 (side chain cleavage enzyme) also known as 20, 22-desmolase, CYP21A2 (21-alpha hydroxylase),

CYP11B2 (aldosterone synthase, 18-hydroxylase), CYP11B1 (11-β-hydroxylase) and CYP19A1 (aromatase).

First, there have been numerous reports showing the inhibition of adrenal cholesterol metabolizing enzyme called CYP11A1 involved in the conversion of mitochondrial cholesterol into pregnenolone.[24-27] In addition, there was also evidence of CYP11A1 inhibition in rat brain mitochondria and rat Leydig cells.[28, 29] Second, another P450 enzyme called $P450_{11B1}$ was shown to be susceptible to inhibition by AG thus lowering the levels of cortisol *in vitro*.[30, 31] Third, the *in vitro* inhibition of P450-dependent 18-hydroxylation of corticosterone to produce aldosterone by aldosterone synthase was reported.[32] Fourth, there is some evidence of inhibition of 21-hydroxylation ($P450_{21}$) responsible for the conversion of progesterone to 11-deoxycorticosterone.[33] Fifth and final target of AG is not the inhibition of adrenal biosynthesis of the cortisol and androgens, but rather estradiol. It has been well documented that AG is capable of inhibiting $P450_{arom}$, an enzyme involved in hydroxylation of androstenedione to estrone and testosterone to estradiol, a key inducer of cell growth in breast cancer cells.[26, 34-36] In fact, AG has been the most widely studied aromatase inhibitor.[37] A study conducted by Harris *et al.* (1983) concluded, based on a 1 week study on 13 post-menopausal patients with progressive advanced breast cancer, that a low AG dose of 125 mg a day was an effective inhibitor of peripheral production of estrogen in post-menopausal patients.[38] It has been reported *in vitro* that $P450_{arom}$ is 10 times more sensitive to inhibition than $P450_{scc}$.[39] Interestingly, the isomeric state of AG

also dictates the degree of estrogen synthesis inhibition. According to a placental microsomal study, 50% inhibition was achieved with only 8 µM of d-AG compared to 310 µM for l-AG.[40]

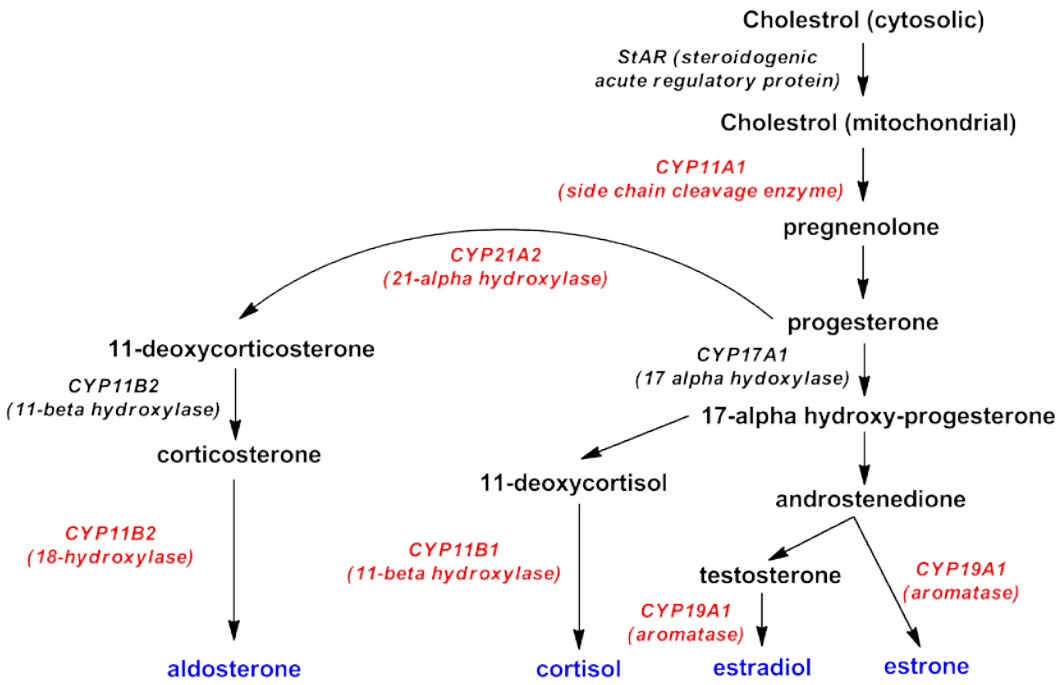


Figure 1.3 Inhibitions of various P450 enzymes by AG

AG is known to directly inhibit 5 P450 enzymes: P450scc (side chain cleavage enzyme), $P450_{21}$ (21-alpha hydroxylase), Aldosterone synthase (18-hydroxylase), $P450_{11B1}$ (11-beta hydroxylase) and $P450_{aromatase}$.

1.1.3.1.2.3.1.1 Mechanism of inhibition

According to Brueggemeier (1994), AG along with other inhibitors such as fadrozole and vorozole are competitive inhibitors that bind non-covalently to the active sites of P450 enzymes. They possess a heteroatom, (i.e. the nitrogen atom of AG's amine group) that interferes with the steroid hydroxylation by binding to the P450 enzyme's heme iron. AG is less enzyme specific which

explains the wide of range of P450 enzyme inhibitions.[41] Similar findings stated that the inhibition depends on the presence of the amino group in the 4'- position of the phenyl ring.[42]

1.1.3.1.2.3.1.2 PKA inhibition

Direct inhibition of the P450 enzymes is not the only way AG affects the steroidogenic pathway. It has been reported that AG can achieve the same goal by inhibiting upstream pathways involved in steroidogenesis. As shown in Figure 1.4, the pituitary gland can secrete hormones such as ACTH and luteotropic hormone (LH) that can bind to receptors located on steroidogenic tissues such as the adrenal gland. The binding of these ligands to their receptors has been associated with an increase in cyclic AMP (cAMP) levels and the activation of protein kinase A (PKA) signalling pathway. The PKA signalling pathway is associated with the activation of cAMP response element-binding protein (CREB) transcription factor involved in transcription of steroidogenesis related genes.[43-47] There are also studies showing the induction of ornithine decarboxylase (ODC), a rate-limiting enzyme involved polyamine biosynthesis, by ACTH.[48] Polyamines play a role in cellular growth and transformation.[49]

A study compared the presence of AG on the ACTH-induced ODC expression levels in gonads and adrenal glands, and concluded that the treatment of AG induced significant reduction in ODC expression. They followed that observation with *in vitro* and a protein kinase A/C activity assay experiments and

found that AG inhibition of ODC expression comes not at the cAMP level but rather through direct inhibition of PKA activity.[50]

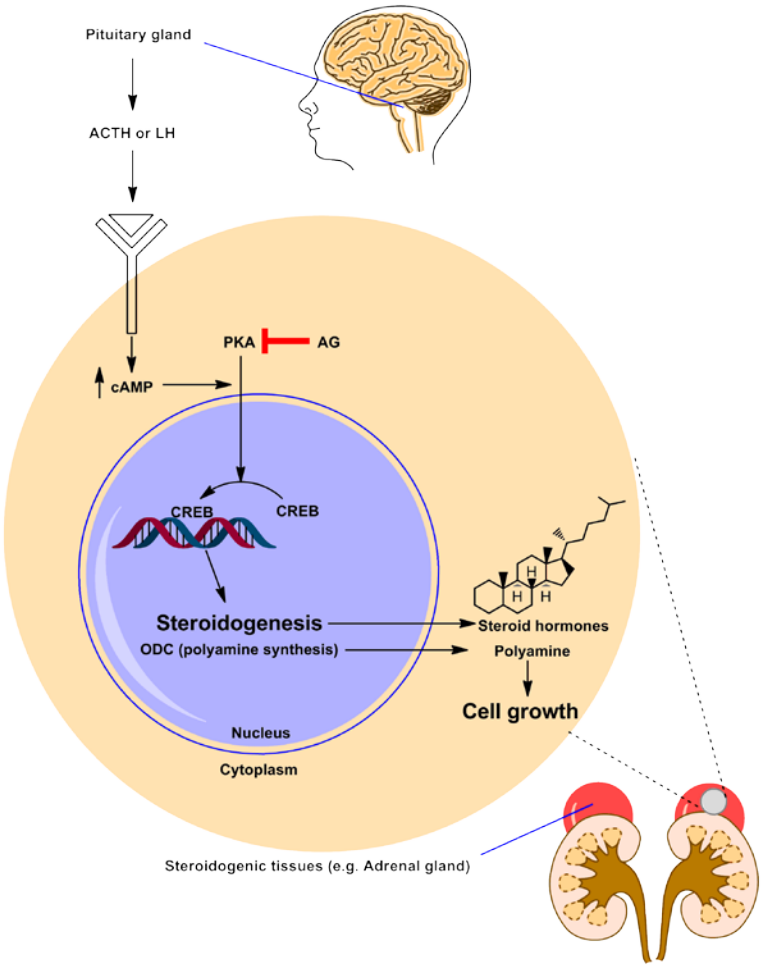


Figure 1.4 Role of AG in PKA-induced steroidogenesis signaling pathway

AG is reported to inhibit PKA activity, preventing it to activate the transcription factor CREB required for transcription of genes involved in steroidogenesis.

1.1.3.1.2.3.1.3 Estrogen dependent breast cancer therapy

Perhaps the most extensive use of AG has been for breast cancer therapy. Breast cancer cells require estrogen as their growth stimuli. AG inhibits estrogen production through inhibiting $P450_{arom}$. According to American Cancer Society's

Breast Cancer Facts and Figures, in 2011, an estimated 230,480 new cases of invasive breast cancer will be diagnosed among women. In 2011, approximately 39,520 women are expected to die from breast cancer.[51]

AG co-administered with hydrocortisone (hydrocortisones were co-administered with AG to account for the additional inhibitory effect of AG on cortisol production) has been a successful regimen for the treatment of estrogen-dependent breast cancer for post-menopausal women.[52-55] Gale (1982) conducted a 14 year study observing 91 women with advancing metastatic breast cancer from 1967-1981. The results of that study showed that the median survival time of the responding patients was 22 months compared to 2 months for non-responding patients and no deaths were related to drug toxicity. The conclusion of that study was that AG provided a response rate equal to those patients undergone the traditional surgical adrenalectomy for the treatment of advanced breast cancer.[56] A study by Smith *et al.* (1981) involved the administration of 250 mg of AG four times a day with 20 mg of hydrocortisone twice a day to 57 patients. The results of that study were 30% and 23% of the patients achieved objective response and stable disease statuses respectively.[57] Lipton *et al.* (1982) conducted a randomized trial with the administration of 1000 mg of AG daily with corticosteroid for 10 months. The result were a 47.5% response rate in the 21 women treated.[58] A study with similar conditions confirmed the results with a 48% response rate in 31 patients.[59]

1.1.3.1.2.3.1.4 Cushing's syndrome

Cushing's syndrome (CS) is a rare syndrome characterized by a prolonged exposure to excessive levels of cortisol. CS can be caused by a cortisol-producing adrenal tumour or excessive secretion of ACTH from a pituitary tumour.[60]

Schteingart *et al.* (1966) was the first group to report on the use of AG in CS in a patient with metastatic adrenal carcinoma.[61] A study by Misbin *et al.* (1976) reviewed the efficacy and tolerability of 66 cases of AG use in CS. AG was able to improve the symptoms of hypercorticism in 13 of the 21 patients with metastatic adrenocortical carcinoma and 4 of 6 patients with ectopic ACTH production due to metastatic carcinomas. All six of the patients with adrenal adenomas showed improvements in their clinical and biochemical status. Finally, 14 out of the 33 patients with bilateral adrenal hyperplasia of pituitary improved as well. Overall, the authors concluded that AG has a potential for controlling the signs and symptoms of excessive adrenocorticoid in patients with CS.[62] A study in Singapore described a man with CS who could not be treated surgically due to his inter-current medical conditions was successfully treated with 750 mg of AG per day. The authors considered AG as a viable non-surgical alternative to treatment of CS.[63]

1.1.3.1.2.4 Gonadal steroid production

As shown in Figure 1.3, pregnenolone is a major precursor to all steroid hormones; it should be no surprise that AG can inhibit gonadal steroid production

by inhibiting the previously discussed $P450_{scc}$ and $P450_{arom}$, needed for the conversion of cholesterol to pregnenolone and testosterone to estradiol respectively. Compared to adrenocortical inhibition, little research has been done on the effect of AG on gonadal steroid production. Nevertheless, Pittman and Brown (1966) reported ovarian enlargement in female rats, Eversole and Thomson (1967) and Zavadil *et al.* (1968) saw oestrous cycle arrest and sterility.[22, 64, 65] Gaunt et al. (1968) confirmed their results and added an overall uterine weight loss as well to the findings. However, when they investigated male rats, they found no evidence of inhibition of testicular function.[66]

1.1.3.1.3 Anti-thyroid effect

Rallison *et al.* (1967) were the first group to report on the effect of AG on thyroid metabolism. They suggested that AG is able to induce the organification of iodine based on evidence from three children's low protein-bound iodine status, and high thyroidal uptake of radio-iodine. Yet, four additional children receiving the drug experienced euthyroid (normal thyroid function) and no abnormalities of protein-bound iodine or radio-iodine uptake were found.[67] The same group also showed signs of anti-thyroidal effect in rats such as thyromegaly (abnormally enlarged thyroid), diminished production of thyroxine (T_4) and di-iodotyrosine and an accumulation of thyroidal inorganic iodide in rats. Structurally similar compounds such as propylthiouracil and aminobenzene derivatives also blocked the organification of iodine. The contradictory findings from the 7 total patients prompted the group to suggest variations in genetic and

individual sensitivity to be the possible explanation.[68, 69] The results from the animal studies were confirmation of a study conducted a year prior that also showed marked increase in thyroid weight and depression of radio-iodine uptake in rats treated with AG.[22] Overall, these results suggest that AG has inhibitory roles in thyroid metabolism as well as goitrogenic properties.

1.1.3.2 Interactions

Over the past decades several drugs have been reported to interact with AG to increase AG's efficacy and alter the efficacy of the interacting drug(s). Melatonin enhances the inhibitory effect of AG on $P450_{arom}$ activity in MCF-7 human breast cancer cells as a result of the decrease in the aromatase mRNA expression.[70] AG is known to diminish the effects of digitoxin, anti-pyrine and blood thinner warfarin. In one study, daily AG dose of 1000 mg increased the plasma clearance rates of warfarin, digitoxin and antipyrine by 81%- 152%. In another study, a 3-5-fold increase in warfarin clearance was observed upon AG administration. The authors suggested the interaction might be due to AG-induced hepatic microsomal enzymes known to accelerate warfarin metabolism.[71] It has also been reported that AG can accelerate the metabolism of dexamethasone by increasing its metabolic clearance rate by 423 L/24 h.[2, 8, 33] The actual mechanism involved has not been studied but most likely involves the up-regulation of P450 enzymes that metabolize dexamethasone.

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1.1.4 Toxicology

AG toxicity remains to be the most serious drawback where 44% of patients treated experienced side effects. The toxicity tends to affect more individuals over 65 years of age.[72]

1.1.4.1 Animal studies

Toxicological animal studies on AG involved introducing the drug by a gavage and measuring several parameters associated with toxicity. Steinetz *et al.* (1985) conducted a reproductive toxicity study in pregnant rats fed 20 mg/kg daily dose of AG. They reported fetal wastage, increased placental weights, and a decreased placental alkaline phosphatase.[73] Ali H *et al.* (1990) fed mice and rats daily 50 mg/kg of AG for 3 weeks and saw 50% fall in both white blood cell and platelet counts in mice but no haematological effects in rats. The authors proposed that the N-hydroxylation of AG occurring in mice and humans but not in rats to be an important step in the activation of the AG's toxic mechanism. The *in-vitro* study in bone-marrow cells in mice also showed 30% decrease in cell colonies of granulocyte-macrophage with 100 µg/mL concentration of AG.[74]

1.1.4.2 Human studies

The side effects reported by clinical studies done on AG are divided into those which are common and those that are very rare. Overall, the common side effects tend to have less of a life-threatening impact than the rare side-effects, which are termed idiosyncratic drug reactions (discussed below).

1.1.4.2.1 Common side effects

The most commonly reported toxicities associated with the intake of AG in humans are skin rash, fever, drowsiness, and central nerves system side effects such as lethargy (fatigue), ataxia (lack of muscle movement coordination), and dizziness). These side effects are usually eliminated upon withdrawal of the drug.[72, 75-77] Findings from a study investigating patients with breast carcinoma that used AG as a cancer hormonal therapy that were also on beta blocker for high blood pressure and dexamethasone for dexamethasone-dependent brain metastatic tumour showed that they developed severe complications.[78] Another study showed that a patient undergoing radiation therapy for bone metastasis that was given AG 6 days prior developed an extensive erythematous maculopapular rash around her face, trunk, and extremities. The rash disappeared once AG treatment was stopped.[79]

1.1.4.2.2 Rare side effects & idiosyncratic drug reactions

Most rare side effects of AG are characterized as idiosyncratic and are often life-threatening.

1.1.4.2.2.1 Background

Idiosyncratic drug reactions (IDRs) or type B adverse drug reactions are defined as ‘an adverse reaction that does not occur in most patients treated with a drug and does not involve the therapeutic effect of the drug’.[80] In fact, the term idiosyncratic means specific to an individual and, in general, it becomes impossible to guess who will develop an IDR to a specific drug. IDRs are not common but their occurrence is unpredictable and can be life threatening. The tendency of a drug to cause IDRs depends on its chemical characteristics as well as individual predisposition such as the individual’s genetics and immunological profile (e.g. the expression of receptors displaying drug-derived antigens on the cell surface). IDRs can have an impact on any organ but the skin, liver, and blood cells have been reported the most. IDRs have two characteristics: IDRs contain a delay between the time of drug exposure to the onset of the symptoms and the risk of IDRs does not appear to be related to an increase in the drug dose.[80]

1.1.4.2.2.2 Challenges

IDRs present a great deal of challenges for the drug development industry because their extremely low prevalence does not allow for their detection in clinical trials. As a result, many drugs have been withdrawn from the market due to serious complications from IDRs well after the drug approval process. Their unpredictable nature further complicates the situation as it makes the mechanistic studies and the establishment of standard human or animal models for IDRs extremely difficult if not impossible. It is most likely that the understanding of IDRs will involve the integration of complex mechanistic and signaling pathways that occur at the cellular level.

1.1.4.2.2.3 The “omics” approach

Given the fact that individual genetic variations influence IDRs, the emergence of ‘omics’ technology has provided a powerful tool in understanding global changes in biomarkers that could potentially explain individual susceptibilities to IDRs. Specifically, the incorporation of epigenomics [the study of epigenetic modifications (i.e. DNA methylation, histone modification and changes to small regulatory RNAs)] could be of potential use to understanding IDRs.

1.1.4.2.2.4 IDRs associated with AG

IDR such as haematological toxicities from AG are reported in patients. They are uncommon but if present have severe consequences on the health of the patients. Several studies have shown that AG damages the bone marrow, and

contributes to several disorders such as thrombocytopenia, pancytopenia, septicemia, leukopenia, neutropenia and agranulocytosis. The exact cause of AG induced bone marrow damage is not known. Bone marrow recovery occurred within 14 days after treatment arrest.[75]

1.1.4.2.2.4.1 Thrombocytopenia

Normal platelet counts range between 150,000 and 450,000 per microlitre. Thrombocytopenia is a condition defined as a platelet count below 150,000 per microlitre.[81] Messeih AA *et al.* (1985) reported that out of the 1333 patients treated with AG and steroid, 12 (0.9%) developed leukopenia and thrombocytopenia after seven weeks of treatment.[82] Young *et al.* (1984) reviewed 17 separate clinical trials and found 22 (1.6%) instances of either thrombocytopenia, neutropenia or pancytopenia among 1345 patients treated with AG.[75] Ragaz *et al.* (1984) also reported one severe and two mild cases (2%) of thrombocytopenia in 141 patients.[83] Buzdar *et al.* (1984) documented three patients out of 163 treated with AG having hepatotoxicity, two (1.2%) of which had severe thrombocytopenia 3 and 5 weeks after the initiation of therapy, respectively. Rapid recovery was observed 3-5 days after discontinuation of treatment.[76] Another study reports severe thrombocytopenia observed in a woman with advanced breast cancer during treatment with low doses of AG.[84] Several other studies have reported thrombocytopenia in their patients treated with AG.[85-88]

1.1.4.2.2.4.2 Pancytopenia

Pancytopenia is defined as a reduction in erythrocytes, leukocytes and platelets counts.[89] A well-known case of pancytopenia was documented by Lawrence B *et al.* (1978) where 1 patient out of 153 was diagnosed with the disease. Other symptoms of the patient included severe bleeding. Pancytopenia was rapidly reversed when the AG was withdrawn.[90] Pancytopenia has also been reported by Young *et al.* (1984).[75]

1.1.4.2.2.4.3 Leukopenia/Neutropenia

Leukopenia is an abnormal reduction in white blood cells. The term is interchangeably used with neutropenia because neutrophil count is the most important indication for risk of infection.[91] Steinberg *et al.* (1993) reported on a 78 year old female given 250 mg of AG with 40 mg hydrocortisone twice daily and increased to 4 times daily for breast carcinoma. After 2 years of therapy, she was admitted to the hospital with fever, chills and diarrhea and diagnosed with neutropenia given her neutrophil count of 0%.[77] AG induced leukopenia has also been reported in numerous other studies.[75, 82, 92-94]

1.1.4.2.2.4.4 Septicemia

Septicemia has been defined as the presence of microorganisms or their toxin in the blood.[95] As discussed, AG lowers the count of neutrophils, the cells responsible for the killing of organisms in the blood. The eventual depletion of neutrophils can allow the survival of many microorganisms in the blood and cause

severe blood infections. In fact, depletion of neutrophils by AG increased the susceptibility to bacterial infection and septicemia.[75] The most severe report on AG induced septicemia came from a case report of a 53 year old woman with reversible bone-marrow insufficiency caused by AG treatment for metastatic breast carcinoma. Besides thrombocytopenia and severe granulocytopenia, the patient reported to have gram positive septicemia and skin infections. With AG withdrawal and antibiotic therapy, the patient had clinical recovery.[88] Messeih *et al.* (1985) also reported patients with septicemia.[82]

1.1.4.2.2.4.5 Agranulocytosis

Agranulocytosis is characterized by an extremely low neutrophil count (<500/μL).[96] It takes usually 1-3 months of treatment with drugs associated with IDRs before agranulocytosis is observed. It is usually asymptomatic and its first indication comes from infections, sore throat and/or fever.[80] A case-control study in Spain showed that the incidence of drug-induced agranulocytosis was 3.46:1 million, the fatality rate was 7.0%, and it increased with age.[97] Vincent *et al.* (1985) conducted a clinical trial on AG induced agranulocytosis of 250 mg dose of AG twice a day which was later increased to four times a day. The findings were that out of 161 patients who received the treatment, three (1.8%) developed the condition.[98] Harris *et al.* (1986) reported two patients (1%) developed agranulocytosis out of 228 patients treated with AG with onset occurring within 10 weeks of starting therapy.[99] AG induced agranulocytosis has also been reported by Caldwell *et al.* (1985) and Mathijs *et al.* (1988).[100, 101] According to

the international agranulocytosis and aplastic anemia study conducted in Israel and Europe, the overall annal incidence of 3.4 per million.[102]

The exact mechanism of drug-induced agranulocytosis is unknown because, as discussed previously, IDRs are unpredictable events during the course of drug therapy. In the study of Harris *et al.* (1986), the authors proposed the likely mechanism might stem from a direct toxic effect on the marrow with poor stem cell reserve or an assault on marrow fat cells since the growth of marrow requires the presence of fat cells. AG is shown to inhibit aromatase activity in human marrow fat cells as well.[99]

1.1.4.3 Antidote and emergency treatment

Since AG inhibits cortisol production (Figure 1.3), in case of blood glucocorticoid deficiency, intravenous infusion of a soluble hydrocortisone should be given within 3 h of intoxication. AG also inhibits aldosterone production (Figure 1.3) responsible for maintaining proper blood pressure. In case of hypotension, an intravenous administration of norepinephrine is recommended. In case of severe intoxication, dialysis may be used.[2]

1.1.4.4 Food and Drug Administration (FDA) status

After its introduction in the USA as anti-convulsant medication in 1960, it was withdrawn from the market in 1966 due to its limited efficacy and unacceptable side effects. It was quickly licensed as an new investigational drug,

for detailed examination of hormone biosynthesis inhibiting properties.[103] However, due to more severe and prevalent toxicities reported in patients AG is currently discontinued by the FDA.[104]

1.2 Cell death

As AG has been documented to produce many side-effects, it is possible that the cytotoxic mechanism is involved in some of these mechanisms. Cytotoxicity, or cell death, can be a component of the adverse drug reactions described above, but this has not been studied for AG.

1.2.1 Definitions of cell death

It has been widely accepted that the definition of a dying cell is to pass a "no-return" phase beyond which the cell will be unable to recover from. This point of no return has been characterized by caspase activation,[105] loss of mitochondrial membrane potential,[106] the permeabilization of the outer mitochondrial membrane[107] and exposure of phosphatidylserine (PS) residues on the outer leaflet of the plasma membrane. Nevertheless, there have been many studies demonstrating that these events do not always accompany cell death.[108-111] Given the confusion in the definition of cell death, the Nomenclature Committee on Cell Death (NCCD) has proposed that a cell is considered dead when any of the following criteria is satisfied: a) the cell has lost its plasma membrane integrity; b) the cell and its nucleus have undergone complete fragmentation; and/or c) its fragments have been engulfed by adjacent cells.[112]

1.2.2 Different forms of cell death

There are many forms of cell death which can be classified according to their morphological appearance (apoptotic, necrotic, autophagic, or mitotic), enzymatological criteria (involvement of nucleases or proteases such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or immunological aspects (immunogenic or non-immunogenic).[112]

1.2.3 Apoptosis

The term ‘apoptosis’ was coined in 1972 to describe a mode of cell death associated with genomic DNA fragmentation in the nematode *Caenorhabditis elegans*.[113] Apoptosis is a cellular death program involving a series of highly regulated and timed events. Apoptotic cells undergo a sequence of distinct morphological changes. Upon apoptosis, the overall cell shrinks, the cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear chromatin condenses and breaks up into fragments. The cells often break up into fragments called apoptotic bodies which are engulfed by neighboring cells (or macrophages). This process ensures the complete “clean up” of cellular debris without the initiation of inflammatory responses.[114]

1.2.3.1 Biological functions

Apoptosis has functions in animal development and deletion of damaged cells. For example, apoptosis is responsible for the proper sculpting of hands and feet during embryonic development. The transition between a tadpole into a frog during metamorphosis involves apoptosis in the tail which eventually disappears in the frog. In the nervous system, the number of nerve connections is constantly adjusted through removal or addition of nerve cells to match the required connections.[114] Apoptosis plays an important role in the quality-control process during the development of animals by eliminating potentially abnormal and hazardous cells. For example, in the immune system of vertebrate apoptosis eliminates T and B lymphocytes that failed to produce useful antigen-specific receptors, or even worse have, produced self-reactive receptors that make the cell dangerous.[114] Of course, apoptosis is balanced with cell division to ensure that the animal tissue is neither abnormally growing nor shrinking. For example, if a part of a liver is removed from an animal, the liver compensates this by increasing cell proliferation to make up the lost cells. Apoptosis is also a highly prevalent event in human bone marrow, the birthplace of most blood cells. Neutrophils are continuously produced and eliminated in large numbers in matter of days. This quick life cycle of neutrophils ensures a rapid supply of neutrophils to fight infections all over the body.[114]

1.2.3.2 Types of apoptotic pathways

There are two main apoptotic pathways in mammalian cells: the extrinsic and the intrinsic pathways. The extrinsic pathway is initiated by the binding of extracellular ligands to death receptors at the plasma membrane. This pathway is used for the removal of unwanted cells during development.[115] On the other hand, the intrinsic pathway is triggered by intracellular events such stress signals (i.e. DNA damage).[116]

1.2.3.3 Apoptotic mechanism: caspase activation and their molecular targets

The apoptotic signal transduction pathway typically begins when an external or an internal stimuli is introduced in the system. This signal triggers the formation and activation of complexes involving adapter proteins. The formed complexes recruit and activate a group of cysteine protease proteins called cysteine-dependent aspartate-directed proteases (caspases), specifically initiatory caspases. Initiator caspases then amplify the apoptotic signal by activating many more effector caspases which then execute the final stage of apoptosis which typically involves the fragmentation of DNA (Figure 1.5).

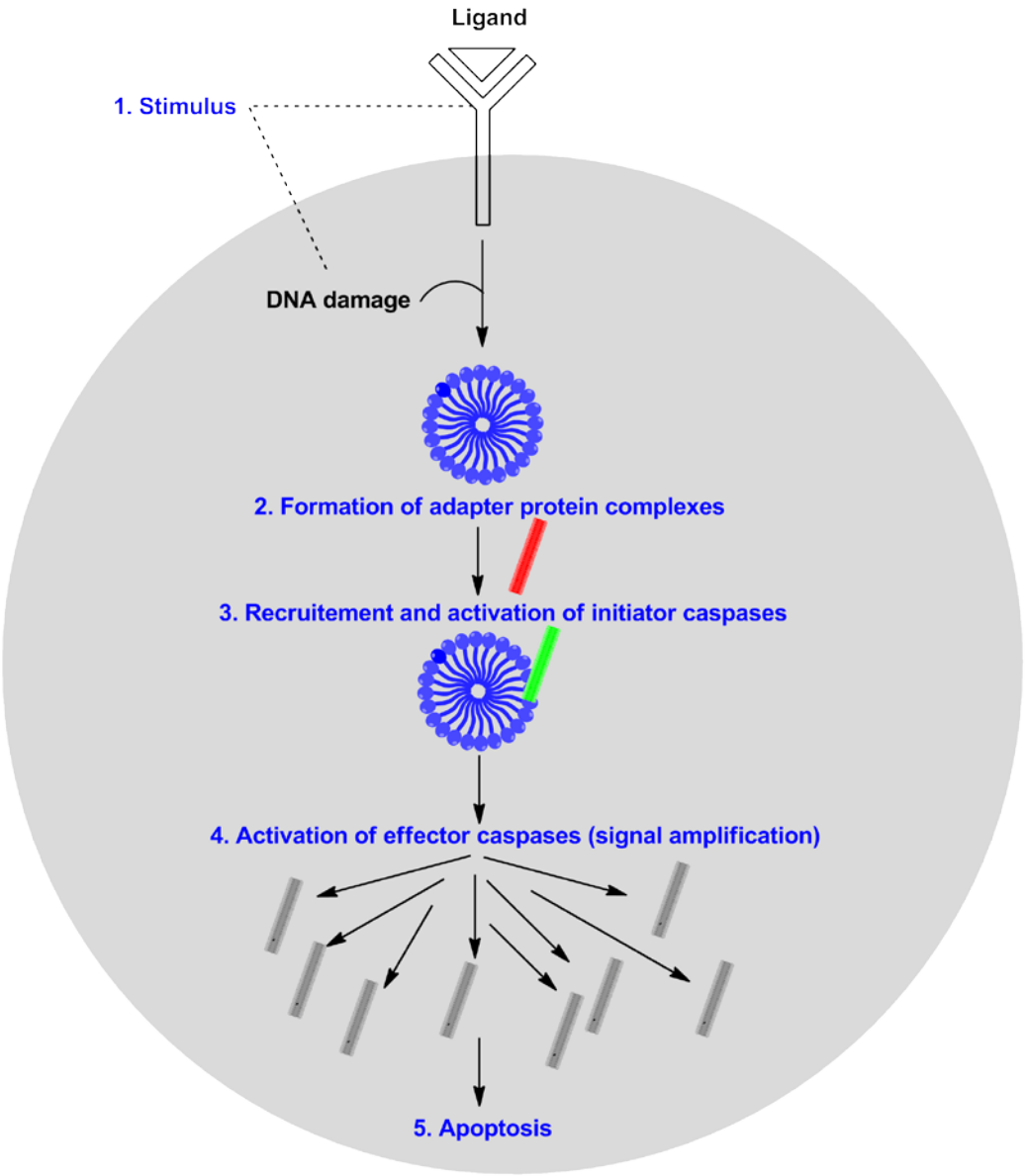

Figure 1.5 An overview of the key steps in apoptosis

1.2.3.3.1 Types of stimuli

The type of stimuli can dictate the type of complexes and caspases which are activated to execute apoptosis. The stimuli can be categorized into external, such as the binding of a ligand to a receptor, or internal, that is generated inside the cell. The extrinsic stimuli, for example, can include the binding of Fas ligand

(FasL) to a Fas receptor (FasR), and the intrinsic signal can include DNA damage. In fact, the cell surface death receptors belong to a family of transmembrane proteins called the tumor necrosis factor (TNF) receptor superfamily. These include Apo-1 (Fas), Apo-3 (TRAMP), TNFR1, and the TRAIL receptor DR4 and DR5. The extracellular domain of all these receptor contains a conserved cysteine-rich repeat.[117]

1.2.3.3.2 Caspases

Caspases can be categorized into non-apoptotic and apoptotic caspases. Non-apoptotic caspases include caspase 1, 4 and 5 which induce "pyroptosis", a form of cell death involving activation of inflammatory cells to be discussed in chapter 1.2.5.6.[118] There are seven known apoptotic caspases in mammalian cells. Of the seven, four (caspase 2, 8-10) are initiator (apical) caspases and three (caspase 3, 6 and 7) are effector (executioner) caspases (Figure 1.6).[119] Caspases, with the exception of caspase 9, are present in the cell as inactivated zymogens (pro-caspases) that require proteolytic cleavage for their activation. The cleavage occurs after a specific aspartate residue. Thus, the cleavage of caspases is seen as a hallmark of apoptosis.[120]

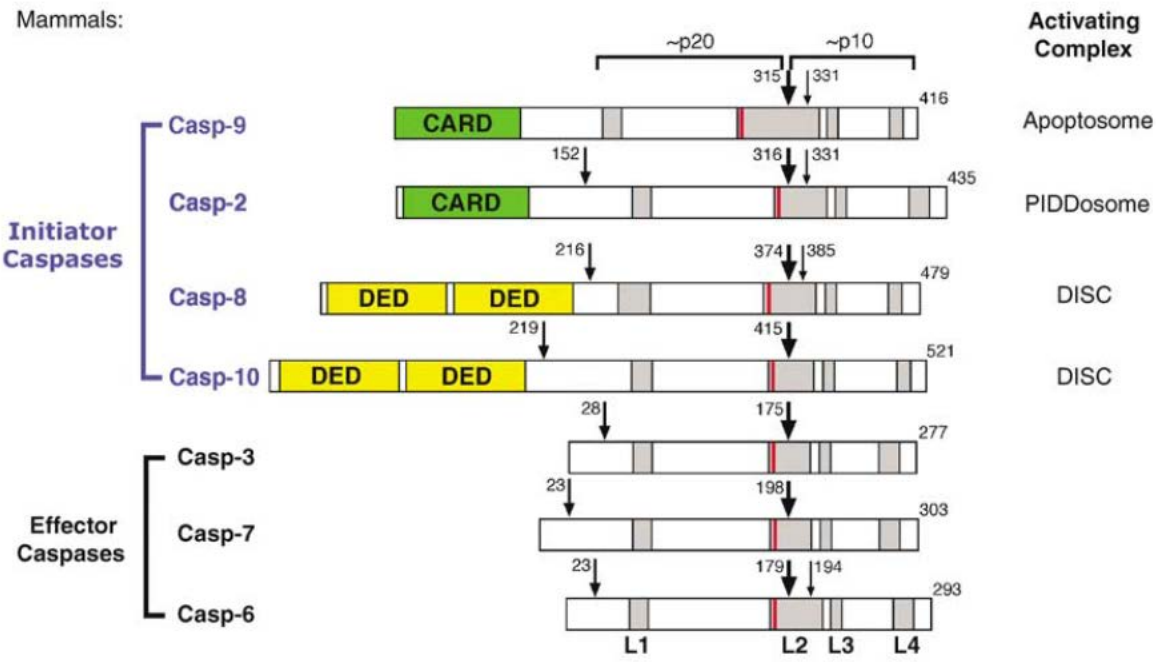

Figure 1.6 Mammalian apoptotic caspases with their activating complexes

Initiator caspases include caspase 2, 8-10, while caspase 3, 6, and 7 are effector caspases. Adapted from Bao and Shi (2007). (Copyright 2007 Cell death and differentiation). [119]

1.2.3.3.3 Activation of initiator caspases by adaptor protein complexes

Initiator caspases are activated by their interaction with adaptor protein complexes which involves their autocatalytic intrachain cleavage.[121, 122] As discussed above, the types of stimuli dictates what type of adaptor protein complex is activated, which subsequently dictates which caspase is activated. In the mammalian cell, there are three major adaptor protein complexes called the p-53 induced protein with a death domain (PIDDosome), the death-inducing signaling complex (DISC) and the apoptosome complex (Figure 1.7). In each case, the initiator caspase is recruited and activated by a specific adaptor protein

complex. The activation of initiator caspases 2, 8, 9 and 10 will discussed in details below.

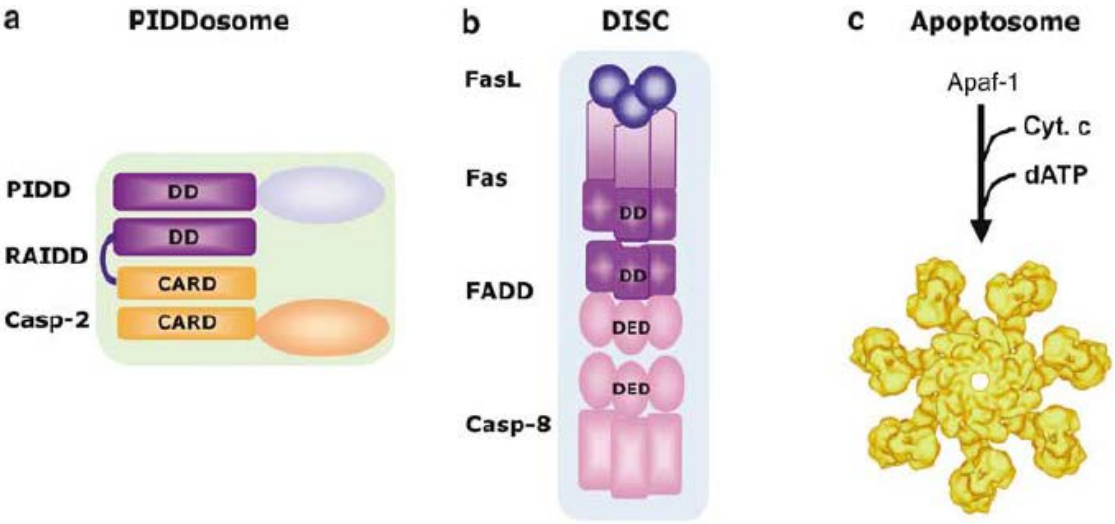

Figure 1.7 Adaptor protein complexes in mammalian cells

The activation of caspase-2, caspase-8, and caspase-9 are mediated by the PIDDosome (a), the DISC (b), and the apoptosome (c) respectively. Adapted from Bao and Shi (2007). (Copyright 2007 Cell death and differentiation).[119]

1.2.3.3.3.1 PIDDosome activates caspase 2

Caspase 2, the second caspase identified in mammalian cells is an initiator caspase involved in the intrinsic apoptotic pathway recruited by the PIDDosome by a stress signal such as DNA damage (Figure 1.8).[123] The PIDDosome is a large protein complex (670 kDa) that contains adaptor proteins called RAIDD and the PIDD. The RAIDD and PIDD interact by their isolated death domain (DD). Caspase 2 and RAIDD interact using their caspase-recruitment domain (CARD) resulting in a dimerization (Figure 1.7a).[124-127] Although there is evidence to suggest that these interactions are crucial for the activation of caspase 2, the exact

molecular mechanism involving its activation by the PIDDosome is unknown. Some suggest that the PIDDosome simply promotes the autocatalytic cleavage of caspase 2 zymogen.

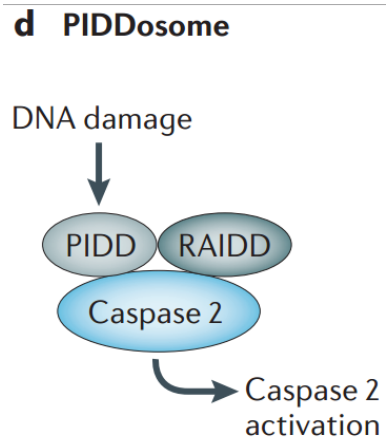

Figure 1.8 PIDDosome activates the initiator caspase 2

Adapted from Hyman and Yuan (2012) with permission. (Copyright 2012 Nature Reviews Neuroscience).[128]

1.2.3.3.3.2 DISC activates caspase 8

The activation of the initiator caspase 8 exemplifies the extrinsic apoptosis pathway. The signal transduction is elicited through the binding of a death ligand called FasL (CD95L) to the transmembrane death receptor Fas (APO-1/CD95).[115] As illustrated in Figure 1.9, the Fas receptor (FasR) contains a DD that dimerizes with the DD of Fas-associated protein with death domain (FADD) adaptor protein. This dimerization between FasR and FADD invites the binding of pro-caspase 8 (and likely 10)[115] to FADD using their death effector domain (DED). The overall result of the interactions between FasL, FasL, FADD, and the pro-caspases is the creation of one large structure known as the DISC. The DISC

executes the cleavage and activation of pro-caspase 8 and perhaps 10 into functional initiator caspases.[115, 129-132] Although there is limited knowledge on the molecular mechanism of caspase-8 activation, studies on isolated capase-8 confirm that dimerization of caspase-8 is crucial to its activation and DISC might facilitate its activation through dimerization.[133-137]

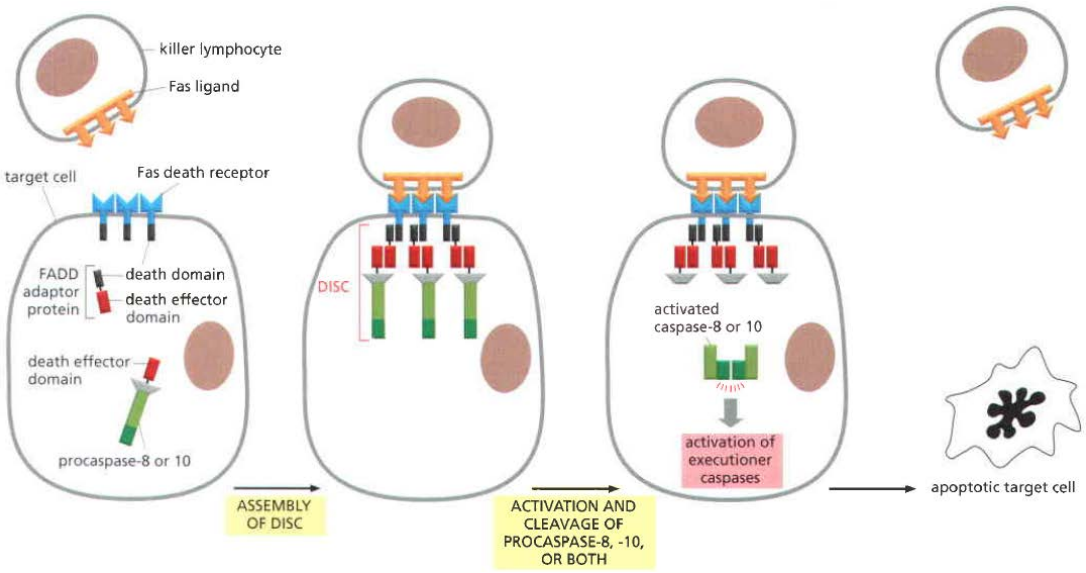

Figure 1.9 DISC activates the initiator caspase 8

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1.2.3.3.3.3 Apoptosome activates caspase 9

The most extensively studied initiator caspase and the only caspase whose activation by adaptor complex has been fully reconstituted *in vitro* is caspase 9. Caspase 9 activation is mediated by a complex called the apoptosome, which includes apoptotic protease activating factor-l (Apaf-1), cytochrome c, and a cofactor dATP/ATP. Apaf-1 is the core component of the apoptosome. It contains three important domains for carrying out its function. First, Apaf-1's N-terminal

CARD domain is used for interaction with the CARD of pro-caspase 9 and is responsible its activation.[138, 139] Second, the nucleotide binding domain is responsible for binding of dATP and together with CARD is responsible for the oligomerization of Apaf-1 in the presence of cytochrome c and dATP. The third domain, C-terminal WD40 repeats is thought to bind cytochrome c.

As shown in Figure 1.10, an apoptotic signal (often described as DNA damaging agents, kinase inhibitors, and activation of cell surface death receptors)[140, 141] allows for the release of cytochrome c from the mitochondria. Released cytochrome c binds to Apaf-1 causing ATP hydrolysis, exposing Apaf-1's CARD.[138, 142] This leads to the oligomerization of multiple Apaf-1 molecules and assembly of the apoptosome.[143-145] Pro-caspase 9 is recruited and is joined to the apoptosome through its CARD domain. Cytochrome c and dATP have been shown to be essential cofactors for the formation of Apaf-1/caspase 9 complex.[138, 146] Once the complete structure is formed, pro-caspase 9 is autocleaved into caspase 9 and activated in a mechanism that is currently under investigation.

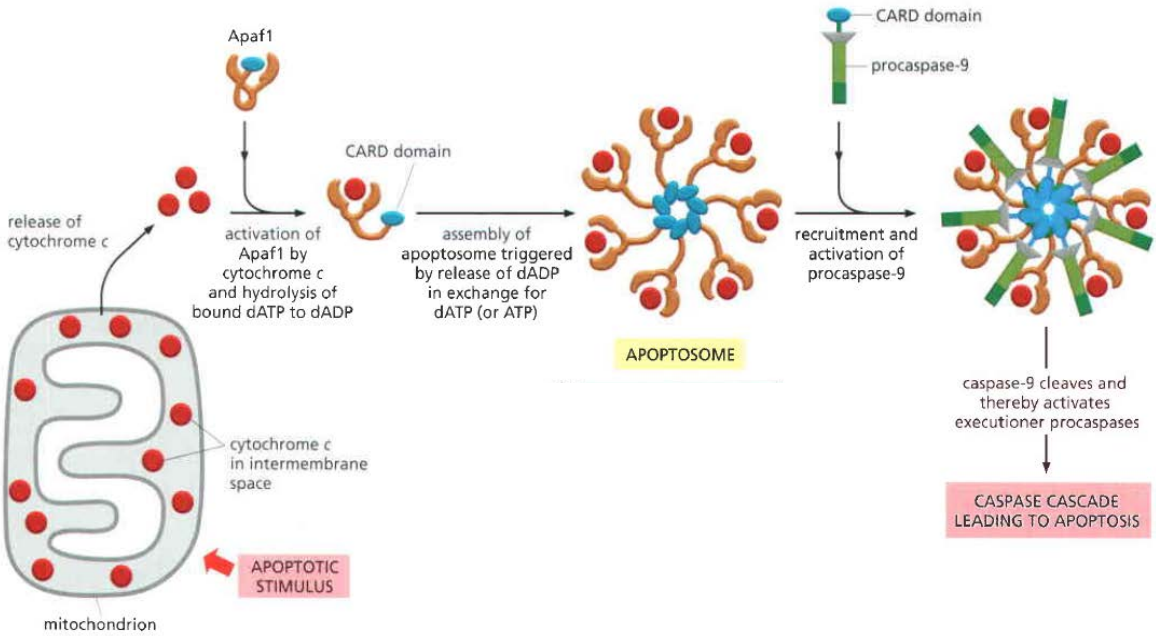

Figure 1.10 Apoptosome activates the initiator caspase 9

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1.2.3.3.3.4 Proposed models of initiator caspase activation

There are three known models of how initiator caspases are activated (Figure 1.11). The first model called the induced proximity model states that the initiator caspases autoprocess themselves when brought into close proximity to one another.[147] This model has been demonstrated by several groups.[148-152] The second model, the proximity driven model, is an improvement over the induced proximity model in that it focuses on the idea of dimerization of initiator caspases as a driving force in their activation. According to this model, the adaptor protein complex serves as a docking station promoting the dimerization by increasing the number of caspases available for dimerization.[153] The third model called the

induced conformation model states that initiator caspases exhibit a higher level of catalytic activity when they are bound to adaptor complexes. According to this theory, binding of the initiator caspase to the complex induces a conformation change that exposes the active site of the caspase thus activating it.[154, 155]

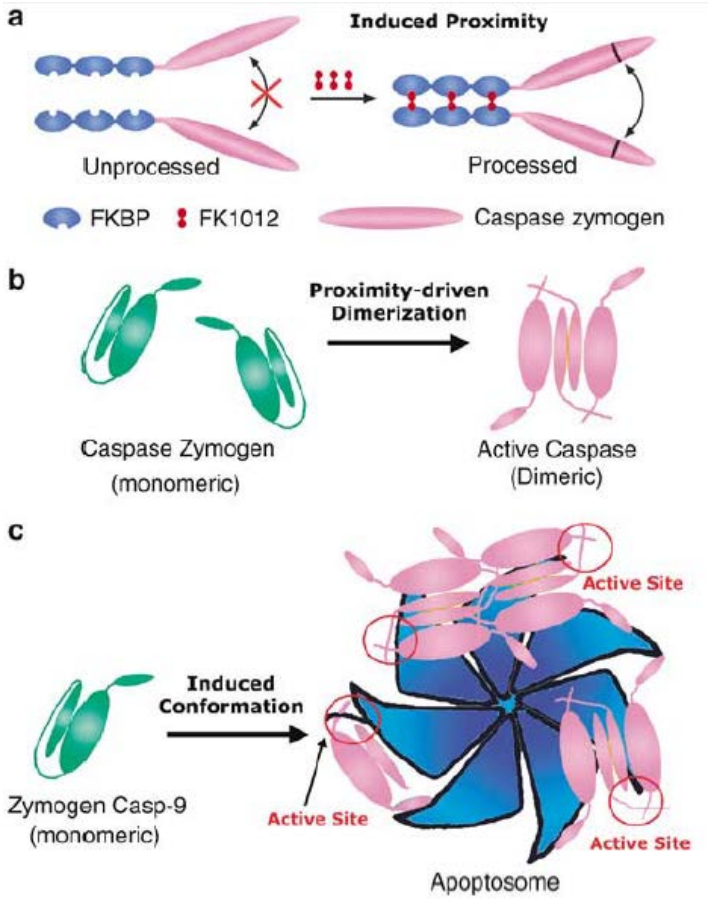


Figure 1.11 Proposed models for the activation of initiator caspases

Initiator caspases can be activated through the induced-proximity model (a), the proximity-driven dimerization model (b), or the induced conformation model (c). Adapted from Bao and Shi (2007). (Copyright 2007 Cell death and differentiation).[119]

1.2.3.3.3.5 Activation of effector caspases by initiator caspases

In contrast to initiator caspases, the activation of effector caspases is rather simple. Effector caspases are activated by initiator caspases through cleavage of an internal aspartic residue dividing the caspases into a small and a large subunit (Figure 1.6).[120, 147] Pop and Salvesen (2009) provide a summary of initiator to executioner caspase activation (Figure 1.12). Initiator caspases 8, 9 and 10 are known activators of caspases 3, 6 and 7.[156] Caspase 2 however remains to be a special caspase in that unlike other initiators which process all executioner caspases, caspase 2 is completely inactive towards other caspase zymogens.[157]

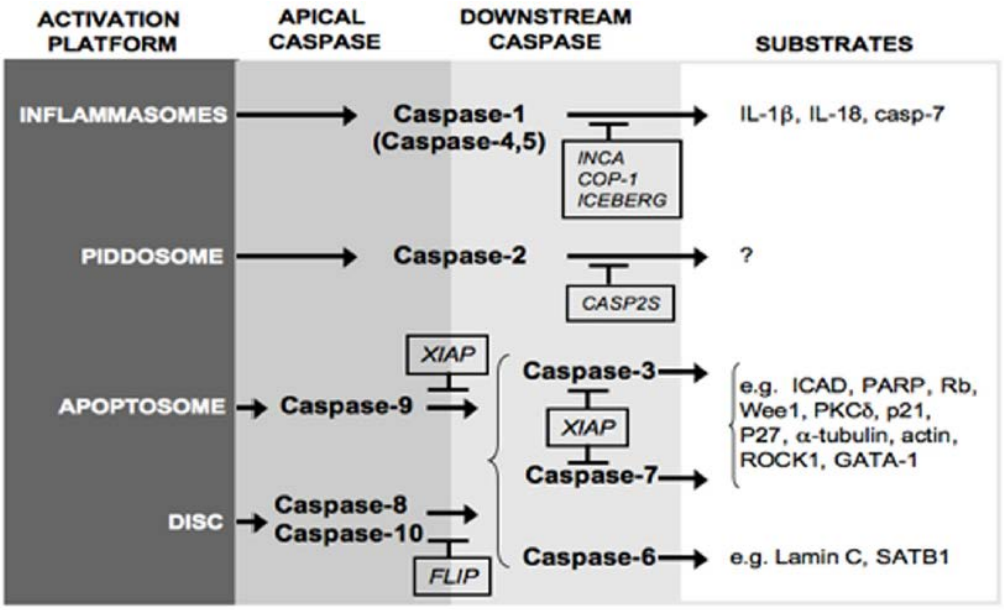


Figure 1.12 Activation of executioner caspases by initiator caspases

This research was originally published in Journal of Biological Chemistry. Pop, C. and Salvesen, G. S. Human caspases: activation, specificity, and regulation. *Journal of Biological Chemistry*. 2009; 284: 21777-81. © The American Society for Biochemistry and Molecular Biology.[156]

1.2.3.3.3.6 Effector caspases execute the final stage of apoptosis

The contribution of each executioner caspase to the execution of apoptosis is still at the speculation stage.[158] Much of the knowledge gained of the execution stage of apoptosis comes from caspase 3 which has been shown to mediate DNA fragmentation (through activation of DNases), inhibit DNA repair and RNA splicing, nuclear membrane condensation, blebbing of the plasma membrane, and cause the loss of apoptotic suppression.[159-165] DNA fragmentation and inhibition of DNA repair have also been reported in caspase 6 and 7.[166, 167] There have been reports of loss of nuclear matrix integrity and nuclear disassembly with activation of caspase 6 which has been a key factor in apoptosis.[168-170]

1.2.4 Necrosis

Necrosis is a form of cell death characterized morphologically by a gain in cell volume, swelling of the organelles, the rupture of the plasma membrane and the loss of intracellular content. Necrosis has been characterized in the past as an uncontrolled form of cell death as supposed to apoptosis which is highly regulated. Nevertheless, there is a growing body of evidence suggesting that necrosis may in fact be regulated by cellular signals.[171, 172] For example, necrosis has been correlated with the induction of death domain and Toll-like receptors such as TNFR1, Fas/CD95, TRAIL-R and TLR3, TLR4 respectively.[112] Unfortunately, there are no universal biochemical changes that can be used to indisputably characterize necrosis. The processes that are responsible for causing necrosis as well as its side effects are unclear. Some of these processes include

production of reactive oxygen species (ROS) as a result of mitochondrial electron uncoupling, mitochondrial and lysosomal membrane permeabilization, lipid degradation and subsequent activation of phospholipases, and finally the increase in cytosolic calcium concentration caused by mitochondrial disruption and activation of non-caspase proteases such as calpains and cathepsins.[112]

1.2.5 Other forms of cell death

Besides apoptosis and necrosis which are the major characterized forms of cell death, there are other forms of cell death (e.g. autophagy, mitotic catastrophe, anoikis, excitotoxicity, Wallerian degeneration, paraptosis, pyrptosis/pyronecrosis and entosis) which have been recently recognized and incorporated into scientific literature.

1.2.5.1 Autophagy

Autophagy is a self-degradation process that involves the autophagosome (a double-membrane vacuole containing the cell's own cytoplasm) fusing with the lysosome to be broken down by hydrolases. Signals such as starvation, ER stress and infections elicit autophagy. Malfunctions in autophagy are involved in the development of pathologies, cancer, and infectious diseases.[173]

1.2.5.2 Mitotic catastrophe

Mitotic catastrophe is a form of cell death characterized by the failure of a cell to undergo proper mitosis. It is usually characterized morphologically by the presence of chromosomal fragments (micronucleation) and multiple nuclei

(multinucleation). However, given that there is no consensus on the use of this term yet, the NCDD recommends characterizing it as cell death proceeding multinucleation.[174-176]

1.2.5.3 Anoikis

The term ‘Anoikis’ was used by Frisch and Francis (1994) to describe a special form of apoptosis induced by loss of cell anchorage to other cells or a substrate.[177] Cell anchorage is crucial for cell survival and is demonstrated in many studies involving fibroblasts,[178] endothelial cells,[179] neuronal cells[180] and other cell types. The NCCD has accepted the use of this term as it has been used in the literature for two decades.

1.2.5.4 Excitotoxicity

Excitotoxicity is a form of cell death caused by continuous excitation of neuronal cells with neurotransmitters such as glutamate which subsequently leads to the opening of the N-methyl-aspartate calcium channel and the release of excessive amount of calcium into the cytosol. The cytosol of cells usually contains very low amounts of calcium because increases in calcium concentration are known to activate various signaling pathways some of which lead to cell death.[181]

1.2.5.5 Wallerian degeneration

Wallerian degeneration is named after the British physiologist Dr. Augustus Desiré Waller, who first described an event where part of axon or the neuron itself degenerates without affecting the cell body.[182] The term is often not described strictly as cell death because the cell body remains functional after the degeneration process has occurred.

1.2.5.6 Paraptosis

Paraptosis was introduced by Sperandio *et al.* (2000) as an alternative form of programmed cell death that was distinct from apoptosis. Paraptotic cell death was characterized by the expression of insulin-like growth factor receptor I and is associated with cytoplasmic vacuolization and mitochondrial swelling in the absence of any other morphological characteristics resembling apoptosis.[183]

1.2.5.7 Pyroptosis/Pyronecrosis

Pyroptotic cell death was first described in the infection of macrophages by *Salmonella typhimurium* involving the activation of caspase 1 also known as interleukin-1b (IL-1b) converting enzyme.[184] The activation of caspase 1 leads to release of IL-18 involved in inflammatory reactions[185, 186] Macrophages undergoing pyroptosis exhibit morphological features of apoptosis and some necrosis.[118] Pyronecrosis is a form of cell death that is distinguished from pyroptosis in that it does not require caspase-1 to induce cell death.[187]

1.2.5.8 Entosis

Entosis is a new cell death modality in which a cell engulfs its live neighboring cell which then dies within the phagosome.[188] The most efficient cells to perform entosis are MCF-7 breast cancer cells.[189]

Chapter 2

Rationale

The previous chapter discussed in detail the various pharmacokinetic and pharmacological aspects of AG as well as its idiosyncratic drug reaction associated with its toxicological profile. It was pointed out that the investigation of IDRs was challenging due to the unexpected and low frequency of events that occur upon drug use. Due to these characteristics, AG induced agranulocytosis remains to be an IDR in which its mechanism is unknown, and animal/human models are yet to be established. As mentioned in chapter 1, there has been progress in understanding IDRs using omics technologies (e.g. epigenomics) because of their powerful and global analytical capabilities. IDRs such as agranulocytosis most likely involve the activation of several complex signaling pathways which lead to changes in expression levels of proteins which are the ultimate down-stream targets for destruction of cells. There are two models for IDRs such as agranulocytosis: the self-nonself model that emphasizes the role of the immune system and the danger model which emphasizes the release of 'danger signals' from injured cells.[190] Following the danger model, we reason that agranulocytosis involves the release of death inducing substrates thereby activating cell death pathways as possible endpoints that might explain the

mechanism. Furthermore, we considered the radical generating property of AG as a focal point in the study because it could be a possible model of AG induced cell toxicity as well as have clinical implications with regards to agranulocytosis. As mentioned previously in chapter 1, AG can utilize MPO protein and H_2O_2 to be activated and produce protein radicals.[14]

We chose the immortalized HL-60 promyelocytic cells as a model of neutrophils because they are the predecessors of neutrophils. Although both cells contain MPO, neutrophils contain 20-30% of MPO found in HL-60.[191] Unlike neutrophils, HL-60 cells can be grown at rapid rates for months, which was necessary for the application of proteomic techniques (described later). Since no *in vitro* model has been validated for agranulocytosis, HL-60 cells can provide a reliable ground for this work.

Therefore, we followed this line of reasoning to design a study that would use proteomic technology to study AG induced toxicity by identifying and characterizing the role of key down-stream proteins which were significantly altered in their expression due to AG and H_2O_2 treatment in HL-60 cells.

2.1 Hypothesis

We hypothesize that:

1. AG metabolism leads to the production of protein radicals in a concentration and time dependent manner. (Figure 2.1)

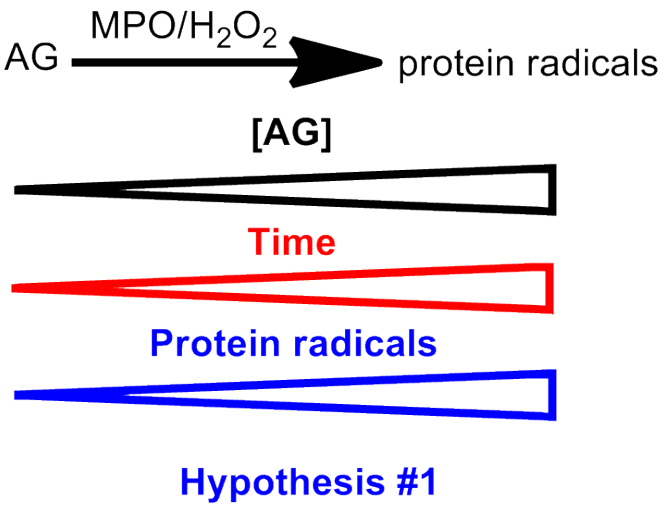


Figure 2.1 Production of protein radicals in a time and AG dose dependent manner by AG, MPO and H_2O_2.

2.

a) AG induces cell death by activating one or more type(s) of cell death signaling mechanism(s) in HL-60 cells in a concentration and time dependent manner. (Figure 2.2)

b) The formation of primary and/or secondary (MPO) radicals generated by MPO is a necessary step in the cell death mechanism. (Figure 2.2)

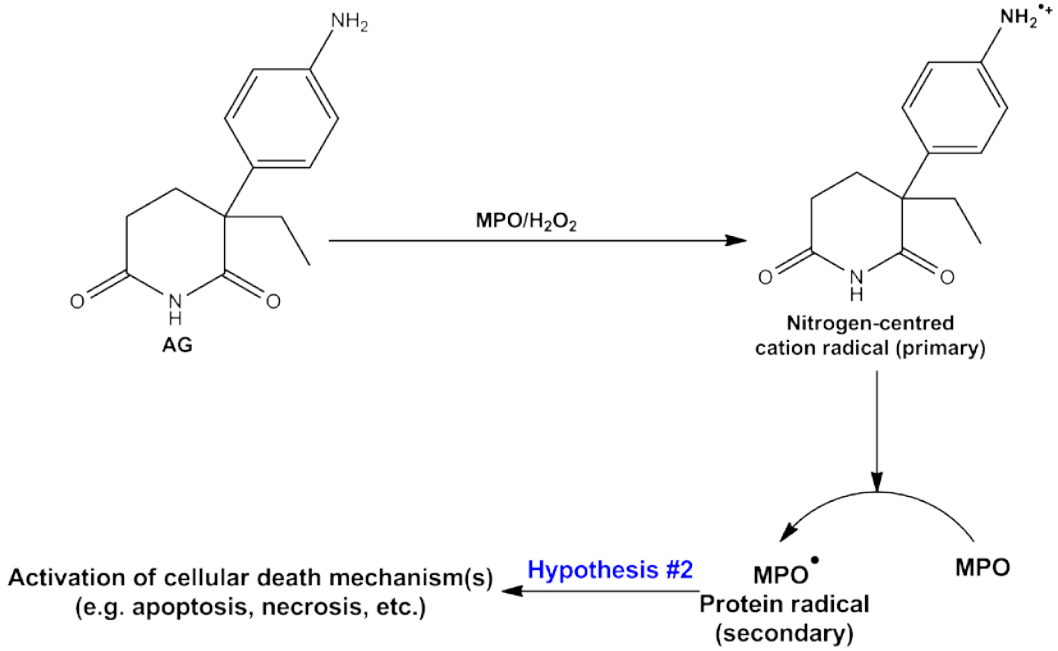


Figure 2.2 Activation of cellular death mechanism(s) by AG through the production of radicals.

2.2 Objectives

The objectives of this study are:

1. Perform immunospin-trapping to detect the presence of protein radicals. The technique involves the use of an agent called 5,5-dimethyl-1-pyrroline N-oxide (DMPO) that traps protein radicals. This protein-DMPO adduct can then be identified using classical SDS-PAGE coupled with an anti-DMPO antibody imuno-detection (chapter 3.3.2).

2. Apply a proteomic technique known as stable isotope labelling in cell culture (SILAC) to investigate the cytotoxicity pathways induced by AG metabolism (chapter 3.3.3) and validating the data using enzyme-linked immunosorbent assay (ELISA) (chapter 3.3.4).

3.

    a) If cell death pathways are involved in AG toxicity, validate the cell death pathways obtained from proteomic through a specific endpoint such as the detection of apoptosis/necrosis markers using fluorescein isothiocyanate (FITC) coupled Annexin-V flow cytometry (chapter 3.3.5).

b) Understand the role of MPO and free radicals in the apoptotic process through using an MPO inhibitor called 4-Aminobenzoic hydrazide (ABAH)

Chapter 3

Materials & methods

3.1 Reagents

3.1.1 Chemicals

AG (Cat No. A609810) was purchased from Toronto Research Chemicals (Toronto, ON). 40% Acrylamide/Bis solution 29:1 (3.3% C) (Cat No. 161-0146) was purchased from Bio-Rad Laboratories (Mississauga, ON). Bovine serum albumin (BSA) (Cat No. A2153), Calcium chloride (Cat No. C1016), Coomassie brilliant blue R (Cat No. B8647), Dublecco's phosphate buffer saline (Cat No. D8537), Glucose (Cat No. G8270), Glucose oxidase (Cat No. G2133), H_2O_2 (Cat No. 95321), Menadione (Cat No. M9429), Propidium iodide (PI) (Cat No. P4170), Sodium bicarbonate (Cat No. S6014), Sodium deoxycholate (Cat No. D6750-25G), and Triton X-100 (Cat No. T8787) were purchased from Sigma-Aldrich Canada Co. (Oakville, ON). DMSO (Cat No. 4100-1), and Sodium chloride (Cat No. 7560-1) were purchased from Caledon Labs (Georgetown, ON). Tris-(2-Carboxyethyl)phosphine (TCEP) (Cat No. 77720), non-reducing lane

marker sample buffer (5X) (Cat No. 39001), HEPES (Cat No. BP310-500) and Sodium dodecyl sulphate (Cat No. BP166-500) were purchased from Thermo Fisher Scientific (Ottawa, ON). Sodium carbonate anhydrous (Cat No. SX0395-1) was purchased from EMD (through VWR Canada, Edmonton, AB). FITC annexin-V (Cat No. 556420) was purchased from BD Biosciences, CA.

3.1.2 Antibodies

3.1.2.1 Primary Antibodies

Rabbit anti-DMPO and anti-SSB antibodies were purchased from Abcam Inc. (Toronto, ON) (Cat No. ab23702 and ab124932, respectively). Rabbit anti-COMT antibody was purchased from Sigma-Aldrich Canada Co. (Cat No. C6870).

3.1.2.2 Secondary Antibodies

HRP conjugated goat anti-rabbit IgG antibody was purchased from Fisher Scientific (Cat No. PI-31460). AP conjugated goat anti-rabbit antibody was purchased from Thermo Fisher Scientific (Cat No. 31340).

3.2 HL-60 cells

HL-60 cells were obtained from ATCC (Cat No. CCL-240, Manassas, VA). The cells were grown in a growing media containing normal RPMI-1640 medium (Gibco ® Reference No. 11875-093), 10% fetal bovine serum (FBS)

(Gibco® Cat No. 12483) and 5% of Antibiotic-Antimycotic (Gibco® Reference No. 15240-062). Cells were maintained in an atmosphere with 5% CO_2 at 37°C, with media change occurring every 2 days. All HL-60 cells used during experiments had a passage number less than 30.

3.3 Experimental methods

3.3.1 Trypan blue exclusion cytotoxicity assay

HL-60 cells were treated in 24-well plates at 3.7 x10^6/mL for 6, 12 and 24 h durations at 5% CO_2 and 37°C with differing concentrations of AG (n=3). At the end of the experiment, sample and trypan blue reagent (Cat No. 17-942E from Lonza) were mixed at 1:1 volume ratio. Cell viability (% of live cells) was measured using TC-10 automated cell counter (Bio-Rad Laboratories). Data was expressed as means ± standard error (SE).

3.3.2 Immuno-spin trapping SDS-PAGE

AG is metabolized by MPO and H_2O_2 (enzyme cofactor) to generate primary nitrogen-centred cation radicals which were detected using EPR.[14] These primary radicals were further shown to create secondary MPO protein radicals using a method known as immunospin trapping. The immunospin trapping method takes advantage of the radical trapping agent DMPO which can covalently bind to radical species forming a DMPO adduct. Using anti-DMPO antibody, this adduct can be identified by western blotting method in cell lysates.[192] (Figure 3.1)

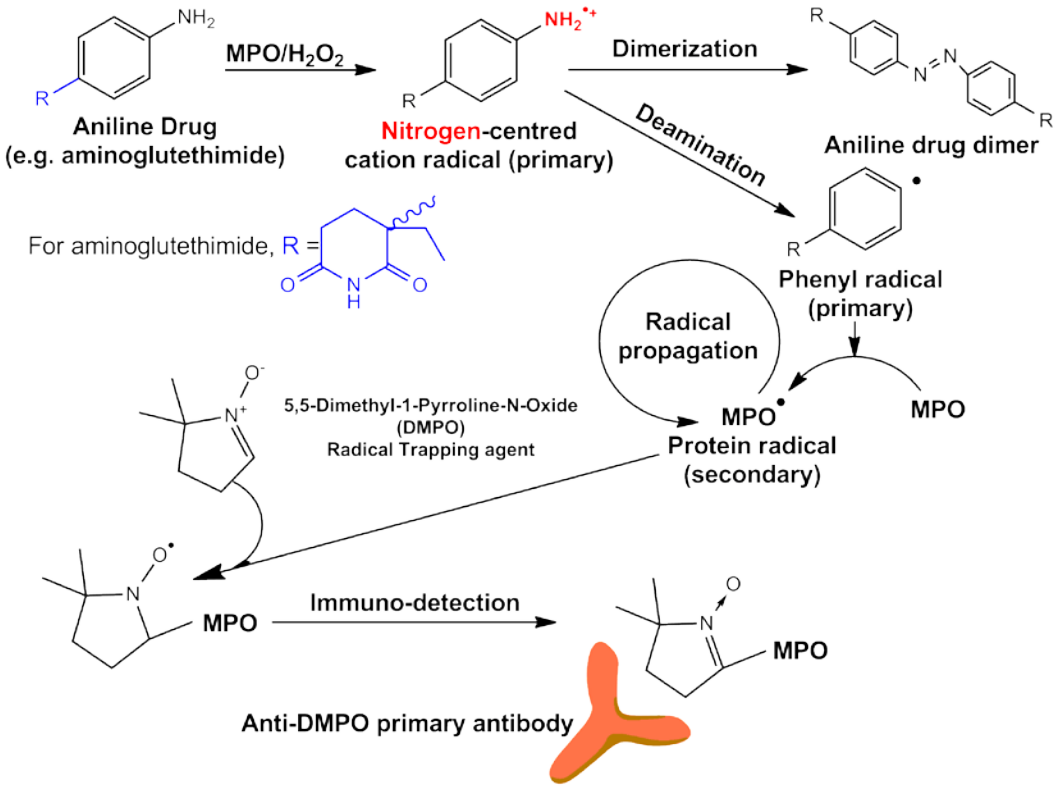


Figure 3.1 Immuno-spin trapping of MPO protein radicals induced by AG

The immuno-spin trapping protocol of Narwaley *et al.* (2011) was followed for our studies.[193] Briefly, HL-60 cells (4.5×10^6/mL, 1 mL) were pre-incubated with DMPO 5 minutes before the addition of AG. The reaction was

initiated with 5 mM glucose/25 mU/mL glucose oxidase (G/GO), which was added last in order to generate H_2O_2. The reaction was briefly shaken and incubated in an incubator at 37°C and 5% CO_2. Cells were lysed using RIPA buffer (0.05 g sodium deoxycholate, 100 µL of Triton X-100, and 10 µL of 10% SDS in 10 mL of PBS). The amount of proteins in the lysed cells was quantified using the bicinchoninic acid (BCA) assay. For loading of samples onto an SDS-PAGE gel, the cell lysates were reduced by 10% TCEP (Thermo Scientific) and denatured for 5 min at 95°C with shaking at 600 rpm. Reduced samples were allowed to cool and were then loaded in equal amounts (30 µg/lane) in a 1.0 mm 10% polyacrylamide gel. The proteins were then transferred from the gel onto a nitrocellulose membrane using a semidry transfer apparatus (Bio-Rad Laboratories). The membrane was blocked overnight at 4°C with 5% BSA in washing buffer (TBS-T). The membrane was then treated with a rabbit anti-DMPO primary antibody (1:5000 in TBS-T) for 1 h at room temperature (RT) with constant shaking to detect DMPO-protein adducts. Afterwards, the membrane was washed four times for a total of 30 minutes and treated with goat anti-rabbit HRP-conjugated secondary antibody (1:10000 in TBS-T) for 1 h at RT. The membrane was again washed four times and treated with chemiluminescence HRP substrate (Millipore Cat No. WBKLS0500) reagent for 2 min and exposed to Fuji super-RX clear blue X-ray film (Fujifilm Cat No. 47410 19250)

3.3.3 Stable isotope labelling in cell culture

3.3.3.1 Cell culturing and incorporation of heavy and light amino acids

HL-60 cells were split into two groups treated either with heavy ($^{13}C_6$) or light ($^{12}C_6$) media. The RPMI-1640 medium without Lysine, Arginine and Leucine was purchased from Sigma Aldrich (Cat No. R1780). To the media was added 0.2 mg/mL of [U12 – C6]-L-Arginine (Part No. 32460) and 0.04 mg/mL of lysine (Part No. 32461) or [U13 – C6]-L-Arginine and Lysine (Cat No. MS10011) from Invitrogen to produce the light and heavy labeled media. 0.05 mg/mL of L-Leucine purchased from Sigma Aldrich (Cat No. L-8000) was added to both media. 10% Dialyzed FBS from Invitrogen (Cat No. 26400-036) was also added to RPMI to complete the growth media. Cells were maintained in a moist atmosphere with 5% CO_2 at 37°C, with media change occurring every 2 days. Cells were allowed to go through 7 passages for incorporation of heavy and light amino acids before treatment with control or treatment reactions as described by Mann *et al*. (2002).[194] (Figure 3.2)

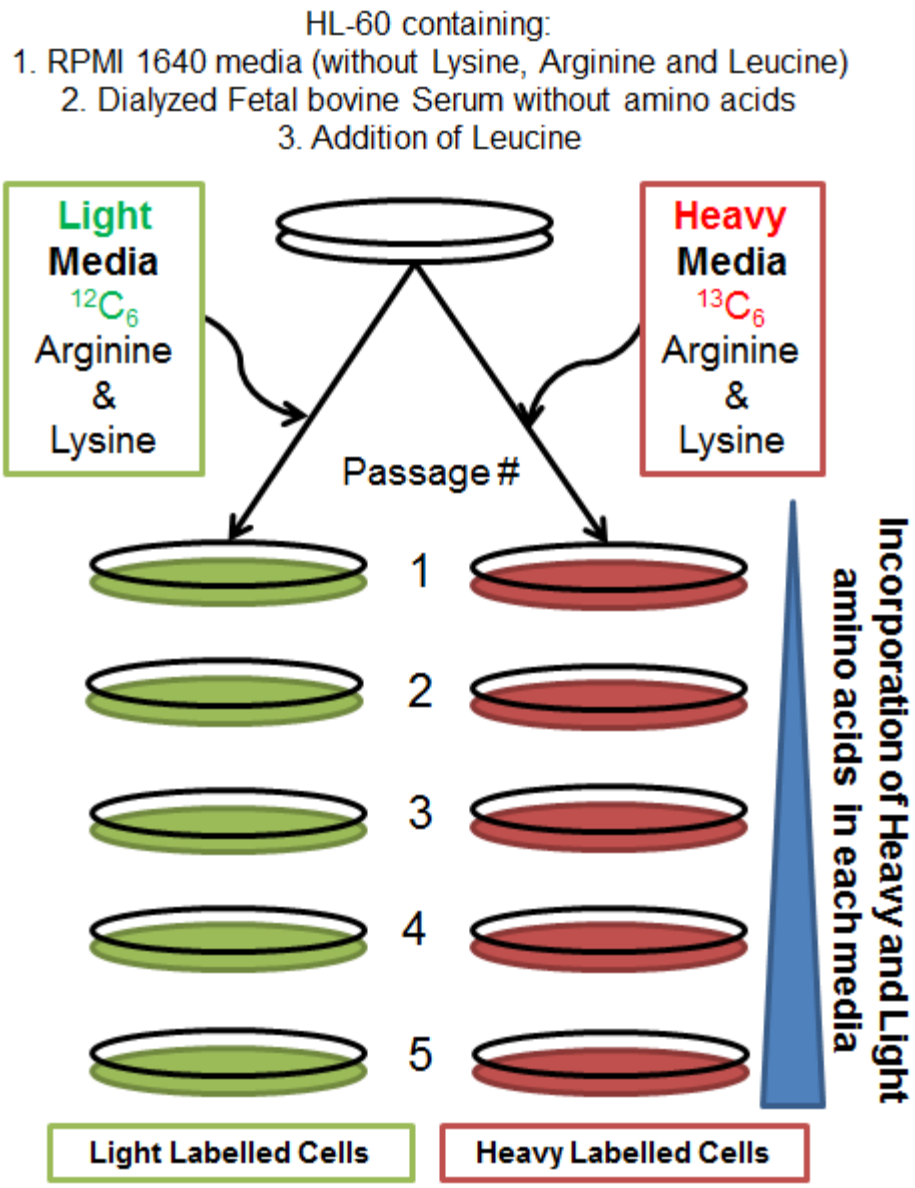


Figure 3.2 Growth of HL-60 cells for SILAC

3.3.3.2 Cell Lysis using RIPA buffer & 1:1 mixing

Heavy and light cells (5.0×10^6/mL, 1 mL) were treated with control (DMSO + 5 mM Glu & 25 mU/mL GO) or the treatment (5 mM AG + 5 mM Glu & 25 mU/mL GO) conditions for 6 h in a moist atmosphere with 5% CO_2 at 37°C. The control light and treatment heavy reactions which are collectively known as

the forward SILAC (sample A) are lysed using RIPA buffer and combined in a 1:1 ratio to produce sample A. The same procedure was applied to treatment-light and control-heavy reactions known as the reverse SILAC to produce sample B. 1:1 mixing was achieved by pre-running the samples on an acrylamide gel, staining it with Coomassie, and using LI-COR Odyssey gel scanner to scan and quantify the intensity of total protein content in each lane. (Figure 3.3)

3.3.3.3 SDS-PAGE & Gel-Digestion

Sample A and B were reduced by a 10% TCEP solution and denatured for 5 min at 95^{o}C with shaking at 500 rpm. Reduced samples were then loaded in a 1.0 mm 10% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R. The lanes corresponding to sample A and B were cut and sent to the Institute for Biomolecular Design at the University of Alberta for in-gel trypsin digestion. (Figure 3.3)

3.3.3.4 Protein identification & Quantification by LC-MS/MS

In-gel trypsin digestion was performed on the samples. Briefly, the excised gel bands were de-stained twice in 100 mM NH_4HCO_3/ACN (50:50). The samples were then reduced (10 mM β-mercaptoethanol 100 mM NH_4HCO_3) and alkylated (55 mM iodoacetamide in 100 mM NH_4HCO_3). After dehydration, trypsin (6 ng/ul) was added to just cover the gel pieces and the digestion was allowed to proceed overnight (~16 h) at RT. Tryptic peptides were first extracted

from the gel using 97% water/2% ACN/1% formic acid followed by a second extraction using a 1:1 mixture of extraction buffer and acetonitrile.

Fractions containing tryptic peptides dissolved in aqueous 25% v/v ACN and 1% v/v formic acid were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (MS) (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100μm inner diameter (300 Å, 5 μm, New Objective). Peptide mixtures were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using 60 min linear ACN gradients from 0 to 45% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60 000 and *m*/*z* range of 400–2000. The ten most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.3 (Thermo Scientific) and the non-redundant reviewed human protein database from Uniprot was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification

and oxidized methionine, ($^{13}C_6$) lysine and ($^{13}C_6$) arginine as dynamic modifications. Quantification of light and heavy SILAC peptides was performed with the Proteome Discoverer 1.3 software. (Figure 3.3). Figure 5.3 of the appendix displays the ESI-MS and MS/MS spectra of HIST1H4A histone protein from both forward and reverse SILAC samples.

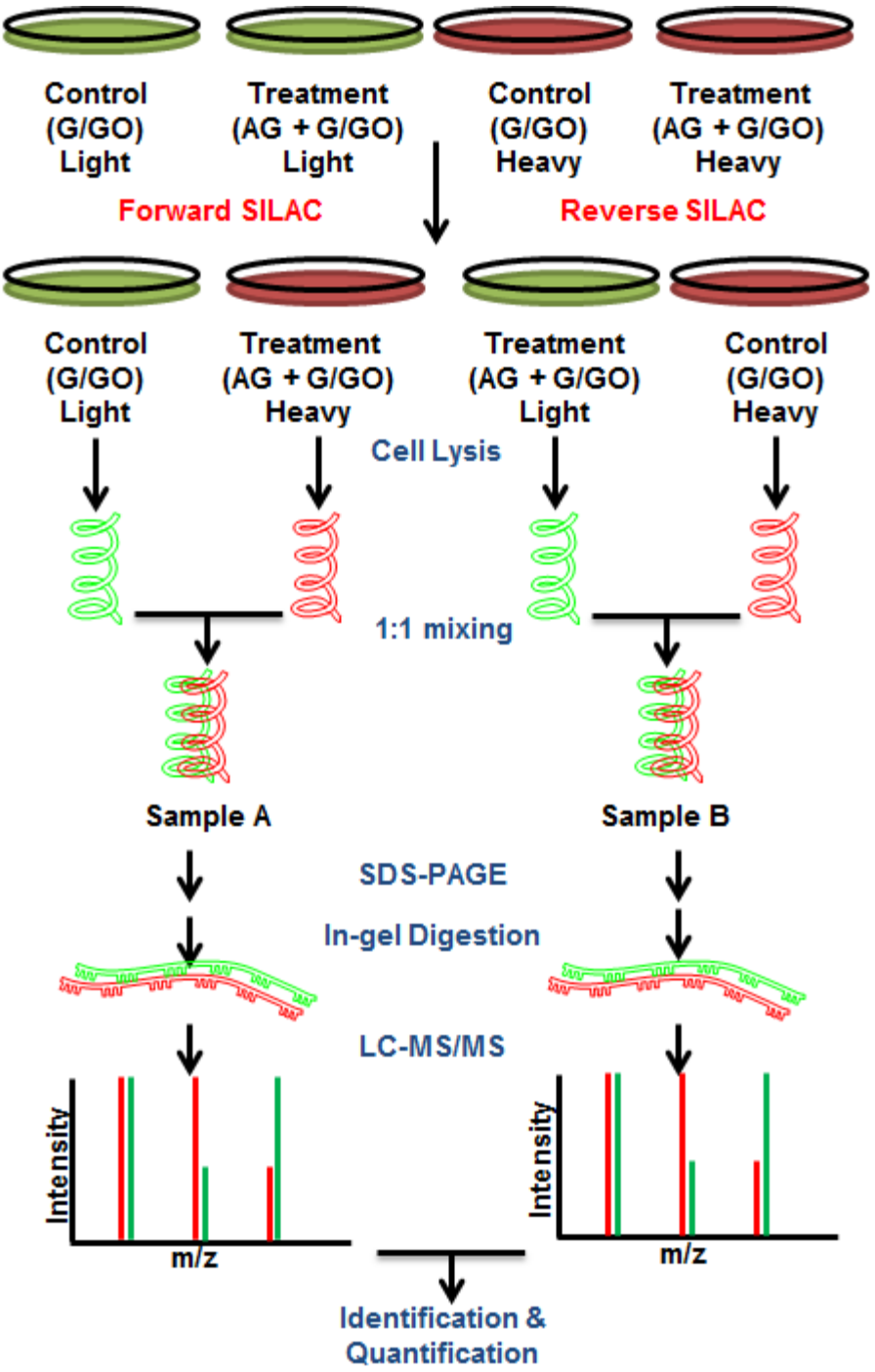


Figure 3.3 Treatment, Cell Lysis, 1:1 mixing, SDS-PAGE, and Gel digestion.

3.3.3.5 Data Processing

Proteome Discoverer 1.3 software was used to extract the high confident peptide MS data into a Microsoft Excel file which contained protein accession number as well as their expression level expressed as C_{13}/ C_{12} ratios. A total of 444 and 442 proteins were identified in sample A and B respectively. The filtering procedure as illustrated in Figure iii goes through the steps in which the proteins of interest are selected from the raw protein population. Proteins were filtered in three steps: First, proteins are separated based on whether they contain a C_{13}/ C_{12} ratio. Second, proteins with a ratio were separated whether they had a z-value above or below 1.96 and -1.96 respectively. Proteins with no ratio underwent manual peptide counting where proteins containing fully opposite labeled peptides found in both in the forward and reverse reactions were selected. The reason that some proteins do not contain a ratio is that their expression level might have changed so drastically that the ratio either becomes zero or close to infinity. Thus, it is necessary to perform a peptide count to select these proteins as potentially hits. Third, duplicate and contaminant proteins were removed from the selection.

The z-value is calculated by taking the $\log_2$ of the protein's C_{13}/C_{12} ratio and subtracting it from the mean $\log_2 C_{13}/C_{12}$ ratios of the total protein population in the sample and dividing it by the standard deviation of the $\log_2 C_{13}/ C_{12}$ ratios. The z-value of each protein corresponds to the number of standard deviations that a particular protein has been up or down-regulated from the mean of the protein population. Any protein above or below a value of +/- 1.96 was set to be

considered a protein of interest. The reasoning behind this is that 1.96 is the 95th percentile in a normal distribution and any value above 95% of all proteins statistically can be considered to be changed significantly. (Figure 3.4)

$$Z = \frac{(\text{Log2 C13/ C12 of protein X}) - (\text{Mean of Log2 for the protein sample})}{\text{Standard deviation of Log2 for the protein sample}}$$

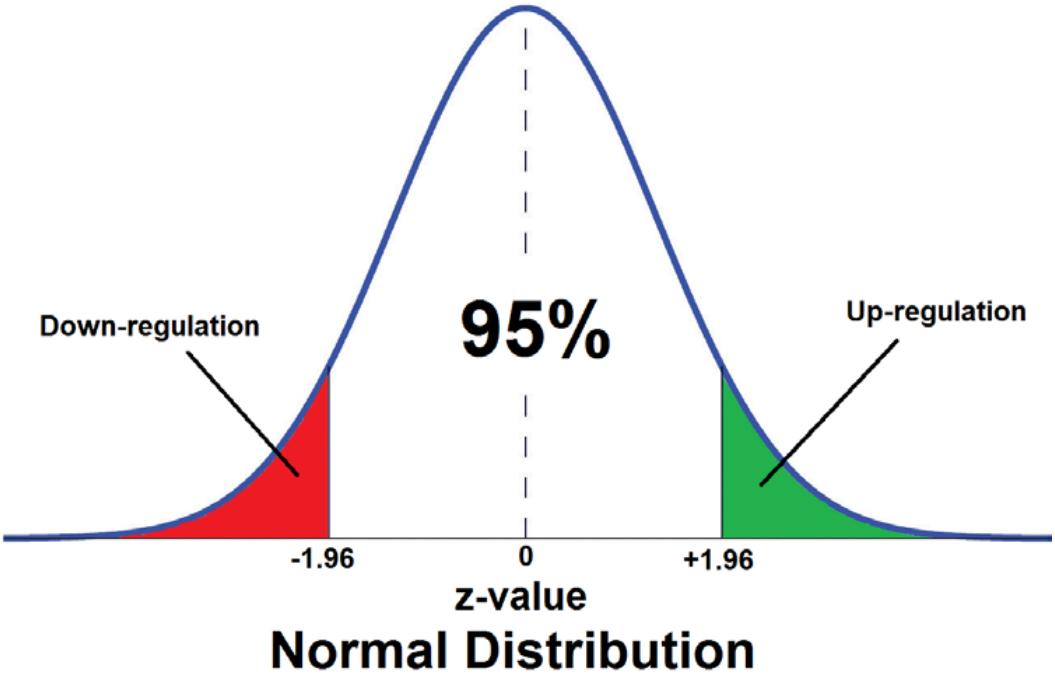


Figure 3.4 The normal distribution

After the fourth step, the resulting 43 proteins were sent to String 9.0 software (http://string-db.org/) for protein-protein analysis. However, most of the pathway analysis was done by manual curation using published peer-reviewed papers. (Figure 3.5)

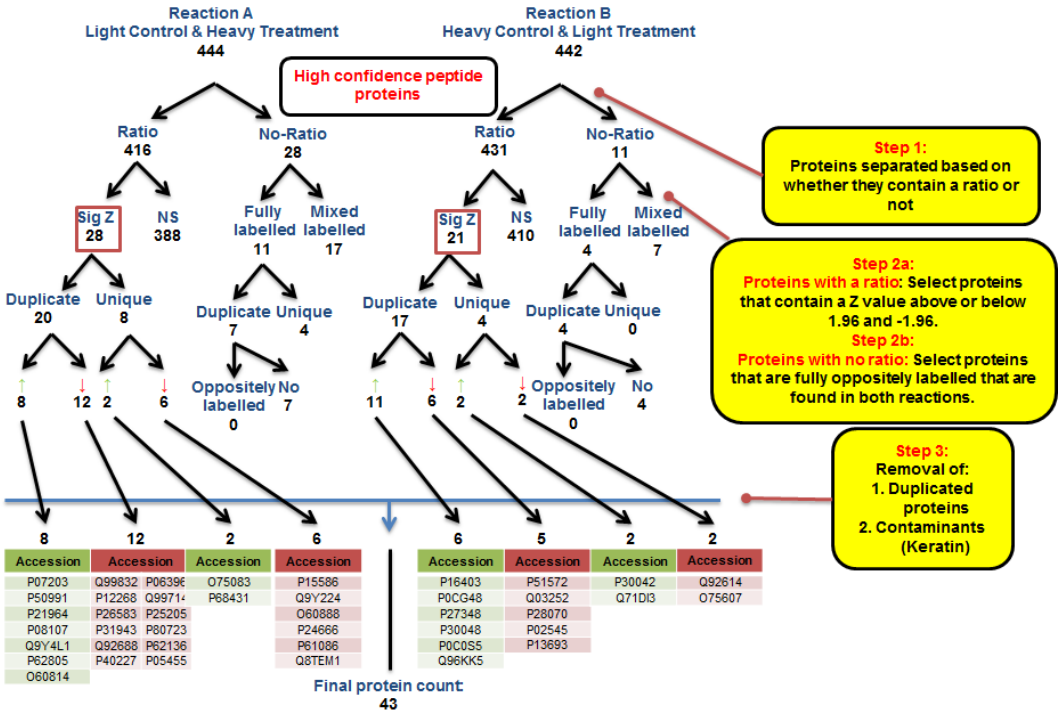


Figure 3.5 Protein filtering schematic

3.3.4 ELISA

A similar experiment to SILAC was run that included the control and the treatment reactions and subsequently quantified using BCA. 1 µg of each sample (100 µL volume) was added to Costar high binding 96 well opaque bottom plates (Fisher Scientific Cat No. 07-200-591). 200 µL of binding buffer (sodium carbonate/bicarbonate solution pH 9.4) was added to samples and allowed to incubate for 2 h at 35^{o}C to allow binding of proteins to the plate. After binding, that plate was washed and 5% BSA blocking solution was added to the wells and allowed to incubate for 2 h at RT. Rabbit anti-COMT primary antibody (Sigma Aldrich Cat No. C6870) in a dilution of 1:500 TBS-T and rabbit anti-SSB primary antibody (Abcam Cat No. ab124932) in a dilution of 1:10000 TBS-T was added

to each well and allowed to incubate overnight at 4^{o}C. The following day, the primary antibodies were removed. The wells were washed 5 times with TBS-T. Following the washing step, AP conjugated secondary goat anti-rabbit antibody was added in a dilution twice that of the respective primary antibodies and allowed to incubate for 1 h at RT. Thereafter, the washing step is once again repeated. After the second wash, Novex® AP Chemiluminescent Substrate (Invitrogen Cat No. WP20002) was added to each protein and allowed to well for 10 minutes. After the incubation, the luminescence (at all wavelengths) of the plates was measured by SpectraMax 5 spectrophotometer. Data was expressed as means ± standard error (SE).

3.3.5 Flow cytometry

Principle

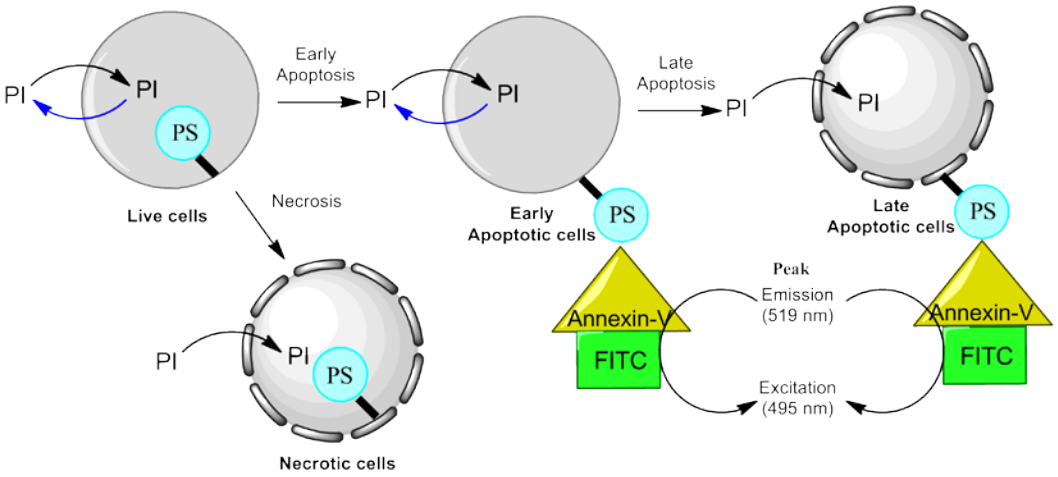
As mentioned in chapter 1, one of the morphological characteristics of apoptotic cells is the exposure of PS residues on the outer leaflet of their plasma membrane which happens to bind annexin-V in the presence of calcium. Flow cytometry is a technique that takes advantage of this characteristic to identify apoptotic cells by using a modified annexin-V coupled to the fluorophore FITC which has a peak emission and excitation of 519 nm and 495 nm respectively. Furthermore, propidium iodide (PI) is a dye that enters cells but is actively exported out of live cells. In combining a FITC annexin-V and PI staining procedure flow cytometry can categorize cells into four different states. First, live

cells will be characteristic of no PI and annexin-V staining. Second, early apoptotic cells will expose their PS residues and thereby be FITC annexin-V positive but they will be able to remove PI from their cytosol and be PI negative. Late apoptotic cells on the other hand will test positive for both FITC annexin-V and PI staining. Necrotic cells, which are not characteristic for PS exposure will be FITC annexin-V negative yet will be PI positive because of their damaged plasma membrane. (Figure 3.6)

Figure 3.6 Principle of Annexin-V-FITC flow cytometry

Protocol

The protocol was adapted from Radziwon-Balicka *et al.* (2013).[195] Briefly,



	Annexin-V	PI
Live cells	–	–
Early apoptosis	+	–
Late apoptosis	+	+
Necrosis	–	+

flow cytometry was performed on HL-60 cells (1.5 x 10^6/mL, 0.5 mL) treated with varying concentration of AG with or without G/GO and the MPO inhibitor 4-

aminobenzoic acid hydrazide (ABAH) for various time points to assess apoptosis. Following the reactions, cells were washed with 1X annexin-V binding buffer [0.01 M Hepes (pH 7.4), 0.14 M NaCl, and 2.5 mM $CaCl_2$] twice at RT. To the pellet was added 100 µL of binding buffer, 5 µL of FITC annexin-V (Cat No. 556420, BD Biosciences, CA) and 10 µL of 50 µg/mL of PI (Cat No. P4170, Sigma Aldrich). The samples were incubated at RT in the dark with constant shaking of 500 rpm for 15 minutes before being diluted with 400 µL with binding buffer and subsequently analyzed. Fluorescence was induced with an argon laser and detected on FL1 (525 nm BP filter) and FL3 (620 nm LP filter) on a Beckman Coulter Quanta SC flow cytometer. A total of 10,000 events were collected per sample. Compensation was performed using Cell Lab Quanta analysis software to account for fluorophore spectral overlap. Three samples treated with 50 µM menadione (MD) for 1 h (an apoptosis inducing agent) were used as negative (cells alone) and positive controls for annexin-V and PI only staining (i.e. cells only treated with one and not the other). Data was expressed as means ± standard error (SE).

3.4 H_2O_2 consumption analysis (appendix)

H_2O_2 consumption was recorded using a TBR4100 Free Radical Analyzer (World Precision instruments, Sarasota, FL) supplied with an ISO-HPO-2 H_2O_2 sensor. The LabScribe 2 PC-based software was used to generate amperometric measurements which were recorded over at RT, and imported into Microsoft

Excel sheet. The rates of H_2O_2 consumption were calculated using standard curves performed for each individual experiment.

3.5 Statistics

Statistical analysis was performed with SPSS version 21.0. A value of $p<0.05$ was considered statistically significant.

3.5.1 Cytotoxicity

One-way ANOVA followed by Tukey's post-hoc analysis was performed for the comparison of mean viability of HL-60 cells between different treatments.

3.5.2 ELISA & Flow cytometry

Independent samples t-test was performed to compare between HL-60 cells exposed with control and treatment.

Chapter 4

Results & Discussion

4.1 Trypan blue exclusion assay

HL-60 cells were treated with varying concentration of AG with or without the presence of G/GO for varying time points. Upon 6 h of exposure there was significant cytotoxicity observed in cells treated with 5 mM AG + G/GO compared to G/GO alone samples ($p<0.05$). At the 2 h time point there was significant cytotoxicity observed in cells treated with 1 and 5 mM AG + G/GO compared to G/GO alone ($p<0.05$). Cells treated with 5 mM AG + G/GO were also significantly different than 1 mM AG + GO which was subsequently different from 0.1 mM AG + G/GO ($p<0.05$). At 24 h of exposure time, there was significant cytotoxicity observed in cells treated with 5 mM AG + G/GO compared to G/GO and 1 mM AG + G/GO alone samples ($p<0.05$). Cells treated with 1 mM AG + G/GO had significantly lower viability than those treated with 0.1 mM AG + G/GO ($P<0.05$). Also, cells treated with G/GO had significantly lower viability than cells lone ($p<0.05$). (Figure 4.1)

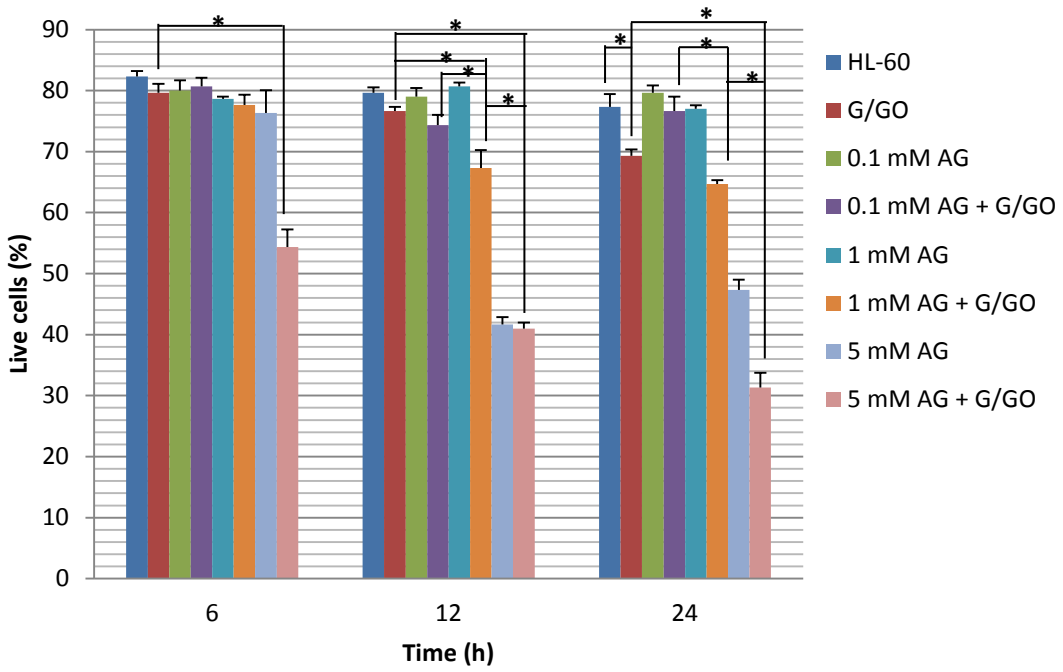


Figure 4.1 Trypan blue cytotoxicity assay on HL-60 cells after 6, 12, and 24 h of treatment with 0, 0.1, 1 and 5 mM AG with or without G/GO.

Tukey's HSD post-hoc test was used to compare between means (n=3). Asterisk (*) correspond to significantly different means ($p<0.05$). Error bar correspond to ± SE.

4.2 Immunospin trapping

HL-60 cells were pre-treated with 50 mM of DMPO before being treated with varying concentration (1-5 mM) of AG with the presence of G/GO for varying time points. There was no generation of protein radicals with cells alone alone (sample 1) or G/GO alone (sample 2). There was a dose and time dependency for the detection of protein radicals (mostly MPO, 75 kDa band) with cells treated with AG + G/GO; the highest amount of protein radicals were

produced at 5 mM AG + G/GO after the 24 h incubation period. The presence of DMPO was necessary for the detection of protein radicals (sample 8). The positive control for anti-DMPO detection was a Metmyoglobin-DMPO adduct provided by the supplier (sample 9). (Figure 4.2)

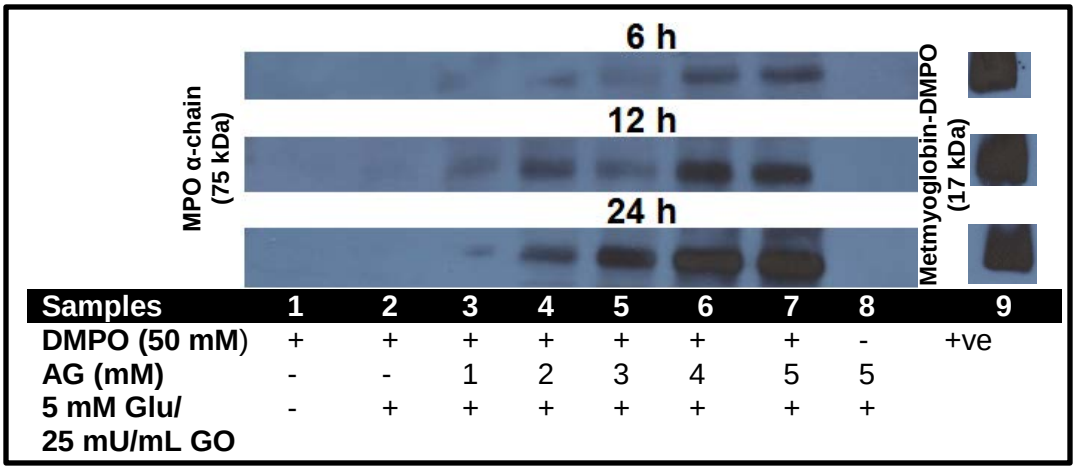


Figure 4.2 MPO radicals are generated by AG and H_2O_2 in HL-60 cells over time and trapped by DMPO using immuno-spin trapping SDS-PAGE method.

HL-60 cells were treated with 50 mM DMPO (sample 1 as negative control), 50 mM DMPO + 5 mM Glu/25mU/mL GO (sample 2 as negative control), 50 mM DMPO + 1 mM AG + 5 mM Glu/25mU/mL GO (sample 3), 50 mM DMPO + 2 mM AG + 5 mM Glu/25mU/mL GO (sample 4), 50 mM DMPO + 3 mM AG + 5 mM Glu/25mU/mL GO (sample 5), 50 mM DMPO + 4 mM AG + 5 mM Glu/25mU/mL GO (sample 6), 50 mM DMPO + 5 mM AG + 5 mM Glu/25mU/mL GO (sample 7), 5 mM AG + 5 mM Glu/25mU/mL GO (sample 8 as negative control) for 6, 12, and 24 hour durations. Positive control contained Metmyoglobin-DMPO (17 kDa). 30 µg of each sample was added per lane.

4.3 SILAC

HL-60 cells were treated with 5 mM AG + G/GO (treatment) and G/GO (control) for 6 h. Approximately 440 high confidence peptide proteins in both the forward and reverse reactions were identified by LC-MS/MS. Among the 440 proteins, only 43 exhibited significant changes in expression levels upon AG treatment. 18 (42%) and 25 (58%) of the 43 proteins were up and down-regulated with the treatment when compared to control, respectively. The protein list and their Z scores are provided in Table 1.

Table 4.1: List of significantly altered proteins obtained from SILAC. Proteins quantified with changes more than 1.96 standard deviations above or below the mean $\log_2 C_{13}/ C_{12}$ ratios of the total protein population in the sample are displayed. Accession number, protein name, z-value are listed. D= Duplicate proteins, U= Unique proteins.

Accession number	Full protein name – (Short name) – (Alternative name)	D/U	↑/↓	Z
Up-regulated Proteins (18)				
Q96KK5	Histone cluster 1, H2ah – (HIST1H2AH)	D	↑	7.6
O75083	WD repeat domain 1 – (WDR1) – (AIP1)	U	↑	6.9
P21964	Catechol-O-methyltransferase – (COMT)	D	↑	6.2
P48739	Phosphatidylinositol transfer protein – (PITPNB)	D	↑	5.3
P68431	Histone cluster 1, H3f – (HIST1H3F)	U	↑	5.1
Q71DI3	Histone cluster 2, H3d – (HIST2H3D)	U	↑	4.5
P62805	Histone cluster 1, H4h – (HIST1H4H)	D	↑	4.2
P30042	ES1 protein homolog, mitochondrial Precursor – (C21orf33)	U	↑	3.7
P07203	Glutathione peroxidase 1 – (GPX1)	D	↑	3.6
P0C0S5	H2A histone family, member Z – (H2AFZ)	D	↑	3.6
O60814	Histone cluster 1, H2bk – (HIST1H2BK)	D	↑	3.4
P50991	Chaperonin containing TCP1, subunit 4 – (CCT4)	D	↑	3.1
P30048	Peroxiredoxin 3 – (PRDX3)	D	↑	2.7
P08107	Heat shock 70kDa protein 1A – (HSPA1A) – (HSP70)	D	↑	2.5
Q9Y4L1	Hypoxia up-regulated 1 – (HYOU1)	D	↑	2.4
P27348	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide – (YWHAQ) – (14-3-3)	D	↑	2.2
P0CG48	Ubiquitin C – (UBC)	D	↑	2.1
P16403	Histone cluster 1, H1c. – (HIST1H1C)	D	↑	2.1
Down-regulated Proteins (25)				
Q99832	Chaperonin containing TCP1, subunit 7 – (CCT7)	D	↓	-2.0
P15586	Glucosamine (N-acetyl)-6-sulfatase – (GNS)	U	↓	-2.0
P26583	High-mobility group box 2 – (HMGB2)	D	↓	-2.0
Q9Y224	UPF0568 protein – (C14orf166)	U	↓	-2.0
P13693	Tumor protein, translationally-controlled 1 – (TPT1)	D	↓	-2.1
P31943	Heterogeneous nuclear ribonucleoprotein H1 – (HNRNPH1)	D	↓	-2.1
P02545	Lamin A/C – (LMNA)	D	↓	-2.1
P28070	Proteasome subunit, beta type, 4 – (PSMB4)	D	↓	-2.2
Q92688	Acidic nuclear phosphoprotein 32 family, member B – (ANP32B)	D	↓	-2.2
O60888	CutA divalent cation tolerance homolog (E. coli) – (CUTA)	U	↓	-2.3
P24666	Acid phosphatase 1, soluble – (ACP1) – (LMW-PTP)	U	↓	-2.4
P40227	Chaperonin containing TCP1, subunit 6A – (CCT6A)	D	↓	-2.4
O75607	Nucleophosmin/Nucleoplasmin 3 – (NPM3)	U	↓	-2.5
P06396	Gelsolin, amyloidosis, Finnish type – (GSN)	D	↓	-2.5
P61086	Ubiquitin-conjugating enzyme E2K – (UBE2K) – (Hip2)	U	↓	-2.5
Q99714	Hydroxysteroid (17-beta) dehydrogenase 10 – (HSD17B10)	D	↓	-2.5
Q92614	Myosin XVIIIA – (MYO18A)	U	↓	-2.6
P12268	IMP (inosine monophosphate) dehydrogenase 2 – (IMPDH2)	D	↓	-2.7
Q8TEM1	Nucleoporin 210kDa – (NUP210) – (gp210)	U	↓	-2.7
P25205	Minichromosome maintenance complex component 3 – (MCM3)	D	↓	-2.8
P80723	Brain abundant, membrane attached signal protein 1 – (BASP1)	D	↓	-2.9
Q03252	Lamin B2 – (LMNB2)	D	↓	-2.9
P51572	B-cell receptor-associated protein 31 – (BCAP31) – (BAP31)	D	↓	-3.0
P62136	protein phosphatase 1, catalytic subunit, alpha isoform – (PPP1CA)	D	↓	-3.1
P05455	Sjogren syndrome antigen B – (SSB) – (autoantigen La)	D	↓	-3.3

4.4 ELISA

The SILAC data was validated using ELISA where two proteins (COMT and SSB) were randomly picked from Table 4.1 1 based on the significance of their Z-value and their expression level was analyzed using the same experimental conditions used for SILAC except the cells were not isotopically labeled. ELISA data showed that the protein COMT was significantly up-regulated in HL-60 cells treated with 5 mM of AG (AG), 5 mM Glu, and 25 mU/mL of GO (G/GO) when compared to G/GO alone (n=3) treated for 6 h ($p<0.05$). The protein SSB (La protein) was shown to be significantly down-regulated (n=6) ($p<0.05$). (Figure 4.3)

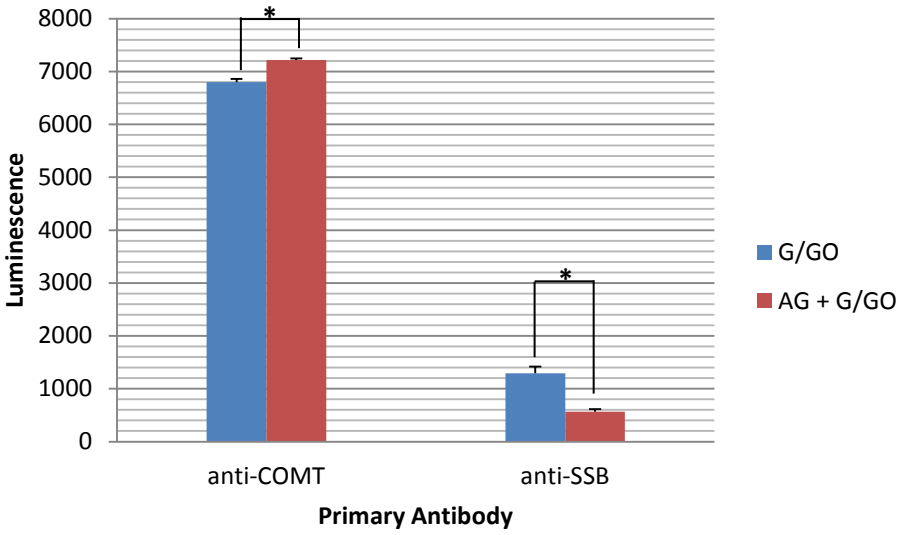


Figure 4.3 ELISA confirms the SILAC data obtained for COMT and SSB (La protein) proteins. Student t-test was used to compare between means (n=3 and 6 for anti COMT and anti-SSB respectively). Asterisk (*) correspond to significantly different means ($p<0.05$). Error bars correspond to ± SE.

4.5 Flow cytometry

Flow cytometry was performed on HL-60 cells treated with the same conditions as SILAC and ELISA and also included a sample containing 100 µM ABAH (MPO inhibitor) to assess the impact of MPO-dependent free radical metabolite production on cell death. 50 µM of menadione was used as a positive control for apoptosis. Upon 1 and even 3 h of treatment, there was no significant difference between the means in cell viability between G/GO, AG + G/GO, and AG + G/GO + ABAH samples in viability, early/late apoptosis or even necrosis; Although a trend was developing that would suggest the treatment with AG was having a some effect on those parameters. Nevertheless, after 6 h of treatment there was a significant drop in viability (approximately 27%, $p<0.05$) in AG + G/GO treated cells when compared to G/GO (Figure 4.4). As a consequence of drug toxicity, there was a significant increase in percentage of late apoptotic (approximately 15%, $p<0.05$) and necrotic cells (approximately 6%, $p<0.05$) but no change in percentage of early apoptotic cells (data not shown). However when cells were pre-treated with ABAH for 1 h, the viability of cells appeared similarly to of G/GO treated cells and had no increase in percentage of late apoptotic or necrotic cells ($p>0.05$) (Figure 4.4-4.6). Cells treated with AG alone did not exhibit toxicity at 1 and 3 h time points, although a slight (but non-significant) toxicity was observed at 6 h when compared to cells alone (data not shown). No toxicity was observed in cells treated with ABAH alone (data not shown). There was a dose dependent response observed as data with 1 mM AG instead of 5 mM showed a profile between G/GO and 5 mM AG + GO (data not shown). A

representative flow cytometry plot is presented in Figure 4.7 (Q1, necrosis; Q2, late apoptosis; Q3, live cells; Q4 early apoptosis).

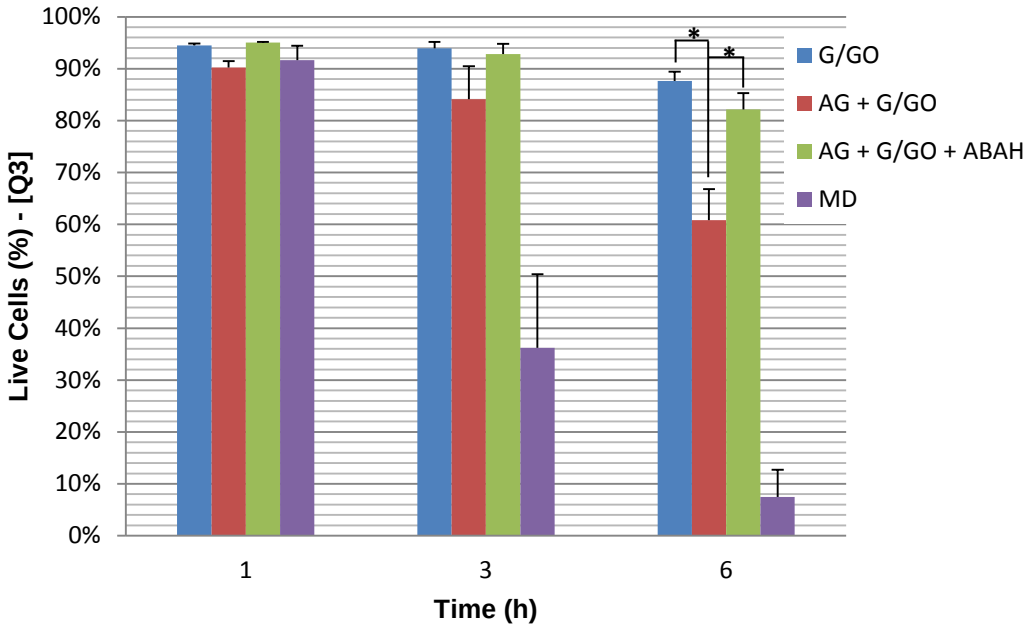


Figure 4.4 Percentage of viable HL-60 cells treated with 5 mM AG, G/GO, 100 µM ABAH and 50 µM MD for 1, 3, and 6 h using flow cytometry. Student T-test was used to compare between means (n=3). Asterisk (*) correspond to significantly different means ($p<0.05$). Error bar correspond to ± SE. MD was used as a positive control. Viable cells are represented on quadrant 3 of a flow cytometry dot plot.

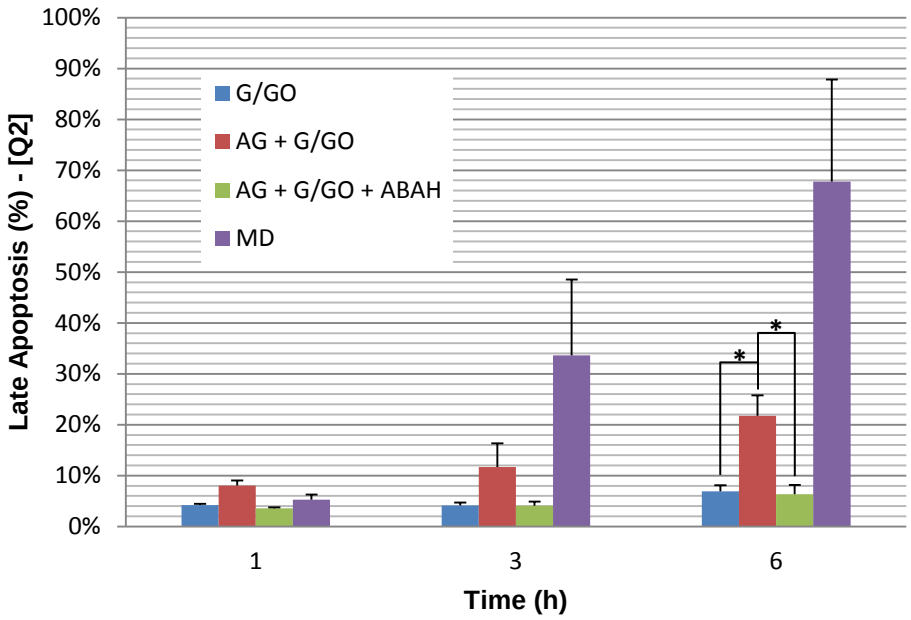


Figure 4.5 Percentage of late apoptosis in HL-60 cells treated with 5 mM AG, G/GO, 100 µM ABAH and 50 µM MD for 1, 3, and 6 h using flow cytometry. Student T-test was used to compare between means (n=3). Asterisk (*) correspond to significantly different means ($p<0.05$). Error bar correspond to ± SE. MD was used as a positive control. Late apoptotic cells are represented on quadrant 2 of a flow cytometry dot plot.

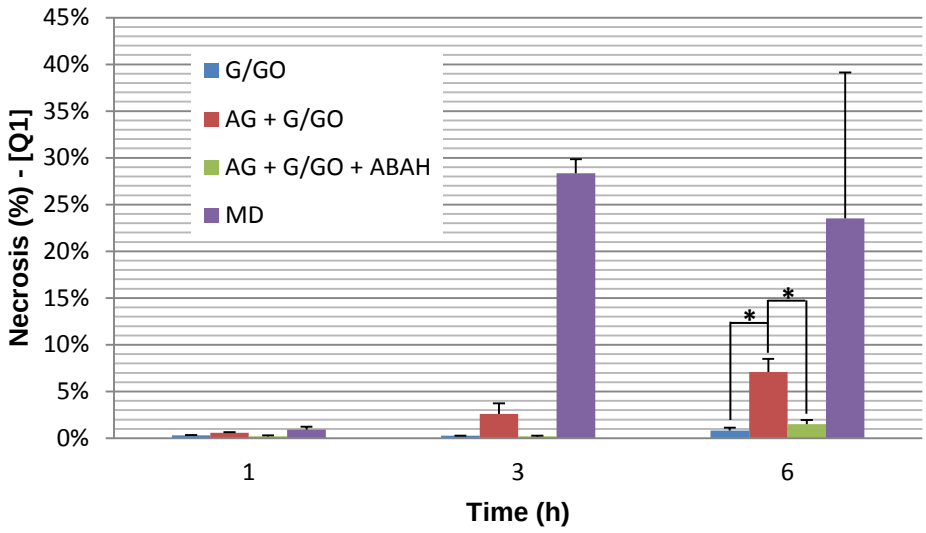


Figure 4.6 Percentage of necrosis in HL-60 cells treated with 5 mM AG, G/GO, 100 µM ABAH and 50 µM MD for 1, 3, and 6 h using flow cytometry. Student T-test was used to compare between means (n=3). Asterisk (*) correspond to significantly different means ($p<0.05$). Error bar correspond to ± SE. MD was used as a positive control. Necrotic cells are represented on quadrant 1 of a flow cytometry dot plot.

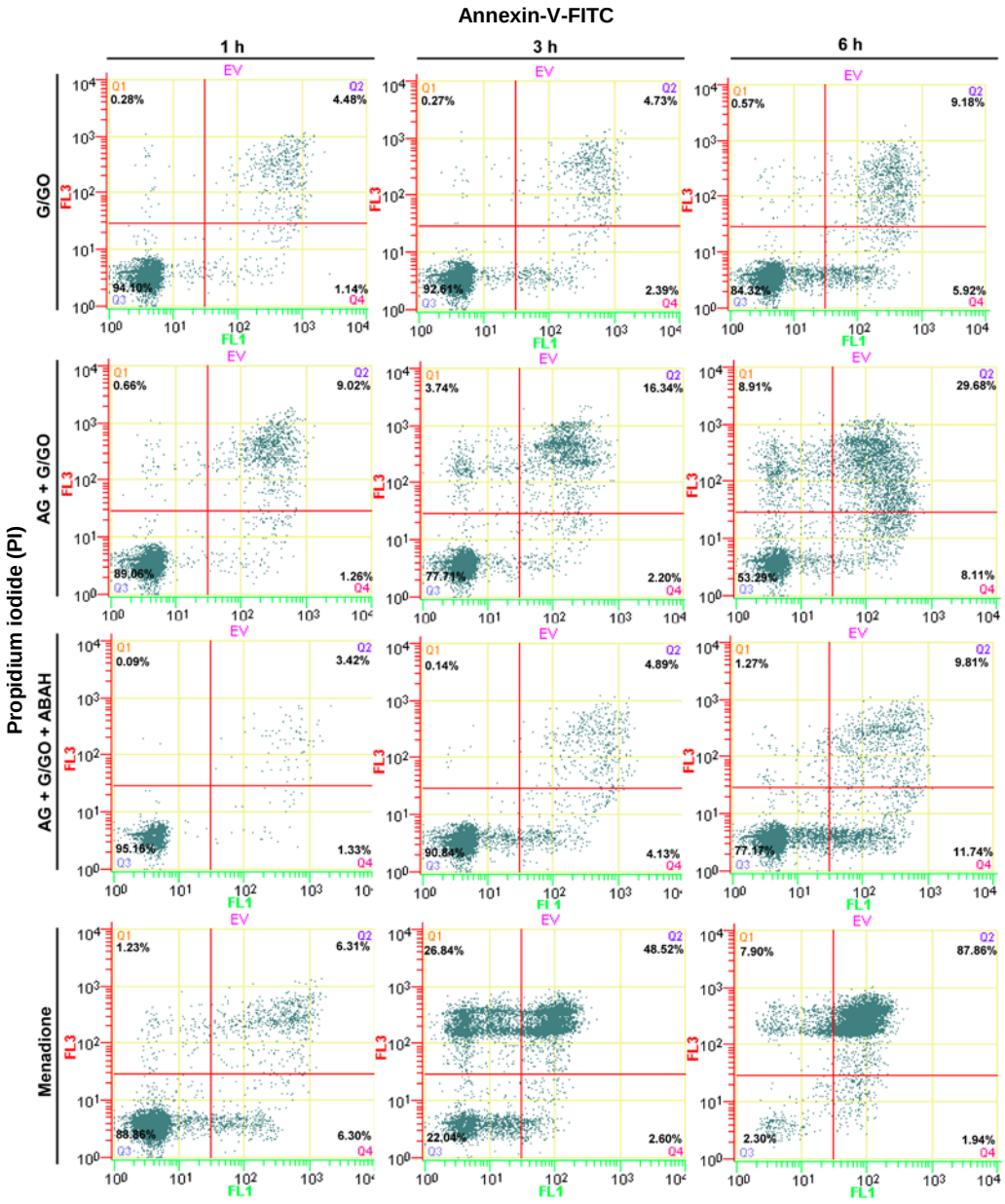


Figure 4.7 Representative flow cytometry dot plots. HL-60 cells were treated with G/GO, 5 mM AG + G/GO, 5 mM AG + G/GO + 100 µM ABAH, and 50 µM MD for 1, 3 and 6 h. MD was used as a positive control. Cells found in quadrants 1, 2, 3, and 4 correspond to necrotic, late apoptotic, live and early apoptotic cells, respectively.

4.6 Discussion

Initial cytotoxicity analysis using trypan blue exclusion assay revealed a significant loss in viability of cells treated with 5 mM AG + G/GO versus G/GO after 6, 12, and 24 h durations ($p<0.05$) and the concentration of AG also dictated the degree of toxicity (Figure 4.1). These results suggested that some form of cell death occurred when AG was introduced that resulted in the loss of viability in HL-60 cells. Subsequent immuno-spin trapping experiments (Figure 4.2) revealed a concentration and time dependent effect on the production of protein radicals which are mainly the α-chain of MPO. The question arose whether there was a link between the MPO radical generating capability of AG and cell death and whether MPO played a significant role in the mechanism. To answer this, a high-throughput quantitative proteomic technique (SILAC) was used to compare the entire proteome of cells treated with AG + G/GO to that of G/GO alone to search for significantly altered down-stream proteins associated with cell death. The conditions for the SILAC experiment were determined based on the concentration of AG that would produce the highest yield of protein radicals that also produced minimal cytotoxicity (by trypan blue uptake). There was however, a need to induce some cytotoxicity in order to ensure that cytotoxic effects of AG are present in cells while at the same time avoid using extremely toxic conditions due to problems in poor sample collection and protein quantification steps. Using these criteria, the treatment was set as 5 mM AG + G/GO and the control as G/GO for a treatment period of 6 h. According to figures 4.1 and 4.2, there was approximately 25% reduction in cell viability in 5 mM AG + G/GO treated cells

when compared to G/GO after 6 h treatment, yet it produced large and comparable amount of MPO radicals to 12 and 24 h time points which were significantly more cytotoxic (greater than 40% loss in viability).

When the proteome between the treatment and control was compared, 43 proteins were identified to be significantly altered in their expression (Table 4.1), among which 16 were associated with cell death pathways. First, there was up-regulation of 5 histone H1 proteins (HIST1H1C, HIST1H2AH, HIST1H2BK, HIST1H3F, and HIST1H4H) which are known inducers of cell death. In one study, purified histone H1 proteins injected in xenotransplanted nude mice with Burkitt's lymphoma showed arrested tumor growth. As was shown by electron microscopy and flow cytometry, the cause of the tumor growth arrest was attributed to severe plasma membrane damage.[196] In a recent study, it was shown that upon DNA damage, histones translocate from the nucleus to the cytoplasm where they increase the permeability of the outer mitochondrial membrane allowing pro-apoptotic molecules such as Smac/Diablo, or Cytochrome c to be released.[197] Therefore, the up-regulation (or perhaps the release of histones from DNA) of histone H1 proteins observed by AG could potentially be a signal for an assault on the cell membrane as well as release of pro-apoptotic substrates leading to apoptosis.

On the note of pro-apoptotic substrate releases from mitochondria, two proteins involved in the inhibition of this process were found to be down-

regulated. First, gelsolin (GSN) is known to inhibit cytochrome c release from mitochondria. GSN accomplishes this by closing a voltage-dependent anion channel thereby inhibiting the loss of mitochondrial membrane potential $\Delta\psi_m$.[198] Changes in the $\Delta\psi_m$ lead to the permeabilization of the mitochondria to solutes (e.g. K^+, Mg^{2+} and Ca^{2+} ions) with a molecular mass less than 1.5 kDa. The accumulation of these solutes and water in the mitochondria leads to the swelling of the mitochondria and the rupturing of outer membrane which causes the release of apoptotic substrates into the cytosol.[199] The release of Cytochrome c has been well established as a marker of apoptosis as it interacts with the previously discussed adaptor molecule Apaf-1 to trigger its ATP-dependent oligomerization and the formation of the apoptosome complex thereby activating the caspase 9/3 pathway.[200] Interestingly, GSN was found to be a cleavable substrate for caspase-3 and was cleaved into 39- and 41-kDa fragments 30 min after apoptosis induction in murine L929 cells. In addition, an *in vivo* study showed that neutrophils extracted from GSN lacking mice exhibited blebbing (irregular bulge in the plasma membrane) and DNA fragmentation, following the initiation of apoptosis.[201] Second, transitionally controlled tumor protein 1 (TPT1) was identified to be a protein down-regulated in tumor reversion (i.e. returning to a non-invasive cell type).[202] Recent studies have confirmed this by reporting its ability to act as an anti-apoptotic protein by inserting into the mitochondrial membrane thereby inhibiting Bcl-2-associated X (Bax) dimerization and degrading pro-apoptotic protein p53 through ubiquitination.[203, 204] Upon apoptosis,

Bax induces the opening of mitochondrial voltage-dependent anion channels thus promoting the release of apoptotic substrates into the cytosol.[205] (Figure 4.8)

Furthermore, we identified 5 inhibitors of apoptosis that were down-regulated by AG thereby supporting a pro-apoptosic response in HL-60 cells. Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B (ANP32B) also known as PAL31 is a caspase-3 inhibitor. In a recent study, it was shown in myeloid leukemia cells that down-regulation of ANP32B enhances caspase-3 activation and apoptosis.[206] The remaining 4 proteins were protein phosphatase 1 (PP1CA), Sjogren syndrome antigen B (SSB), Ubiquitin-conjugating enzyme E2K (UBE2K) also known as HIP2 and Acid phosphatase 1 (ACP-1) also known as LMW-PTP. In a study, human neutrophils treated with oleic acid, an inhibitor of PP1CA, were shown to be subjected to apoptosis.[207] The depletion of the RNA binding protein SSB also known as La protein using small interfering RNA technology was shown to reduce cell proliferation.[208] UBE2K or Hip2, promotes the degradation of mature Smac, a pro-apoptotic molecule that inhibits X-linked inhibitor of apoptosis protein (XIAP) thereby blocking cell death.[209] Finally, acid phosphatase-1 (ACP1) is an 18-kDa protein that removes phosphate groups off of tyrosine phosphorylated proteins. ACP1 was shown to be a positive regulator of tumor onset and growth in *in vivo* animal models.[210] Overall, key regulatory proteins involved in the inhibition of apoptosis by inhibiting the release of pro-apoptotic protein have been down-regulated by AG. (Figure 4.8)

The plasma and the nuclear membrane were also found to be affected by AG treatment. These membranes are key structures that are condensed and fragmented as a result of apoptosis.[113] We found nuclear envelope proteins LMNB2 and LMNA as well as nuclear pore complex NUP210 (gp210) to be down-regulated in AG treated cells. Disruption of the nuclear lamina can have catastrophic effect on the structural integrity of the nucleus as well as the vital chromosomes that are cell's master switch to adapt to environmental changes.[211, 212] Furthermore, a novel member of the myosin superfamily called MYO18a was also found to be down-regulated suggesting plasma membrane fragmentation. MYO18a was shown to be involved in actin reorganization, and migration of epithelial cells.[213] (Figure 4.8)

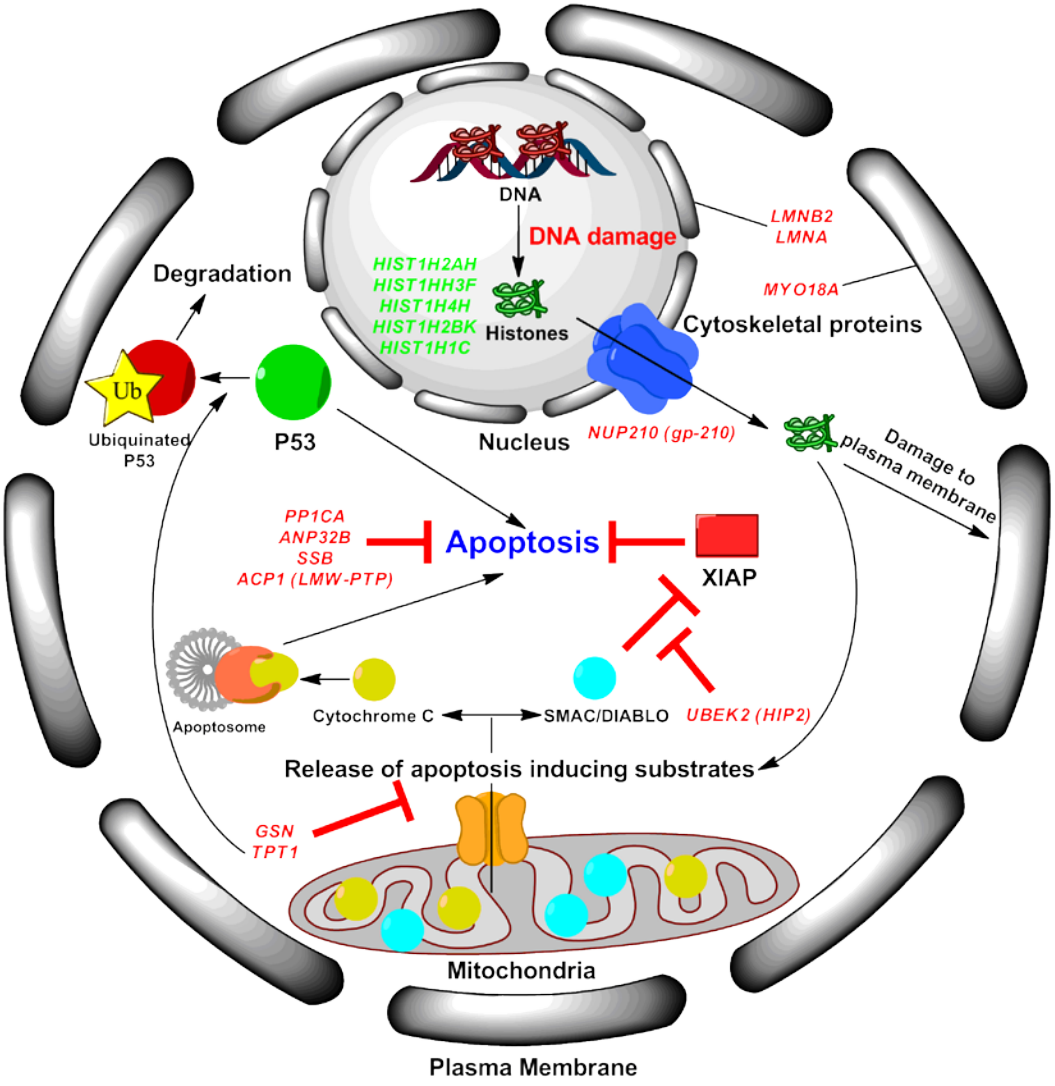


Figure 4.8 An illustration of significantly altered proteins due to treatment with AG and their link to cell death.

The validity of the SILAC data was confirmed by measuring the expression levels of two proteins with significantly altered expression (SSB and COMT) through ELISA. As shown in figure 4.3, HL-60 treated cells with 5 mM AG + G/GO demonstrated a significant up and down-regulation in COMT and SSB proteins, respectively, when compared to G/GO control ($p<0.05$). This data correlated with the Z-scores that were derived from the C_{13}/C_{12} ratios of LC-MS-

MS peptide analysis. The COMT and SSB proteins had a Z score of +2.6 and -3.3, respectively. In other words, the treatment resulted in a change in their expression level at 2.6 and 3.3 standard deviations above and below the average expression of most proteins when compared to control, respectively (Table 4.1).

The data obtained from SILAC prompted a deeper investigation into apoptosis as an endpoint of AG toxicity. As a result, the annexin-V flow cytometry was employed to detect two parameters of cell death: PS exposure on the outer leaflet of the plasma membrane and the presence of PI inside the cells (see chapter 3.3.5). The results showed significantly more late apoptosis (15%) and necrosis (6%) in AG treated cells than control after 6 h treatment, which complemented and reaffirmed the results obtained from SILAC. In addition, the results showed significantly less viable cells (27%) which are comparable to the trypan blue assay (25%). The inhibition of MPO enzymatic activity by ABAH played a major role in inhibiting the cytotoxicity caused by AG: the levels of both late apoptosis and necrosis of AG + G/GO + ABAH treated cells were similar to that of G/GO alone. This suggests that AG's metabolism by MPO is a major contributor to AG toxicity. A previous study showed that ABAH inhibited protein radicals induced by AG metabolism, suggesting a link between AG-induced radical and cell death mechanisms.[14] However, the exact causative effect in cell death has yet to be identified. This could be the nitrogen centred AG radical, the MPO radical, or a by-product down-stream the MPO metabolism pathway. Free radicals can lead to oxidative stress which is known to damage macromolecules

and can cause the triggering of signaling mechanisms that lead to apoptosis. AG nitrogen centred free radical appears to lead to MPO protein radical formation. If this protein radical is not repaired, it can lead to impairment of function and/or formation of large protein aggregates which are cytotoxic.[214]

4.7 Conclusion

The data obtained from proteomic and flow cytometry provide a new foundation for AG cytotoxicity mechanisms and have broader implications to overall aromatic amine toxicity, and potentially IDRs such as drug-induced agranulocytosis. The role of MPO in the peroxidative metabolism of AG as well as apoptosis and necrosis in HL-60 cells characterizes free radical species as significant players in the process.

4.8 Future studies

Although SILAC provided invaluable information on AG cytotoxicity through identifying possible down-stream targets, there is yet to be mechanistic studies carried out on the matter. For example, it was interesting to see that seven histone proteins, five of which involved in cell death, were up-regulated upon AG treatment; however, the exact up-stream signaling pathways (i.e. DNA damage) activated that elicited such response remains to be investigated. DNA damage can be probed using the single-cell gel electrophoresis also known as the comet assay which can measure DNA stranded break in eukaryotic cells.[215] Since apoptosis is characterized by DNA fragmentations, southern blot agarose gel electrophoresis

can be used to analyze DNA fragmentation patterns. Therefore, future studies should focus on providing a mechanistic framework for AG toxicity. This can be accomplished through first, the identification of up-stream proteins that interact with AG and second, understanding the interplay of these up-stream proteins with the already identified down-stream targets. Since free radical production by MPO was an important marker of cell death, it would be of great interest to investigate the exact down-stream targets of MPO radical itself.

Future studies should focus on including ABAH in SILAC to understand the changes in the proteome specifically to cell death bio-markers when free radical generation is inhibited. SILAC should also be done in different time points to better understand how time changes the proteome. There should also be a shift from the *in vitro* HL-60 model to a more clinically relevant *in vivo* model using a label free method called spectral counting. Spectral counting, unlike SILAC, does not use isotopically labelled amino acids to quantify protein expression between control and treatment. In spectral counting, control and treatment samples are individually subjected to LC-MS/MS analysis where quantification involves the comparison of peaks intensity between the same peptide. Spectral counting will allow for a better understanding of how the results obtained in our HL-60 model relate to neutrophils of animal models. Specifically, it would be important to know if clinical doses of AG would elicit the same apoptotic down-stream targets in neutrophils and whether inhibiting MPO would impact neutrophil count.

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Chapter 5

Appendices

5.1 Continuous generation of H_2O_2 using G/GO

H_2O_2 is produced continuously by the use of G/GO and O_2 using the reaction below:

D-Glucose + O_2 → D-glucono-1,5-lactone + H_2O_2

5.1.1 Initial rate of H_2O_2 production in RPMI-1640 media

The initial rate of H_2O_2 production was measured using 5 mM glucose and 25 mU/mL of GO (G/GO) in RPMI-1640 media with constant mixing for 6 hours. After collecting and plotting the data, the slope of the linear portion of the data corresponding to the first hour of three independent experiments was obtained. These values measured an increase or change in the voltage per second (Δ V / sec) which was converted to per minute (Δ V / min). Standard curve plots were done for each experiment where changes in voltage were recorded with known amount of H_2O_2. The rates in Δ V / min were converted to μM H_2O_2 using the standard curve values. The amount of GO used in each reaction equated to approximately 1.48×10^{-4} g and total volume for each reaction was 1 mL. Using these values, the rate of H_2O_2 production was calculated to be 15.51 ± 1.45 μmol H_2O_2/mg GO/min (Table 5.1).

Table 5.1 Rate of H_2O_2 production.

	y = mx + b	R^2	m (Δ V / min)	1 V (μM H_2O_2)	Rate of production (μmol H_2O_2/mg GO/min)
1	y = 0.0008x + 0.6684	0.9927	0.054	50.48	18.42
2	y = 0.0005x + 0.5438	0.9932	0.030	69.29	14.05
3	y = 0.0004x + 0.5643	0.9852	0.024	86.78	14.07
				Mean	15.51
				S.E	1.45

5.1.2 Overall production of H_2O_2 over 6 h

The overall production of H_2O_2 was recorded over a 6h period using the same procedure as chapter 5.1.1 except the reaction was done inside an incubator (37^{o}C and 5% CO_2) to imitate the actual experimental conditions. Figure 5.1 illustrates the standard curve for the experiment where a change in one voltage corresponds to approximately 63.97 μM of H_2O_2.

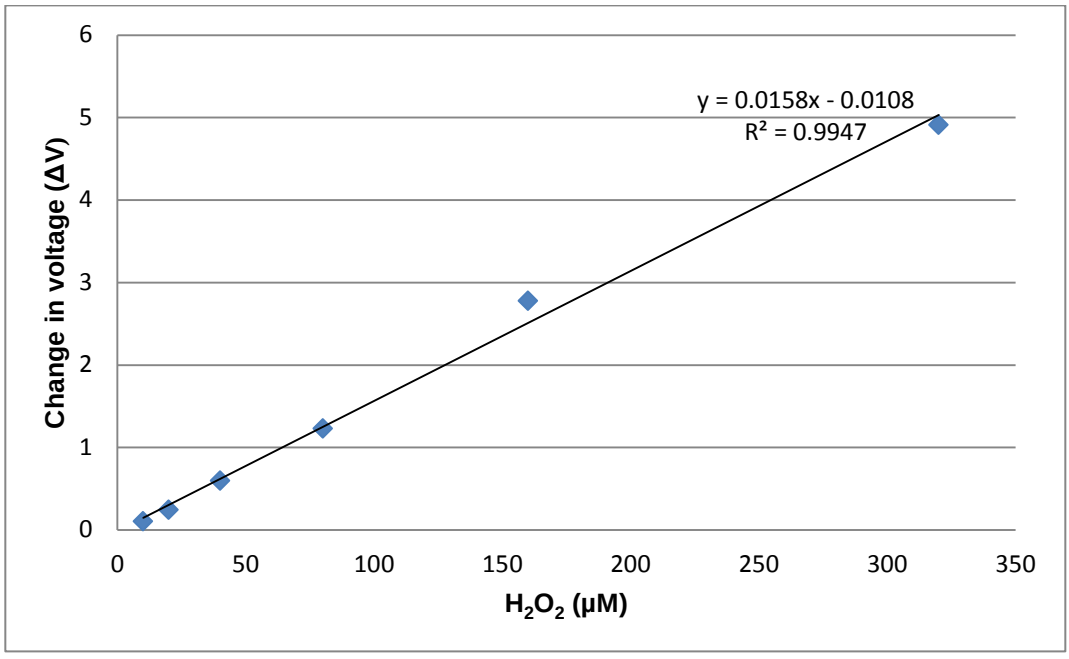

Figure 5.1 Standard curve for H_2O_2.

Figure 5.2 shows the recorded levels of H_2O_2 produced over 6h duration in RMPI-1640 media only, media containing 15.67 nM of MPO (rough estimate found in 1 x 10^6/mL of HL-60 cells), and in cells (1 x 10^6/mL, 1mL). Media containing G/GO produced roughly 1278 µM of H_2O_2 in 6h duration. This was slightly more than a reaction containing G/GO containing MPO which 1166 µM of H_2O_2 suggesting some consumption of H_2O_2 by MPO itself. However, when G/GO was run in cells, no more than 17 µM of H_2O_2 was produced suggesting its consumption by the cell's enzymes (i.e. catalase). (Figure 5.2)

When 5 mM of AG was introduced into the G/GO reaction containing MPO, there was a sharp decrease in the amount of H_2O_2 (15.5 µM) after 6 h suggesting its metabolism by MPO. However, when the MPO inhibitor ABAH was added to AG + G/GO, the levels of H_2O_2 (1223 µM) were similar to the G/GO condition after 6 h confirming the role of MPO in catalysis of H_2O_2 with the presence of AG. (Figure 5.2)

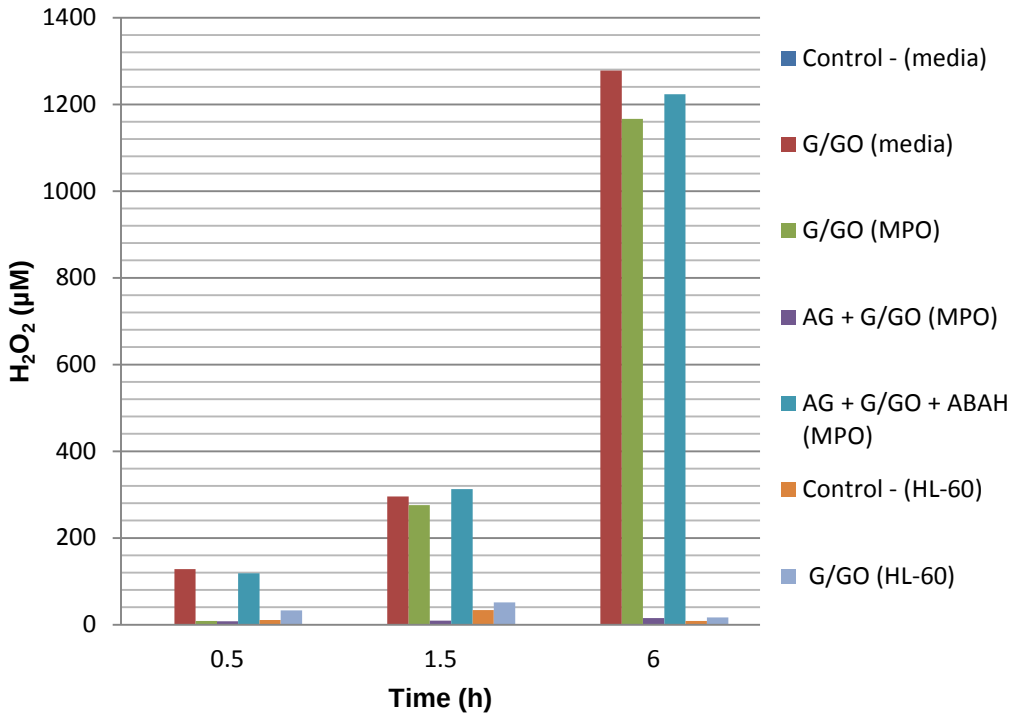

Figure 5.2 Production of H_2O_2 over 6 h.

5.2 ESI-MS and MS/MS spectra

Figure 5.3 is representative ESI-MS and MS/MS spectra showing AG-induced up-regulation of HIST1H4A histone protein. Shown are the MS for the [M+2H]2+ ions of HIST1H4A peptide ISGLIYEETR and ISGLIYEETR* ('R*' designates the heavy arginine) from the forward (A) and reverse (B) SILAC experiments. Depicted in (C) and (D) are the MS/MS for the [M+2H]2+ ions of ISGLIYEETR and ISGLIYEETR*, respectively.

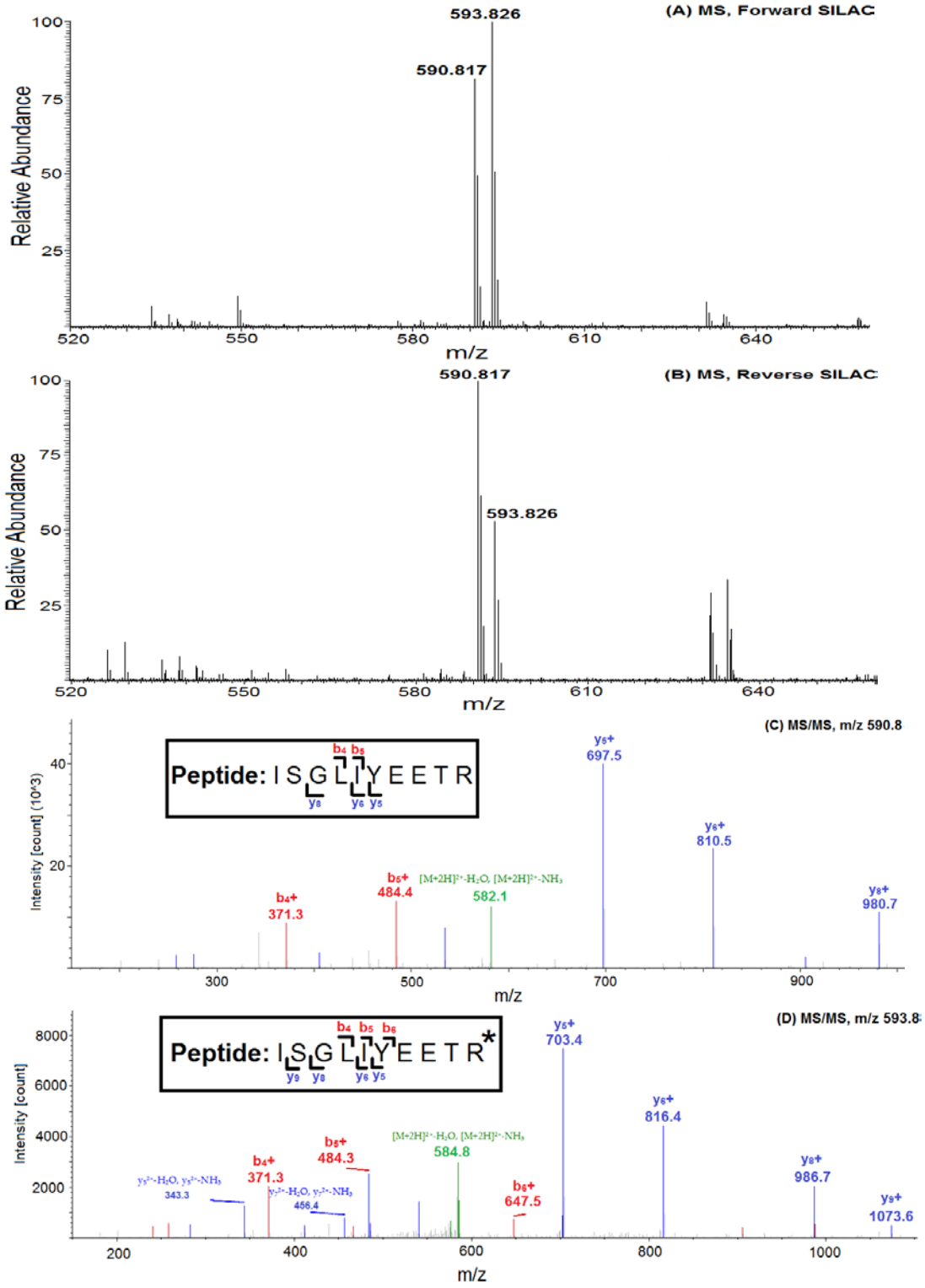

Figure 5.3 ESI-MS and MS/MS spectra.

R* designates the heavy arginine