“Chemical Reactivity and Biological Activity of Bethoxazin, an Industrial Microbicide”

by

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Abstract

Bethoxazin is a broad spectrum industrial biocide with commercial applications as a material preservative; however its mechanism of action has not been investigated. In this study, the chemical reactivity of bethoxazin towards biologically important nucleophiles was assessed with UV-Vis spectroscopy. Bethoxazin reacted with molecules containing free sulfhydryl groups such as glutathione and human serum albumin but not with amino, acetate or phenol containing compounds. Bethoxazin was shown to potently inhibit the growth of the K562 human cancer cell line with an IC_{50} value in the micromolar range. The sulfydryl fluorescent label ThioGlo-1 was used to investigate the biological effects of bethoxazin in K562 cells and explore its mechanism of action. Bethoxazin inhibited the formation of covalent adducts in K562 cells between the free sulfhydryl group of biomolecules and ThioGlo-1, implying that bethoxazin reacts with molecules containing free sulfhydryl groups. Likewise, when glutathione was depleted in K562 cells, by buthionine sulfoximine, high concentrations of bethoxazin were able to inhibit the formation of covalent adducts between sulfhydryl biomolecules and ThioGlo-1. The growth inhibition assay (MTS) was used to investigate the effect of continuous bethoxazin treatment versus wash out in K562 cells. The MTS assay revealed a reduction in the potency of bethoxazin due to the wash out effect, suggesting that the growth inhibition effects of bethoxazin are likely not due to glutathione depletion. A two-colour flow cytometry analysis of bethoxazin treated K562 cells for eight hours demonstrated that bethoxazin provokes necrosis induced cell death in K562 cells. Taken together, these experimental results demonstrate that the reaction of bethoxazin with proteins containing an accessible sulfhydryl group is more likely to be the mechanism of action of the cell growth inhibition effects rather than glutathione depletion.

Acknowledgments

I would like to thank my advisor Dr. Neal Davies for his supervision and support throughout my Master's program. His guidance and encouragement kept me going.

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Many thanks to the graduate chair Dr. Hope Anderson, as well as Mr. Brian Barth, and Dr. Matthew Decter for their sincere efforts and help throughout my Master program. Their professional advice and kindness were highly supportive in helping me to fulfill the program requirements.

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The continuous support, care, and encouragement that I received from my friends, colleagues, and family was what kept me strong to overcome the difficulties I faced during my program.

Special thanks to my parents, siblings, and my son for inspiring me and being around me at the times I need them most.

Dedication

This project is dedicated to my beloved parents, siblings, and my adorable son who were always supportive and caring.

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Abbreviations

DNA: deoxy ribonucleic acid

kDNA: kinetoplast plasmid DNA

UV-Vis: Ultraviolet Visible

GSH: Glutathione

HSA: human serum albumin

DMSO: dimethyl sulfoxide

Tris: tris(hydroxymethyl)aminomethane

pH: the negative of the logarithm of hydrogen ions concentration

α-MEM: alpha minimum essential medium

PBS: phosphate buffer saline

FBS: fetal bovine serum

IC_{50}: 50% inhibitory drug concentration

$t_{1/2}$: half-life of a chemical reaction

SE: Standard error

SD: standard deviation

BSA: bovine serum albumin

FI: fluorescence intensity

MTS: *(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)*

Nu: nucleophile

X: represents a leaving group

PS: phosphatidylserine

Log P: the negative of the logarithm of partition coefficient

Annexin-V FITC: Annexin-V-fluorescein isothiocyanate

PI: propidium iodide

min: minutes

hr: hour

h: hour

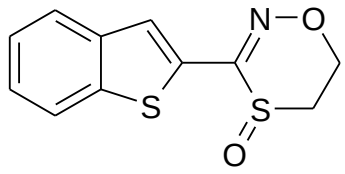
Chapter 1: Introduction

1.1. Bethoxazin

During drug discovery screening of various compounds provided by Chemtura Corporation (Elmira, ON, Canada) to our laboratory, a compound was identified that exhibited potent cytotoxic effects in the chronic myeloid leukemia K562 cell line with an IC_{50} value of 1 µM. This same compound also showed potent activity against a large number of cancer cell lines in the US National Cancer Institute 60-cell screens (unpublished data). The identified compound is bethoxazin (Figure 1), a broad spectrum oxathiazine industrial microbicide that Chemtura licensed to Janssen Pharmaceutica (Bosselaers J et al, 2003) for commercial development. Bethoxazin is used to protect materials such as wood from decaying by micro-organisms due to its antifungal and algaecide actions (Bosselaers J et al, 2003). Other applications of bethoxazin also include incorporation into paints, coatings, adhesives, sealants and plastics (Bosselaers J et al, 2003; Nicholas D, 2004).

The mechanism of action by which bethoxazin exerts its biocidal effects has not been investigated and there have been no published reports on its activity against cancer cells. There was a study on the effects of bethoxazin against various bacterial strains (Wallace D F & Dickinson D J, 2004) and another study that has shown growth inhibition effects against yeast (Chee G L et al, 2012). The chemical reactivity and biological effects of bethoxazin have not been further delineated. Most of the reported studies on bethoxazin in the literature have been focused on its spectrum of activity towards microorganisms for commercial use (Bosselaers J et al, 2003; Williams G & Bacon M 2006; Bruns et al, 2013; Davis R A et al, 1998; Van Der Flaas, M A J B, 2003).

Figure 1: Chemical structure of bethoxazin. Chemical name: 3-(1-benzothiophen-2-yl)-5,6-dihyro-1,4,2-oxathiazine 4-oxide. Molecular formula: $C_{11}H_9NO_2S_2$. Molecular weight: 251.33 g/mol. Partition coefficient: log 10 P o/w = 2.7 (Bosselaers J et al, 2003).



In addition to showing potent inhibitory activity against K562 cells and yeast BY4742 cells (Figure 2A, unpublished data, Chemtura (Elmira, ON, Canada); 2B), bethoxazin also inhibited the catalytic decatenation activity of human and yeast topoisomerase IIα enzyme (Figure 3 A, B) with IC_{50} values in the micromolar range (IC_{50} graphs not shown). Since topoisomerase II is an essential nuclear enzyme that regulates the topology of DNA and an important target for a number of clinically important anti-cancer drugs such etoposide, it was of interest to study bethoxazin in greater detail to understand its cytotoxic mechanism of action, and to explore its potential use as a clinical anticancer agent. Studying the mechanism of action of bethoxazin is a critical aspect of its potential utility as a pharmaceutical agent. Due to some initial scientific findings, it was of interest to investigate the mechanism by which bethoxazin exhibits its cell growth inhibition effects in K562 cancer cells.

Figure 2: (A) Bethoxazin inhibits the growth of K562 cells at an IC_{50} of about 1 µM. The graph shows the growth inhibitory effects on K562 cells after a 72 hours incubation measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. The absorbance was measured at 490 nm using a molecular devices plate reader (unpublished data). (B) Bethoxazin inhibits the growth of BY4742 yeast cell line at an IC_{50} of about 11 µM (Chee et al, 2012).

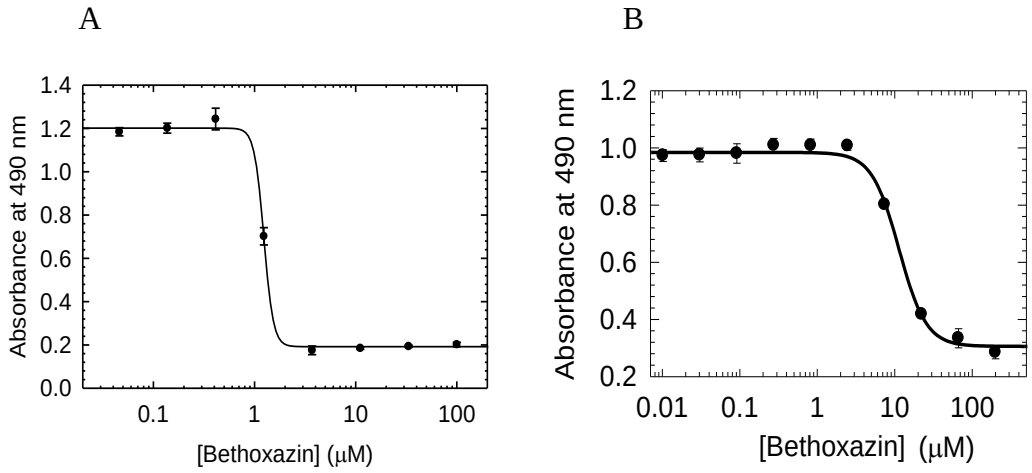
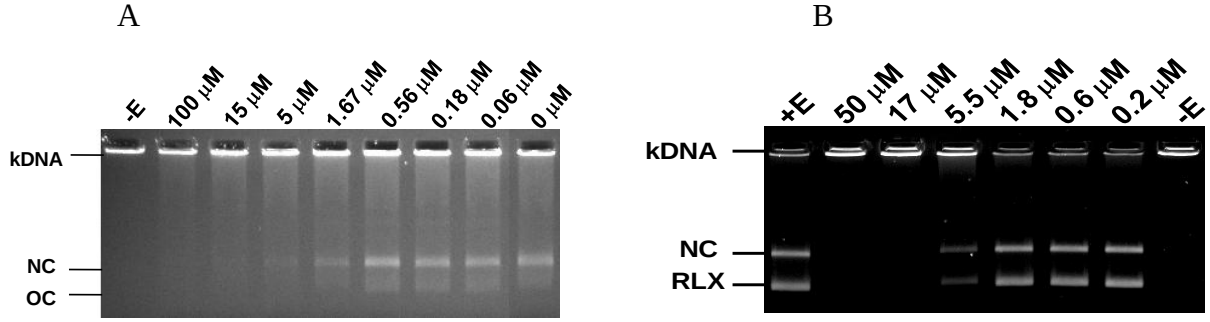


Figure 3: Fluorescent image of ethidium bromide-stained agarose gel shows that bethoxazin inhibits the catalytic decatenation of human topoisomerase II enzyme (A) (unpublished data) and yeast topoisomerase II enzyme (B) (Chee et al, 2012)..



Bethoxazin exhibits exciting drug likeness properties which makes it a potentially interesting drug candidate for further development. It has a high favorable acute oral LD_{50} value of 3631 mg/kg in rats of (Bosselaers J et al, 2003) which is much higher than the LD_{50} of

acetylsalicylic acid (aspirin) in rats which is 200 mg/kg. (MSDS, NSF international, Ann Arbor, MI). Furthermore, bethoxazin has a molecular weight of 251.33 g/mole, a partition coefficient value of about 2.7, and is potent at the target site with an IC_{50} of 1 μM. These properties were attractive features to further investigate bethoxazin's chemical and biological effects and its possibility to be developed into a pharmaceutical agent.

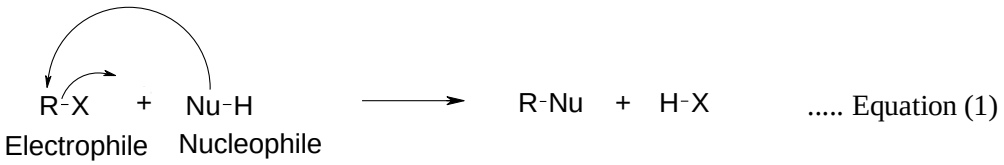
Due to the interesting scientific findings on bethoxazin in our laboratory and the lack of published literature on bethoxazin, this study was conducted. The aim of this study is to assess the chemical reactivity of bethoxazin and further understand its mechanism of action. Understanding the chemical reactivity of bethoxazin would provide a greater mechanistic understanding of its reaction with biological molecules, its potential safety, and its practical applications. Understanding the mechanism of action of bethoxazin may also provide some insight into its clinical efficacy, safety, duration of action, and pharmacological formulations. The ultimate goal of this study is to further advance the possible development of bethoxazin as a drug.

1.2. Stability of bethoxazin

The chemical structure of bethoxazin in Figure 1 reveals a 1,4, 2-oxathiazine 4-oxide ring system that is uncommon in commercially available products and has not been closely studied (Chee G L et al, 2012). A study of the chemical reactivity of bethoxazin would provide greater understanding of the chemical and biological effects in K562 cancer cells. Since bethoxazin is resistant to hydrolysis at pH below 9, but unstable at pH values between 9 to 11 (Bosselaers J et al, 2003), the instability of bethoxazin in aqueous media at pH values above 9 could be due to the predominance of the hydroxyl anions (OH-), which may react with an electron deficient

(electrophilic) center in bethoxazin. Based on this observation, it is hypothesized that the C3 of the oxathiazine oxide ring in bethoxazin (Figure 1) may be susceptible to attack by electron rich (nucleophilic) molecules. This is due to the presence of electronegative oxygen (O1) and a strong electron withdrawing sulfoxide group at position 4. Therefore, the reactivity of bethoxazin towards various functional groups that are present in biological systems was examined. The results of these preliminary chemical reactions revealed that bethoxazin reacts with nucleophilic compounds, indicating that bethoxazin is electrophilic.

Since bethoxazin was shown to behave as an electrophile in preliminary chemical reactions with selective nucleophiles, and the chemical reactivity of the oxthiazine structurally related compounds have not been reported in the literature, it would be interesting to evaluate examples of electrophilic compounds previously reported. The term electrophile (electron lover) refers to a compound that is attracted to an electron rich group (nucleophile) and tends to accept electrons in a chemical reaction. Reactions between electrophiles and nucleophiles take the general equation (equation 1):



..... Equation (1)

Where the nucleophile (Nu, electron pair donor) attacks an alkyl group (R) which is attached to a leaving group (X) leading to the formation of a new bond between (R) and (Nu), and a new bond between the hydrogen and the leaving group.

Electrophilic-nucleophilic reactions are present in biological systems. Electrophiles are attracted to nucleophilic biological molecules such as proteins, peptides, amino acids and DNA. It is important to study electrophilic-nucleophilic reactions because they can lead to a pharmacological drug response, toxic effect, or activation of a prodrug. For example, the anti-neoplastic or cytotoxic effect of many anticancer agents is due to the direct interaction between an anti-cancer agent with various biological molecules (Casarett L, Klaassen C D, Doull J, 2008; Foye W O et al, 2008). However, some anti-cancer agents are prodrugs that are activated by metabolizing enzymes such as Cytochrome P450 to generate electrophilic metabolites, which react with biological nucleophiles (Casarett L, Klaassen C D, Doull J, 2008).

As bethoxazin was shown to behave as an electrophile, and potently inhibit the growth of human K562 cells as well as microorganisms, examples of electrophilic therapeutic agents will be discussed. The emphasis will be on their chemical reactivity towards biological molecules and their mechanism of action. Understanding the chemical reactivity of electrophilic agents in clinical use or in clinical trials may provide some insight into how bethoxazin might exert its growth inhibition properties in K562 cells.

1.3. Electrophilic therapeutic agents

1.3.1. Electrophilic drugs in clinical use

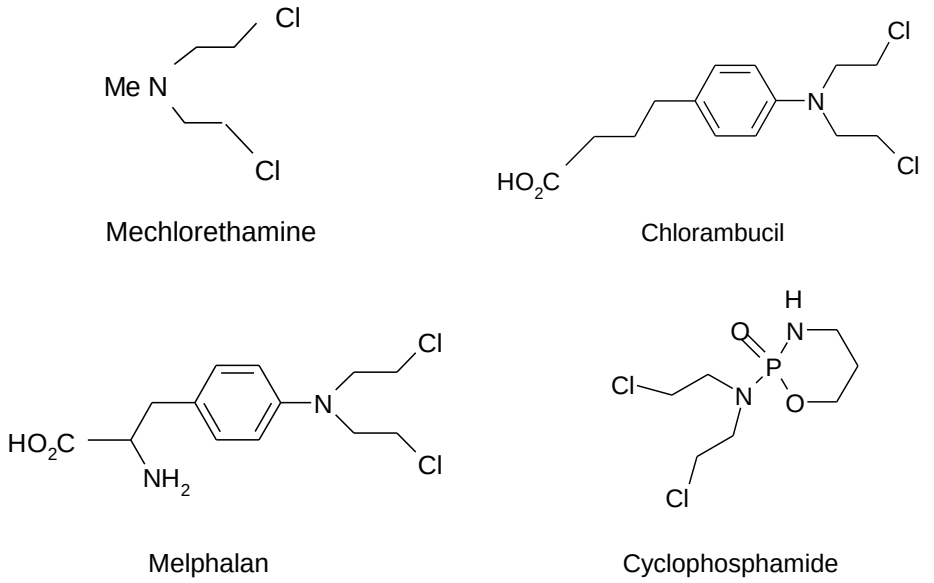
1.3.1.1 Electrophilic alkylating agents

Alkylating agents are compounds that involve the transfer of an alkyl molecule to another molecule (Jerry M., 1985). They are classified according to their character as electrophilic or nucleophilic.

Electrophilic alkylating agents involve the transfer of an alkyl cation (carbacation) to an electron rich species (nucleophile) such as alcohols, carboxylic acids and thiols (Izbicka E & Tolcher A W, 2004). These agents are often toxic due to their ability to alkylate DNA (Ahluwalia V K & Chopra M, 2009). The alkylation of DNA leads to improper coiling, uncoiling of DNA or the inability to be processed by enzymes leading to cytotoxicity.

1.3.1.1. A Nitrogen Mustards

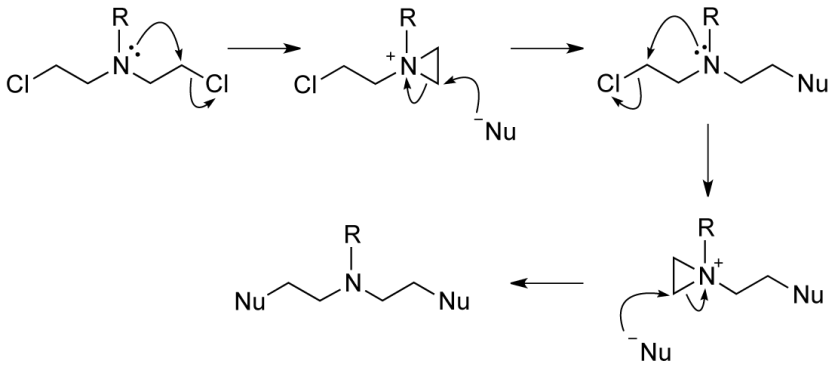
Figure 4: Examples of nitrogen mustards in clinical use (Denny W A, 2001).



Nitrogen mustards are electrophilic alkylating agents. Examples include: mechlorethamine, chlorambucil, melphalan, and cyclophosphamide (Figure 4) (Denny W A, 2001). These agents alkylate DNA at the N7 of guanine in DNA by forming a cyclic cationic intermediate followed by a nucleophilic attack (Panthananickal A et al, 1978; Palmer B D et al, 1990; Niculescu-Duvaz

I et al, 1989, Kohn KW et al 1994). The reaction between mechlorethamine, as an example of nitrogen mustards, and a nucleophile is demonstrated in Figure 5.

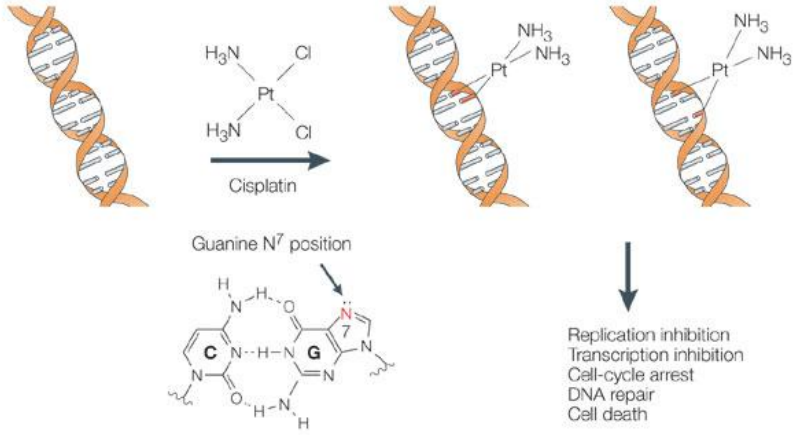
Figure 5: The mechanism of action mechlorethamine that cross-links DNA strands (http://www.atdbio.com/content/16/Nucleic-acid-drug-interactions).



1.3.1.2 Cisplatin

Cisplatin is an anti-cancer agent that acts by cross-linking DNA (Figure 6) (Ahluwalia V K & Chopra M, 2009). Cisplatin was shown to react with sulfhydryl, methionine, histidine, and amino acids with the nitrogen side chain due to its electrophilicity (Ivanov A I et al, 1998; Hagrman D et al, 2003; Sadowitz P D et al, 2004). Cisplatin strongly inhibited the catalytic activity of topoisomerase II α (Hasinoff B et al, 2004) and antagonized the formation of a fluorescent adduct between topoisomerase II α and the sulfhydryl-reactive maleimide reagent ThiGlo-1 Based on previous observations, it was suggested that the cell growth inhibition effect and the antitumor activity of cisplatin could be partially due to the inhibition of topoisomerase II α by reacting with the sulfhydryl groups of the enzyme. Therefore, the reaction of cisplatin with DNA and topoisomerase II could be related to its electrophilicity (Hasinoff B et al, 2005).

Figure 6: The mechanism of action of Cisplatin with DNA (www.sciencegeist.net).



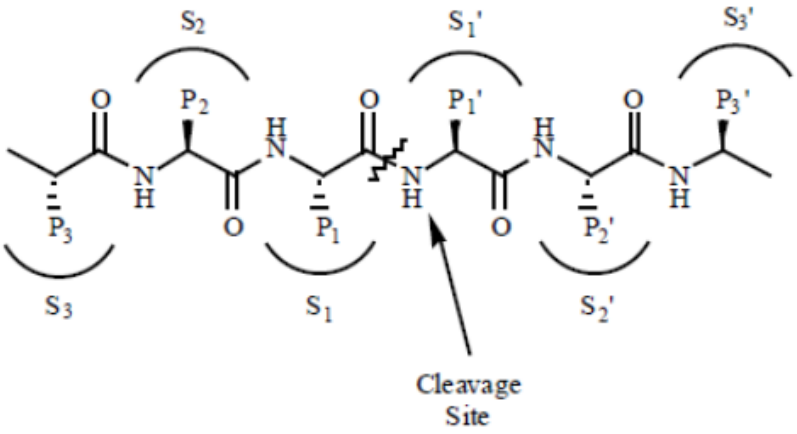
1.3.2. Electrophilic Therapeutic Agents in Clinical Trials

1.3.2.1. Cysteine Protease Inhibitors

Proteases are proteolytic enzymes that hydrolyze the peptide bond of proteins. They control most physiological processes by regulating the activation, synthesis, and turnover of proteins. Proteases are classified according to the catalytic residue that affects hydrolysis into 5 classes: aspartic, serine, cysteine, metallo and threonine. They bind to their substrates or inhibitors in a groove or a cleft where hydrolysis of the amide bond occurs. Figure 7 shows the protease binding sites (S) and the substrate/inhibitor residues (P) where prime represents C- and non-prime represents N-side of cleavage site (Abbenate G & Fairlie D P, 2005). Cysteine proteases contain a cysteine residue at the active site. The thiol of cysteine residue of the enzyme attacks the carbonyl carbon of the scissile amide bond through a Michael addition reaction resulting in the cleavage of the amide bond. Cysteine protease inhibitors interact with the non-prime subsites of the enzyme and inhibit their normal function in a mechanism that is similar to their

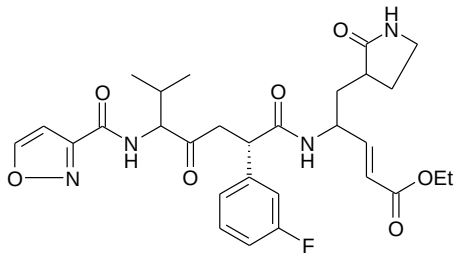
amide bond hydrolysis. Two examples of cysteine protease inhibitors in clinical trials will be discussed: an antiviral agent (Rupintrivir) and an anti-inflammatory agent (Pralnacasan) (Leung-Toung R et al, 2002).

Figure 7: Proteases bind to their substrate/ inhibitor in a groove or a cleft where the amide bond is cleaved. The figure shows the substrate/inhibitor residue (P) and the protease binding site (S). Prime represents C-terminal and non-prime represents the N-terminal of the cleavage site (Abbenate G & Fairlie D P, 2005).



1.3.2.1.1. Rupintrivir

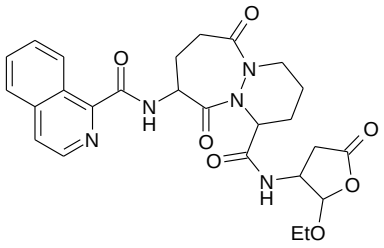
Figure 8: Chemical structure of Rupintrivir (Abbenate G & Fairlie D P, 2005).



Rupintrivir (AG7088) (Figure 8) is an antiviral agent that was developed for the treatment of the common cold virus. It targets the human rhinovirus 3C protease by forming an irreversible covalent bond with the active cysteine of the enzyme (Matthews D A et al, 1999). Rupintrivir was shown to potently inhibit the protease activity of enterovirus 71 3C protease by forming a covalent bond between the sulfur atom of cysteine 147 of the enzyme and the electrophilic β-carbon of the alky ester in the inhibitor (Wang J et al, 2011). Rupintrivir had reached phase III clinical trials in 2003, but its development was ceased due to poor bioavailability in animal studies (Abbenate G & Fairlie D P, 2005). Presently, more bioavailable derivatives of rupinrivir are being investigated (Dragovich P S et al, 2003; Dragovich P S et al, 2002, Leung-Toung R et al, 2006).

1.3.2.1.2. Pralnacasan

Figure 9: Chemical structure of Pralnacasan (Abbenate G & Fairlie D P, 2005).



Pralnacasan (VX-740) (Figure 9) is the first orally available interleukin converting enzyme (ICE) inhibitor, developed by Vertex Pharmaceuticals, which entered phase III clinical trials (Randle J CR et. al, 2001; Abbenate G & Fairlie D P, 2005). It is anti-inflammatory agent that was developed for the treatment of rheumatoid arthritis. It was designed to inhibit ICE protease, which is responsible for the hydrolysis of the pro-inflammatory cytokines and interleukins IL-1β and IL-18. The expression of IL-1β and IL-18 is dramatically increased in response to inflammatory stimuli Thus, inhibiting the ICE enzyme could be beneficial in the management of inflammatory disorders such as rheumatoid arthritis (Randle J CR et. al, 2001).

Pralnacasan is formulated as a prodrug that is activated *in vivo* (Figure 10). The carbonyl carbon of the scissile amide bond of Pralnacasan becomes susceptible to nucleophilic attack by the thiol residue of cysteine in the ICE enzyme leading to the formation of a reversible complex (Figure 11) (Abbenate G & Fairlie D P, 2005).

In 2003 toxicological animal studies demonstrated liver abnormalities after prolonged administration (9 months) of Pralnacasan at high doses therefore it was discontinued (Vertex

Press Release, Nov. 2003). New derivatives of VX-740 are currently under investigation (Vertex Press Release Jan. 2004; Abbenate G & Fairlie D P, 2005).

Figure 10: Pralnacasan (compound **6**) is delivered as an acetal prodrug which is rapidly hydrolyzed to the active form (compound **7)** *in vivo* (Wang Z et al, 2010).

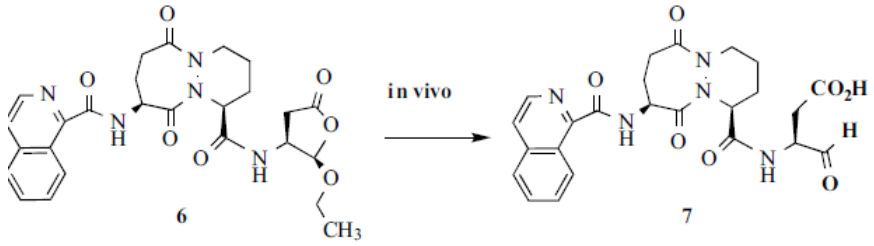
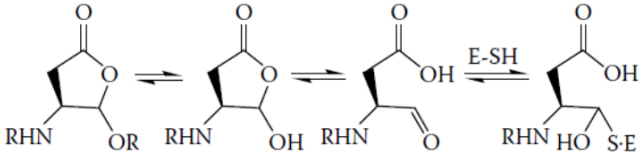


Figure 11: The basic mechanism of cysteine protease reversible inhibition (O'Brien T & Linton S D, 2009).



1.3.3. Electrophilic Therapeutic Agents in Experimental Studies

1.3.3.1. Electrophilic Agents that Target Thiols in Biomolecules

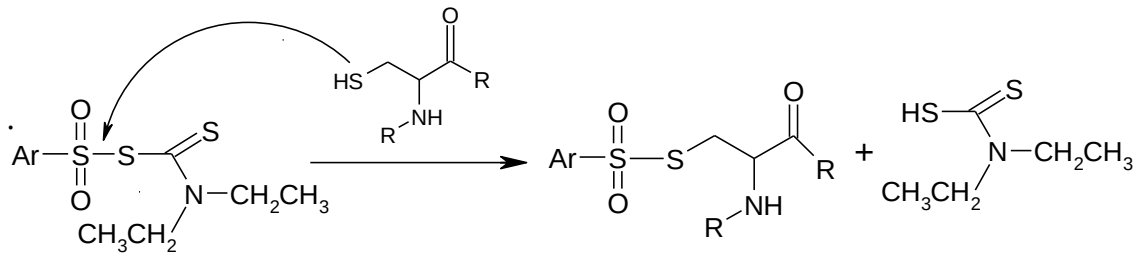
Thiol or sulfhydryl groups are important functional groups that are found in biological molecules such as glutathione and the amino acid cysteine. The thiol moiety is an important target for designing novel pharmacological drugs due to its ability to participate in oxidation-

reduction reactions, co-ordinate transition metal ions, and its nucleophilic nature to react with electrophiles. Modification of the cysteine residue by chemical reactions will result in various pharmacological or biological consequences. (Scozzafava A et al, 2001).

1.3.3.1.1. Antitumor agents that targets β-tubulin

Scozzafava et al 2001 investigated the reactivity of a series of arylsulfonyl-N, N-dialkyl-dithiocarbamate derivatives towards cysteine and glutathione in order to identify derivatives that might react with the cysteine residue of β tubulin. β-tubulin is an important protein constituent of microtubules, which provide cytoskeleton and facilitate the movement of cellular organelles. Moreover, tubulins are important constituents of the mitotic spindles, which are important during cell division (Medina J C et al, 1998; Medina J C et al, 1999; Shan B, 1999). Some of these derivatives have shown strong tumour cell growth inhibition against various cell lines. Furthermore, Scozzafava A et al 2001 also confirmed that such derivatives inhibit tubulin polymerization *in vitro*. It was suggested that the tumour cell growth inhibition could be due to the modification of the cysteine residue of β tubulin (Figure 12).

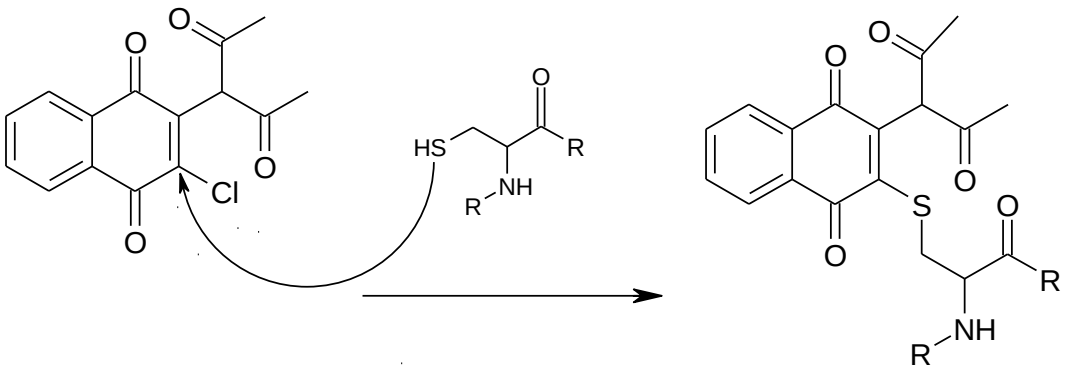
Figure 12: Modification of the cysteine residue of β tubulin by an alkyl/arylsulfonyl-N, N-diethyl-dithiocarbame derivative (Scozzafava A et al, 2000; Scozzafava A et al, 2001).



1.3.3.1.2. Anti-HCMV Agents in Experimental Studies

Human cytomegalovirus (HCMV) is a virus that can cause serious opportunistic infections in immunocompromised patients (Baum E Z et al, 1996). Various derivatives of 2-chloro-3-substituted naphthoquinone were developed to target HCMV protease, which is an essential enzyme for the capsid assembly and maturation of the virus (Holwerda B C, 1997; Ertl P et al., 1999). These agents were shown to inactivate the HCMV protease by modifying a cysteine residue of the enzyme (Figure 13) (Ertl P et al., 1999, Baum E Z, 1996).

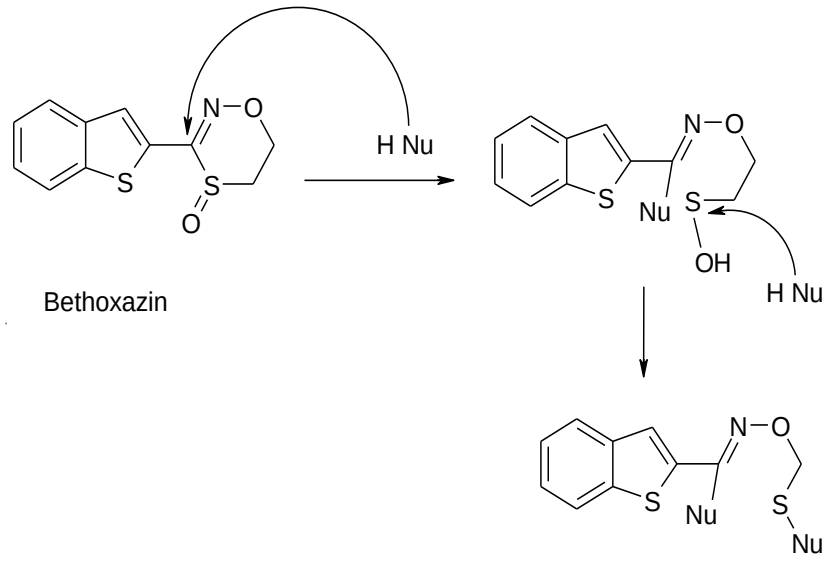
Figure 13: Inactivation of HCMV protease by a 2-chloro-3-substituted naphthoquinone derivative (Ertl, P et al., 1999).



1.4. Hypothesis

The previous examples of electrophilic therapeutic agents have demonstrated that their electrophilic reactivity towards various nucleophilic biological molecules such as DNA and cysteine residue of proteins plays an essential role in their cytotoxic effect. Thus, this observation had led us to speculate that bethoxazin may behave similarly. It is hypothesized in this study that bethoxazin could exert its growth inhibition effects in K562 cells due to its chemical reactivity electrophilicity (Figure 14).

Figure 14: The hypothesized mechanism of nucleophilic attack on bethoxazin.



1.5. Project Objectives

The objectives of this study are to:

1. Assess the chemical reactivity of bethoxazin

2. Explore its mechanism of action

3. Determine if it is a suitable candidate for further pharmaceutical development

1.6. Experimental Design

The chemical reactivity of bethoxazin was evaluated in vivo by UV-Vis spectroscopy to predict the possible functional groups that bethoxazin might react with in biological systems. Subsequently, the chemical reactivity of bethoxazin was evaluated in K562 cells using a fluorescent label. The cell viability of K562 cells was determined by trypan blue assay. The growth inhibition effects of bethoxazin were investigated by the MTS assay and the form of cell death induced by bethoxazin in K562 cells was investigated by flow cytometry.

Chapter 2: Materials and Methods

2.1. Materials

Bethoxazin was a gift from Chemtura Corporation (Guelph, ON). Reagents used as nucleophiles were glutathione (GSH), human serum albumin (HSA), sodium acetate, phenol, and alanine methyl ester and were purchased from Sigma-Aldrich (Oakville, ON). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit was purchased from Promega (Madison, WI). Hanks balanced salts buffer was from Sigma-Aldrich (Oakville, ON). α-MEM and PBS buffer were obtained from Gibco BRL (Burlington, ON). FBS was purchased from Invitrogen (Burlington, ON). Coomassie Bradford Protein assay kit was obtained from PIERCE (Rockford,IL). ThioGlo-1 was sourced from Covalent Associate (Corvallis, OR). ApoAlert Annexin V- FITC kit Catalog No. 630109 was obtained from Clontech (Mountain View, CA).

2.2. Cell Culture

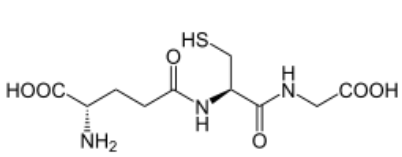
Human leukemia K562 cells, obtained from the American Type Culture Collection (Rockville, MD), were thawed from a -80 ˚C freezer in a 37°C water bath for two minutes or until completely thawed. The contents of the vial were transferred into a clean conical tube, topped up with 15 ml of fresh pre-warmed α-MEM supplemented with 10% FBS and antibiotics, and centrifuged at 1000 rpm / min. The pellet was resuspended and the cell suspension was transferred into a clean 75 ml tissue culture flask and placed in a humidified 37°C incubator (5% CO_2). The cells were maintained as suspension cultures in α-MEM supplemented with 10% FBS at a humidified 37°C incubator (5% CO_2).

2.3. Methods

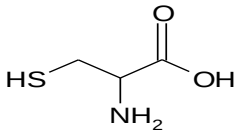
2.3.1. Chemical Reactivity Studies

The reactions between bethoxazin and various nucleophiles were carried out at room temperature and monitored by Cary 1 spectrophotometer (Varian, Mulgarave, Australia) at fixed times. The reactions were initiated by adding 100 µM of bethoxazin (in DMSO) to 1ml of 20 mM Tris buffer solution at pH 7.4 in a 1 cm stoppered silica cell, followed by the addition of the nucleophile (at 1-5 molar equivalents, Figure 15). In the reaction of HSA with bethoxazin, HSA was directly dissolved in Tris buffer followed by the addition of bethoxazin.

Figure 15: The chemical structures of selected nucleophiles that were used to assess the chemical reactivity bethoxazin.

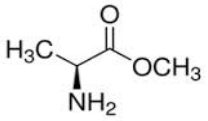


Glutathione (GSH)

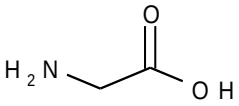


Cysteine

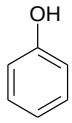
Human Serum Albumin (HSA)



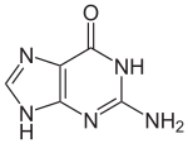
Alanine methyl ester



Glycine



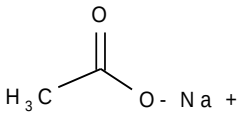
Phenol



Guanine

CH3-OH

Methanol



Sodium Acetate

2.3.2. Sulfhydryl binding of bethoxazin in normal and GSH depleted K562 cells

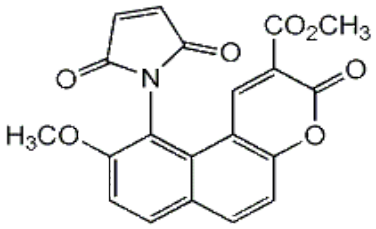
Normal human leukemia K562 cells or GSH depleted K562 cells were treated with various drug concentrations for 2 hours to determine its electrophilic reactivity towards sulfhydryl

containing biomolecules such as glutathione (GSH) and proteins. A maleimide fluorescent reagent 10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-2-carboxylic acid methyl ester (ThioGlo-1, Figure 16) was used to label biomolecules containing a free sulfhydryl group (Langmuir et al, 1996; Tygarajan K et al, 2003). For the same set of experiments the cell viability was assessed and the total protein concentrations were also measured.

2.3.2.1 Bethoxazin-ThioGlo-1 Experiment

A. Bethoxazin-ThioGlo-1 Experiment in Normal K562 cells

Figure 16: Chemical Structure of ThioGlo-1. ThioGlo-1 (10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-2-carboxylic acid methyl ester) is a maleimide reagent, producing a highly fluorescent product on its reaction with sulfhydryl group present in peptides and proteins (Langmuir et al, 1996).



K562 cells were grown to confluence in 10% FBS α-MEM media in a humidified incubator (5% CO_2). On the day of the experiment, the cells were counted using a haemocytometer or using TC10 cell counter from Bio-Rad (Mississauga, ON) then centrifuged at 13,000 rpm. α-MEM media was removed and the cells were resuspended in Hanks buffer. Four million cells were seeded in petri dishes (at a concentration of 1 million cells/ml). The cells were then treated with six different concentrations (0.4, 1.2, 3.7, 11, 33, 100 μM) of bethoxazin for two hours. The compound was then washed away twice using Hank's buffer. The cells were resuspended in 20mM Tris buffer pH 7.4 and lysed by freezing (-80˚C) and thawing (37˚C) five

times. The lysed cells were centrifuged at 13,000 rpm and the supernatant was collected. Each well of a clear bottom black 96 well plate was seeded with 0.4 million cells equivalent of supernatant. 2 µl of ThioGlo-1 (1 mM stock solution in DMSO, Figure 16) was added to the supernatant in 20 mM Tris buffer pH 7.4 to give a total volume of 100 µl/well and incubated at room temperature for 10 minutes. The fluorescence intensity was measured using FLUOstar Galaxy microplate reader from BMG Labtechnologies GmbH (Offenburg, Germany). The percent at which bethoxazin inhibits the binding of ThioGlo-1 to the free sulfhydryl molecules relative to the control untreated cells was calculated based on the florescence intensity readings of the untreated versus bethoxazin treated K562 cells as demonstrated in equation (2).

% ThioGlo-1 inhibition= $100 - [(FI_{\text{bethoxazin treated}} - FI_{\text{blank}}) / (FI_{\text{untreated}} - FI_{\text{blank}})] * 100$ … Equation (2)

B. Depletion of GSH levels in K562 cells by BSO

K562 cells were grown to confluence in 10% FBS α- MEM media in a humidified incubator (5% CO_2). On the day of the experiment, the cells were counted using a haemocytometer then centrifuged at 13,000 rpm. α-MEM media was removed and the cells were resuspended in α-MEM . One million cells were seeded in 25-ml T-flask to give a cell suspension concentration of 0.25 million cells/ml. Cells were treated or untreated with buthionine sulfoximine (BSO) at 50 µM or 150 µM for 24 or 48 hours. After 24 or 48 hours of BSO treatment, cells were washed away twice using Hank's buffer. The cells were resuspended in 20mM Tris buffer pH 7.4 and lysed by repeated freezing (-80˚C) and thawing (37˚C) five times. The lysed cells were centrifuged at 13,000 rpm and the supernatant was collected. Each well of a clear bottom 96 well black plate was seeded with 1.2 million cells equivalent of supernatant. 2 µl of ThioGlo-1

(1 mM stock solution in DMSO) was added to the supernatant in 20 mM Tris buffer (pH 7.4) to give a total volume of 100 µl/well and incubated at room temperature for 10 minutes. The fluorescence intensity was measured and the percent that GSH was reduced by BSO treatment relative to the control untreated cells was calculated by equation (3).

% GSH reduction= $100 - [(FI_{BSO\ treated} - FI_{blank}) / (FI_{control} - FI_{blank})] * 100$ ….. Equation (3)

C. Bethoxazin- ThioGlo-1 experiment in GSH depleted K562 cells

K562 cells were treated with 50 µM of BSO for 48 hours prior to bethoxazin addition. On the day of the experiment, α-MEM media was removed and the cells were resuspended in Hanks buffer. Four million cells were seeded in petri dishes (at a concentration of 0.5 million cells/ mL). The cells were then treated or not with six concentrations (0.4, 1.2, 3.7, 11, 33, and 100 µM) of bethoxazin for two hours. The cells were resuspended in 20mM Tris buffer pH 7.4 and lysed by freezing (-80˚C) and thawing (37˚C) five times. The lysed cells were centrifuged at 13,000 rpm and the supernatant was collected. Each well of a clear bottom 96 well black plate was seeded with supernatant volume corresponding to 1.2 million cells. A greater number of cells were used for the BSO-treated experiment because 1.2 million untreated control cells were required to give a FI reading significantly higher than the background. 2 µl of ThioGlo-1 (1 mM stock solution in DMSO) was added to the supernatant in 20 mM Tris buffer pH 7.4 to give a total volume of 100 µl/well in the 96 well plate and incubated at room temperature for 10 minutes. The fluorescence intensity was measured and the percent inhibition of ThioGlo-1 binding to the free sulfhydryl molecules relative to the control untreated cells was calculated using equation (1) in section 2.3.2.1 A.

2.3.2.2. Cell viability assay by trypan blue

For the same set of cells that was used for the ThioGlo-1 experiment, the viability of the cells was assessed. The assay was done by mixing 250 µl of cell suspension, in Hanks buffer, with 50 µl of trypan blue 0.4 % (Freshney R, 1987). Then 11 µl of the blue suspension was injected to a haemocytometer and observed under the microscope. The cell viability was also determined using a TC10 cell counter to analyze the cell mixture containing 10 µl aliquot of the single cell suspension and 10 µl of trypan blue. The cell viability was calculated based on the number of healthy cells compared to the total number of the cell population after 2 hours of drug treatment. The same trypan blue assay procedure was followed for both normal and GSH depleted K562 cells.

2.3.2.3. Cell protein concentration measurement

A. Bradford assay standard curve

BSA stocks were prepared in water (4 mg/ml, 0.4 mg/ml and 0.04 mg/ml). In a clear 96 well plate, the required volume of water, and 200 µl of Coomasie reagent were added to each well to give a final volume of 250 µl/well. The reaction was allowed to sit at room temperature for 10 minutes. The absorbance readings were taken at 595 nm using a Molecular Devices (Menlo Park, CA) microplate reader. The absorbance (subtracted from blank) at 595 nm was plotted against the protein concentration to obtain the standard curve (Figure 21).

B. Determination of protein concentration using the standard curve

The protein concentration of the supernatant of K562 cells treated with various concentrations of bethoxazin from the ThioGlo-1 experiment (Section 2.3.2.1 A) was used to determine the total cell protein concentration. In a clear 96 well plate, approximately 4 µl of the supernatant

(28,000 cells) were taken out of each sample then mixed with water and 200 µl of Coomassie blue reagent in each well to give a final volume of 250 µl/well. An adjustment of the supernatant volume was used for cells treated with 100 µM and 33 µM of bethoxazin. The mixture was incubated at room temperature for 10 minutes and the absorbance reading at 595 nm was taken using a Molecular Devices microplate reader. Using the standard curve previously obtained, the protein concentration of each sample was determined.

2.3.4. Effect of continuous bethoxazin treatment versus washing of bethoxazin on the IC_{50}

K562 cells were counted (10.5 million), using a TC10 cell counter, centrifuged at 1300 rpm for 5 minutes then resuspended in (20 ml) Hanks balanced salts buffer to give a cell concentration of 0.5 million cells/ml. The cell suspension was divided into two sets of microtubes ("wash out" set and "continuously treated" set) each tube containing 0.5 million cells in 1 ml. The "wash out" set was treated only once with 7 different concentrations of bethoxazin (0.27, 0.8, 2.5, 7.4 22.2, 66.7, 200 µM and two no drug controls) while the "continuously treated" set was continuously treated with bethoxazin. Initially, both sets of cells were treated with 7 different concentrations of bethoxazin for 2 hours at 37˚C. Afterwards, the cells were centrifuged at 1300 rpm for 5 minutes and resuspended in (1 ml) of α-MEM. The continuously treated set was treated with bethoxazin for 20 min whereas the "wash out" set was not treated. The cells were centrifuged and resuspended in α-MEM media and the "continuously treated" set was treated with bethoxazin for another 20 minutes, while the "wash out" was not treated. Both sets of cells were then counted, centrifuged and resuspended in 1 ml of α-MEM. 6000 cells, from each microtube of both sets of cells, were seeded in each well of a 96 well plate to give a final volume of 100 µl. The "continuously treated set" in the 96 well plate was treated with the same seven different concentrations of bethoxazin. On the other hand, bethoxazin was not added to

the "wash out" set. Both sets of cells were incubated at 37 ˚C for 72 hours. Then 10 µl of MTS reagent was added to each well and further incubated for 3 hours at 37˚C. The absorbance readings at 595 nm were read using a Molecular Devices microplate reader. Four replicates were measured at each drug concentration, and the median inhibitory concentration of drug (IC_{50}) values and their SEs for growth inhibition were obtained by fitting the data from the absorbance-drug concentration plot to a four parameter logistic equation using Sigma plot software (Jandel, San Rafael, CA) (Hasinoff B et al, 1997).

2.3.5. Determination of the type of cell death induced by bethoxazin in K562 cells by flow cytometry

Flow cytometry analysis was used to determine whether bethoxazin induces cell death through apoptotic or a necrotic mechanism. The analysis was done by simultaneously measuring green (Annexin-V-FITC) fluorescence and red (propidium iodide) fluorescence (Hasinoff B et al, 2006). The Annexin V-FITC apoptotic kit was used to determine the binding of Annexin-V-FITC to the phosphatidylserine (PS) present on the outer cell membrane.

K562 cells were grown to confluence in α-MEM supplemented with 10% FBS. On the day of the experiment, the cells were counted using a TC10 cell counter. Cells were centrifuged at 1300 rpm for 5 min and resuspended in α-MEM to give a concentration of 0.5 million cells/ml. Cells were treated or untreated for 8 hours with 10 µM of bethoxazin at 37 ˚C. The cells were collected by centrifugation at 1300 rpm then washed twice with PBS followed by one wash with 1X binding buffer and finally resuspended in 200 µl of 1X binding buffer. The cells were stained with propidium iodide and Annexin-V-FITC for 10 minutes in the dark. 300 µl of 1X binding buffer were added to bring the reaction volume to 500 µl prior to analysis by Beckman Coulter flow cytometer. MoFlo^{TM} XDP software from Beckman Coulter (Mississauga, ON) was used to analyze the flow cytometry data.

Chapter 3: Results

3.1. Spectrophotometric studies of the reaction of bethoxazin with nucleophiles

Based on the hypothesis that bethoxazin has the potential to act as an electrophile, the chemical reactivity of bethoxazin towards biologically relevant nucleophiles was spectrophotometrically examined. In this experiment, a UV-Vis spectrophotometer was used to monitor the rate of the chemical reaction. The study provides insight into the ability of bethoxazin to form covalent adducts with biomolecules such as peptides, proteins, and DNA.

Bethoxazin reacted rapidly (less than 5 minutes) with glutathione (GSH, five equivalents) in 20 mM Tris buffer (pH 7.4) (Figure 17). The reaction of bethoxazin with GSH displayed an isosbestic point indicating the formation of a single absorbing product. A much slower reaction was observed between bethoxazin and human serum albumin (HSA) in 20 mM Tris buffer (pH 7.4) (Figure 18). The later reaction took more than five hours to complete.

Figure 17: Spectrophotometric study of the reaction of bethoxazin with GSH. The spectra were recorded before the addition of GSH and at various intervals after the addition of GSH. Hypsochromatic spectral changes were observed when 100 µM of bethoxazin was allowed to react with 500 µM of GSH in 20 mM Tris buffer (pH 7.4, 25 ˚C).

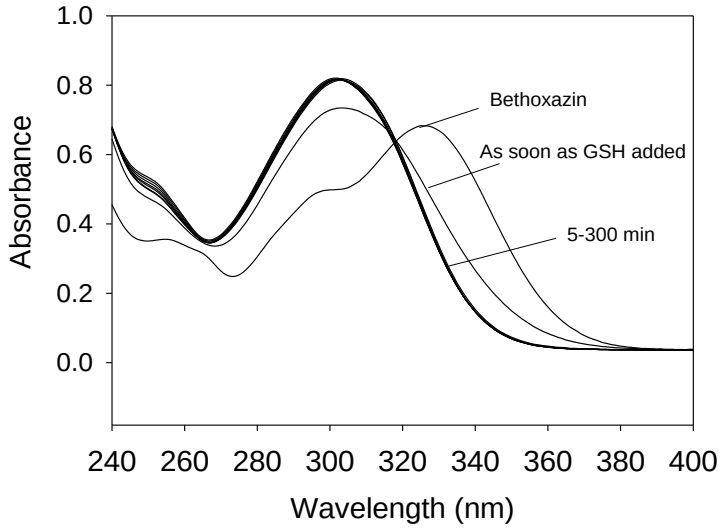
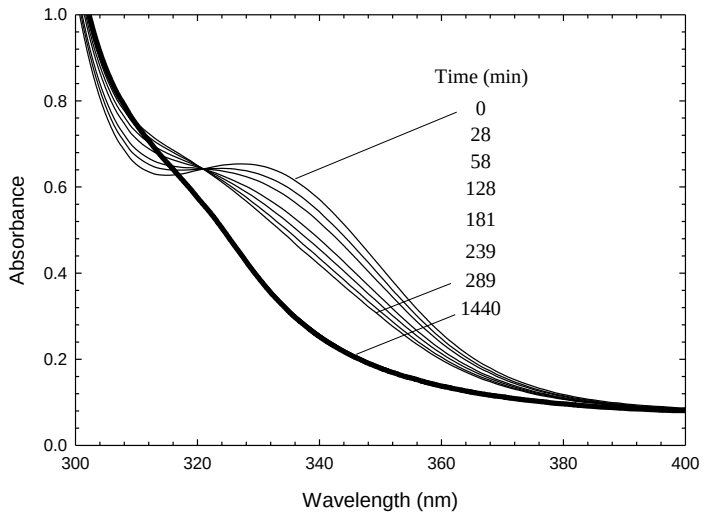


Figure 18: Spectrophotometric study of the reaction of bethoxazin with HSA. The spectra were recorded before the addition of bethoxazin to HSA (in Tris buffer) and at various time intervals after the addition of bethoxzin. Spectral changes were observed when 100 µM of bethoxazin was allowed to react with 500 µM of HSA in 20 mM Tris buffer (pH 7.4, 25 ˚C).



Despite bethoxazin's reactivity towards sulfhydryl groups, bethoxazin did not react with five equivalents of alanine methyl ester, phenol, or sodium acetate in 20 mM Tris buffer (pH 7.4) as there were no observed spectral changes over one day**.**

3.2. Sulfhydryl binding of bethoxazin in normal and GSH depleted K562

3.2.1. Effects of bethoxazin on the binding of free sulfhydryl molecules to ThioGlo-1

ThioGlo-1 is a malemide fluorescent reagent that yields high fluorescence upon covalent binding with sulfhydryl molecules (Langmuir et al., 1996; Tyagarajan K et al, 2003). The fluoresence intensity (FI) emitted due to the binding of ThioGlo-1 to sulfhydryl molecules can be measured using a fluorescence plate reader. It is expected that if bethoxazin reacts with free sulfhydryl groups in K562 cells then FI of bethoxazin treated cells will be less than FI of cells not treated with bethoxazin.

In this experiment, untreated K562 cells resulted in high fluorescent reading due to the formation of fluorescent adducts upon reaction between ThioGlo-1 and the free sulfhydryl groups present in glutathione and other biomolecules. Cells that were treated with bethoxazin at concentrations of 11 µM and lower resulted in high FI similar to non-treated cells. Conversely, K562 cells treated with high concentrations of bethoxazin (33 and 100 µM) resulted in low FI.

The percent of ThioGlo-1 inhibition by bethoxazin relative to control was calculated by equation (2) in section **2.3.2.1 A**:

$$\% \text{ ThioGlo-1 inhibition} = 100 - [(FI_{\text{bethoxazin treated}} - FI_{\text{blank}}) / (FI_{\text{untreated}} - FI_{\text{blank}})] * 100 \quad \ldots \text{Equation (2)}$$

From the above formula, there is a notable inverse relationship between the fluorescence intensity (FI) and the % of ThioGlo-1 inhibition relative to untreated control cells. Figure 19

(blue bars) shows the % ThioGlo-1 inhibition by bethoxazin relative to control. Cells that were treated with high concentrations of bethoxazin (33 and 100 µM) inhibited the binding of free sulfhydryl biomolecules to ThioGlo-1 by 78-91%, while the cells that were treated with 11 µM or lower of bethoxazin resulted in about 6-27% inhibition.

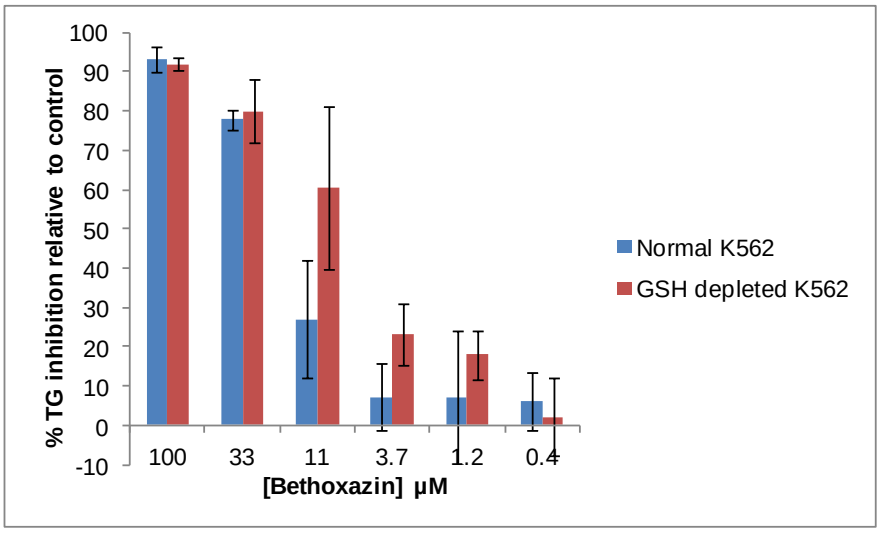
As the stability studies revealed that bethoxazin reacts with GSH, it was of interest to investigate whether GSH depletion is the route by which bethoxazin induces cell killing in K562 cells. Thus, cells depleted of GSH were used to study the effect of bethoxazin on sulfhydryl binding using ThioGlo-1 as previously examined for normal K562 cells. Normal K562 cells were first treated for 48 hr with 50 µM buthionine sulfoximine, a γ-glutamyl cysteine synthetase inhibitor that blocks synthesis of GSH (Pastore A et al, 2003). BSO treated cells gave a lower FI reading because BSO inhibits the synthesis of GSH, resulting in less free sulfhydryl molecules to be labeled by ThioGlo-1. This treatment decreased the GSH levels by ~72 % relative to untreated control (92% cell viability). The % GSH reduction relative to control was calculated by equation (3) in section 2.3.2.1 B:

$$\% \text{ GSH reduction} = 100 - [(FI_{\text{BSO treated}} - FI_{\text{blank}}) / (FI_{\text{control}} - FI_{\text{blank}})] * 100 \quad \text{..... Equation (3)}$$

The BSO pre-treatment was followed by two hours treatment of bethoxazin at various concentrations (experiment section 2.3.2.1 C) as shown in Figure 19 (red bars). When GSH was depleted by BSO in K562 cells, bethoxazin still inhibited the binding of free sulfhydryl molecules to ThioGlo-1 in a similar way to that observed with normal K562 cells that were not depleted from GSH as shown in Figure 19 (red bars). The % ThioGlo-1 inhibition by bethoxazin relative to control in GSH depleted K562 cells was calculated using Equation (2) above.

In this study, the number of cells equivalent of supernatant seeded in clear bottom black 96-well plate was adjusted because BSO treated cells gave much lower FI reading than the untreated cells. Since GSH concentrations were reduced by ~72 % relative to untreated control cells (~28% of the level of GSH in untreated controls) following 50 µM BSO treatment for 48 hr, three times the number of cells/well were seeded (1.2 million cells/well) in this experiment to be equivalent to 0.4 million normal cells in terms of the GSH amount (section 2.3.2.1 C). The FI reading of 1.2 million BSO treated K562 cells was in the range of 17,000 units, whereas that of 0.4 million cells of normal K562 cells was about 30,000 units. Theoretically it is expected that three times the number of BSO treated cells should result in FI reading close to 30,000 units but experimentally this was not evident. This is likely due to the FI measuring system which is not accurate enough to detect changes in cells that have ~72 % reduced GSH levels since the 17,000 units is not large enough from the background reading (~6000 units). This observation also explains the high error bars when calculating the % ThioGlo-1 inhibition by bethoxazin relative to untreated controls (Figure 19, red bars).

Figure 19: Percentage of ThioGlo-1 inhibition by bethoxazin relative to control in normal and GSH depleted K562 cells. The result is based on 3 experiments with each experiment done in triplicates. N = 3 Mean ± SD.



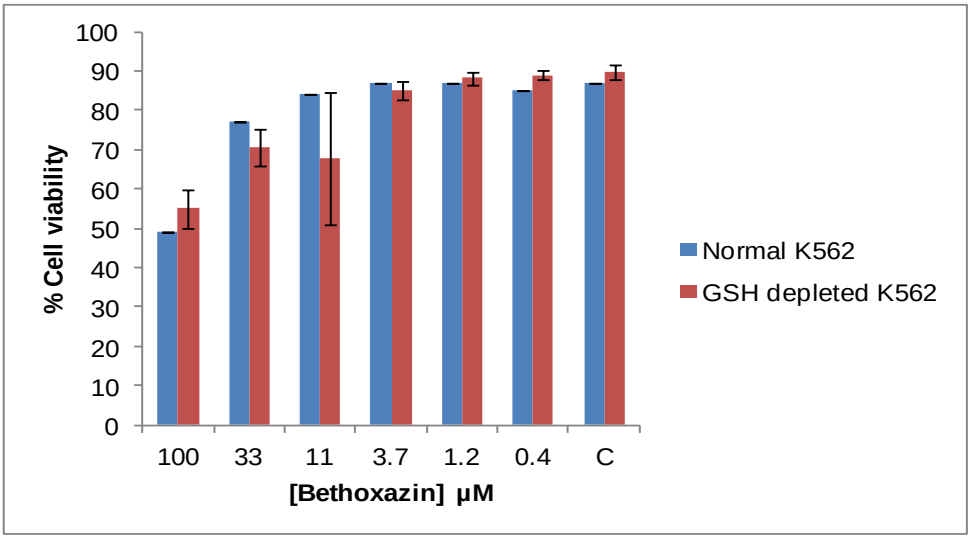
3.2.2. Effects of bethoxazin on cell viability

Cell viability of the cells treated with bethoxazin was assessed by trypan blue assay. Trypan blue is a negatively charged dye that is able to penetrate cells with a leaky cell membrane but not cells with intact cell membranes (Shen X et al, 2012). When the cells are treated with high concentrations of bethoxazin, it is expected that the membrane becomes leaky and allows trypan blue to penetrate through the membrane and the cell stains blue. Healthy cells will appear unstained under the microscope.

The result of this experiment showed that a 100 µM concentration of bethoxazin treatment in normal K562 cells for two hours resulted in a 50% cell viability. When the cells were treated with 33 µM of bethoxazin the cell viability was about 78%. Bethoxazin concentrations of 0.4-11 µM resulted in cell viability of 85-90% cell viability (Figure 20, blue bars). These findings suggest that 100 µM was the IC_{50} for this assay. When GSH was depleted in K562 cells, the %

cell viability at all bethoxazin concentrations were comparable to those of normal K562 cells (Figure 20), suggesting that bethoxazin potency in GSH-depleted cells was comparable to that in normal cells.

Figure 20: Percentage of viable cells following a 2 hour treatment of bethoxazin at different concentrations. The result is based on three separate experiments with each experiment done in duplicates. N = 3 Mean ± SD.



3.2.3. Cell protein concentration measurement

A Bradford assay was used to determine the total protein concentration of the supernatant that came from K562 cells treated with bethoxazin in sections 2.3.2.1 A and 2.3.2.1 C. The Bradford assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs resulting in a colour change from red to blue (Bradford, M M, 1976; Yang F et al, 2008). Bovine Serum Albumin (BSA) is a standard protein. Each concentration of BSA is expected to give a certain absorbance value. Plotting the BSA concentrations versus

Absorbance will result in a standard curve (Figure 21). The standard curve was used to determine the concentrations of protein in the supernatant that were treated with bethoxazin after measuring the absorbance of the samples using a microplate reader (section 2.3.2.3 A).

The purpose of measuring the cell protein concentration here is to confirm that ThioGlo-1 inhibition, which is calculated through the fluorescence intensity, is due to the sulfhydryl binding of bethoxazin to the free sulfydryl groups of biomolecules. This observation will rule out that the low fluorescence intensity reading is due to loss of cellular contents from leaky damaged cells during the washing steps of the bethoxazin-ThioGlo-1 experiment (section 2.3.2.1 A). A low fluorescence intensity reading may result from either low cell count or a small number of free sulfhydryl molecules to form adducts with ThioGlo-1 due to their binding to bethoxazin. The total protein concentration measurements after 2 hours of bethoxazin treatment are expected to be the same for all the samples regardless of the concentration of bethoxazin. An explanation for this experimental observation is that the total protein concentration is a measure of the total number of cells in a sample; so equal numbers of cells are expected to produce equal total protein concentration measurements. The result of this assay demonstrates that the protein concentration of the supernatant of cells treated with various concentrations of bethoxazin and untreated cells is equivalent (Figure 22).

Notably, in this experiment, K562 cells treated with 100 and 33 µM of bethoxazin initially resulted in lower total protein concentration measurements compared to cells that were untreated or treated with lower bethoxazin concentrations. This is likely the consequence of high concentrations of bethoxazin that induce leaky membranes and thus the loss of cellular contents during the experiment. Cells treated with 100 and 33 µM of bethoxazin required a supernatant volume to be increased by 2 and 1.5 fold respectively to yield a protein absorbance

value comparable to other samples. As a result, the same volume adjustments were made to supernatants of cells treated with 100 and 33 µM of bethoxazin in the ThioGlo-1 experiment discussed in section 2.3.2.1 A. When the fluorescence intensity of the supernatants was measured following the volume adjustment, it was almost the same as before the adjustment. If the fluorescence intensity following the adjustment was increased it would suggest that the low fluorescence intensity readings following bethoxazin treatment are likely to be due to cell loss during the experiment which does not appear to be evident in these experiments.

These results imply that the total number of cells among all supernatants of cells treated or untreated with bethoxazin is the same. This observation further suggests that the reduced fluorescence intensity measurements following bethoxazin treatment are due to sulfhydryl binding of bethoxazin to the free sulfhydryl groups of biomolecules instead of cell loss during the washing steps of the experiment in section 2.3.2.1 A.

Figure 21: BSA standard curve to measure the total cell protein concentration.

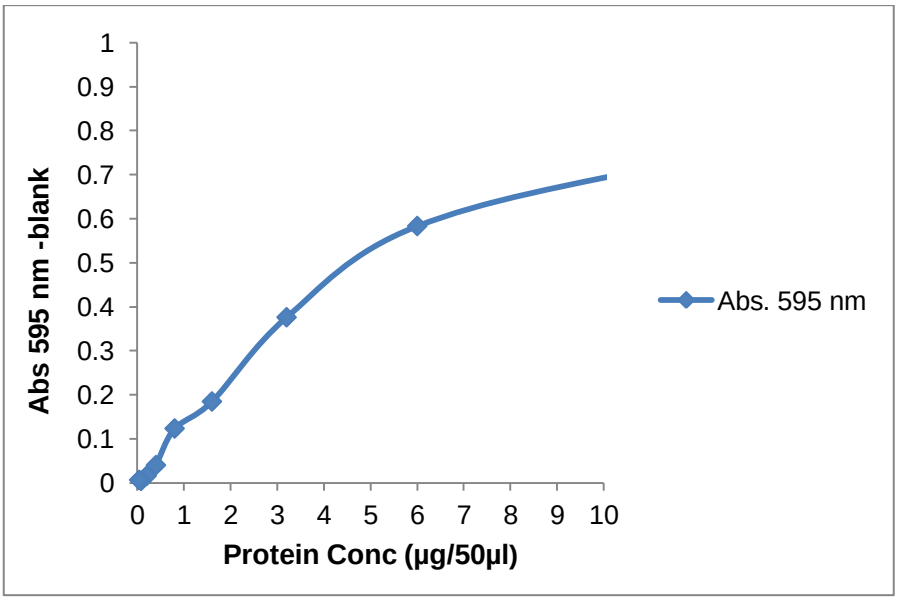
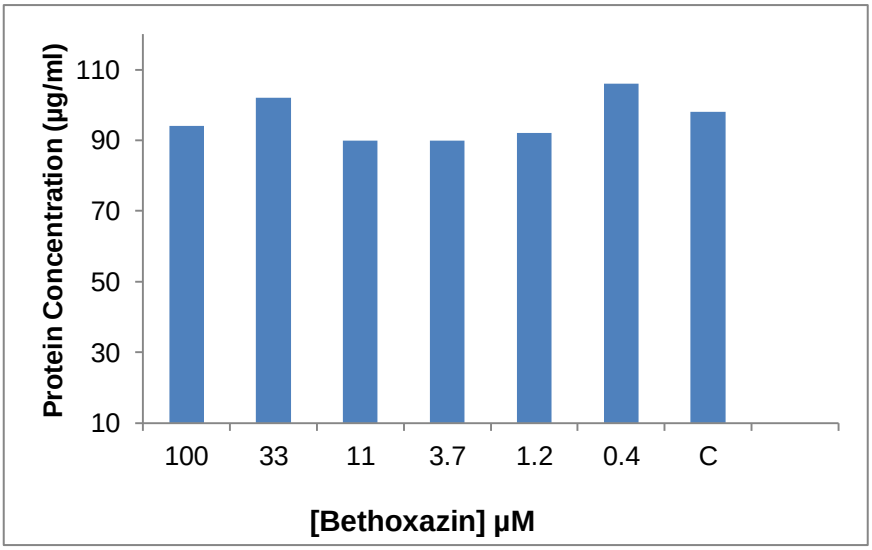


Figure 22: The total protein concentration of the supernatant of bethoxazin-treated K562 cells. The result is bases on three experiments with each experiment done in triplicates followed by extrapolation from the BSA standard curve (Figure 21).



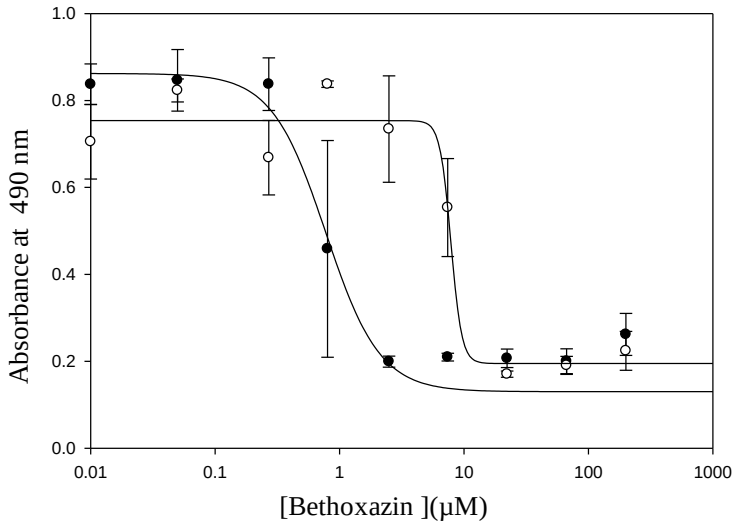
3.3. Effect of continuous treatment versus wash out on the IC_{50} of bethoxazin

A cell growth inhibition assay (MTS) was used to determine the IC_{50} after 72 hours of incubation. In this assay, the MTS reagent *(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)* measures the ability to reduce mitochondrial succinyl dehydrogenase in viable cells (Stamatiou R et al, 2012). When the MTS reagent, is added to viable cells, the enzyme is reduced resulting in a purple colour due to a formazan complex formation. Thus, healthy cells will yield high absorbance values. The quantity of the formazan complex formation is directly proportional to the number of viable cells (Malich G, 1997).

Bethoxazin was shown to inhibit the growth of K562 cells with an IC_{50} value of about 1 µM. To explore whether GSH depletion is the major cause of bethoxazin cytotoxicity, the washing experiment was performed. In this experiment, the growth inhibition assay (MTS) was used to

compare the effects of bethoxazin in two sets of K562 cells: continuously treated cells and washed out cells as explained in section 2.3.4. Bethoxazin demonstrated a 10 fold greater cytotoxicity to cells that were continuously treated than the washed out cells with an IC_{50} values of ~ 0.7 µM and ~ 7 µM respectively (Figure 23).

Figure 23: The growth inhibitory effects of bethoxazin on K562 cells that were continuously treated (•) or washed out (o) after 72 hours of incubation measured by MTS. The values shown represent 3 experiments with each experiment done in quadruplicates. N= 3 mean ± SE.



3.4. Determination of the type of cell death induced by bethoxazin in K562 cells by flow cytometry

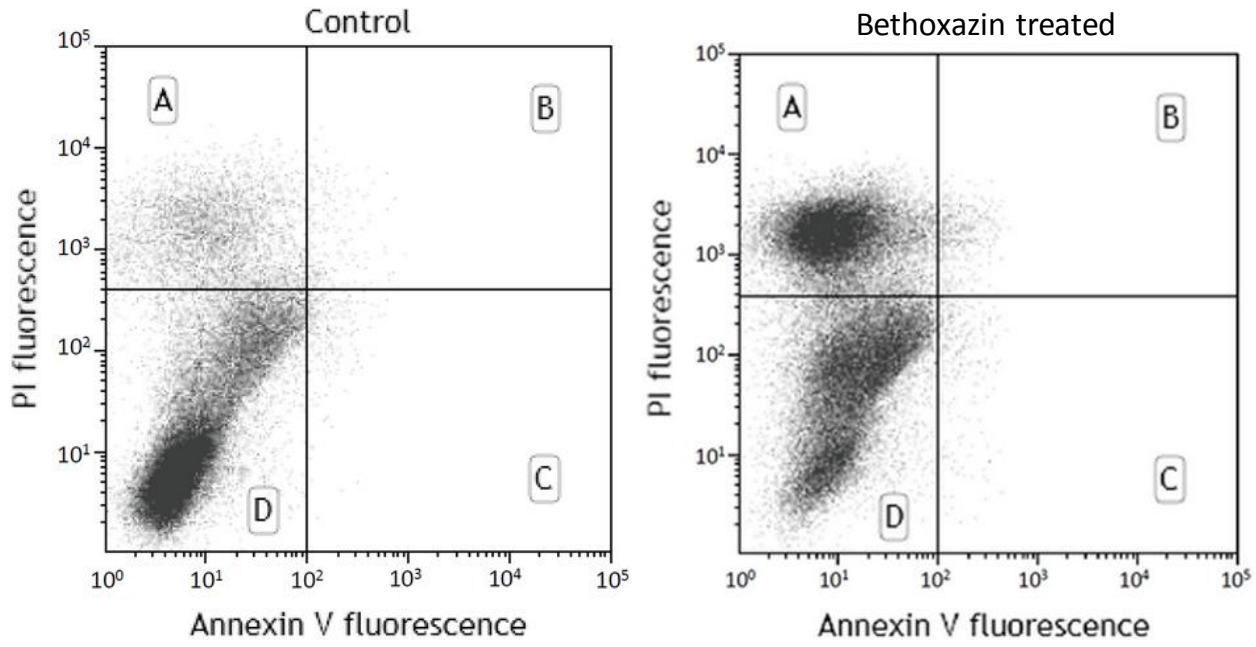
To determine the form of K562 cell death induced by bethoxazin treatment, Annexin-V-fluorescein isothiocyanate (FITC) / propidium iodide (PI) two-colour fluorescence flow cytometry analysis was performed (Hasinoff B et al, 2006). Cell death can result from an apoptotic or necrotic process. Apoptotic cells undergo physiological changes, one of which is the change in the location of phospholipids on the cell membrane. Phosphatidylserines are one of the phospholipids that are localized in the inner layer cell membrane of non-apoptotic cells. In the early stages of apoptotic cells, these phosphatidylserines move to the outer surface of the

cell membrane and become exposed to the extracellular environment (Kang YJ et al, 2000; Martin SJ et al, 1995, Fadok VA et al, 1993). When phosphatidylserines are exposed they can be detected by Annexin-V-FITC, which is a fluorescent dye that has a strong binding affinity towards phosphatidylserines (Kang YJ et al, 2000). Thus, Annexin-V-FITC stained cells appear in both the lower right (C) and upper right (B) quadrants of Figure 24.

When Annexin-V-FITC is used in combination with propidium iodide, a DNA binding fluorescent dye, necrotic cells can be distinguished from apoptotic cells. Since the membrane of necrotic cells is leaky, it allows propidium iodide to permeate and bind with DNA (Hasinoff et al. 2006). Thus, cells in the upper left (A) quadrant were necrotic only; cells in the upper right quadrant were both apoptotic and necrotic (B) (Figure 24).

Treatment of K562 cells with 10 μM bethoxazin for 8 hours reduced the percentage of viable cells by about 41% (lower left quadrant, D) and increased the percentage of necrotic only cells (upper left, A) quadrant) by approximately 39% compared with the untreated control. Conversely, there was no major difference in the percentage of apoptotic only cells (lower right quadrant, C) or the apoptotic/necrotic cells (upper right quadrant, B) (Figure 24).

Figure 24: Bethoxazin treatment of K562 cells induces necrosis in K562 cells as determined by annexin V- fluorescein isothiocyanate (FITC)/propidium iodide two colour fluorescence flow cytometery. The figure shows two colour flow cytometry scatter plots of untreated control (left) and bethoxazin treated cells (10 µM) for 8 hours. The lower left quadrant represents viable cells (D), the upper left quadrant represents only necrotic cells (A), the lower right quadrant represents apoptotic only cells (C), and the upper right (B) quadrant represents both apoptotic and necrotic cells.



Chapter 4: Discussion

The results of bethoxazin stability studies demonstrate that bethoxazin reacts only with sulfhydryl containing compounds such as GSH and HSA, but not with alanine methyl ester, sodium acetate, or phenol in Tris buffer (pH 7.4). The reaction of bethoxazin with HSA was much slower than the reaction of bethoxazin with GSH ($t_{1/2}$=2.1 minutes, Chee G L et al, 2012). This could be attributed to the fact that HSA is a large protein molecule containing one free sulfhydryl group that is more sterically hindered compared to a low molecular weight peptide such as GSH with an accessible sulfhydryl moiety.

Chee G L et al, 2012 reported that bethoxazin also reacted with cysteine in 20 mM Tris buffer at pH 7.4 (Chee G L et al, 2012). Furthermore, bethoxazin did not react with glycine, guanine, phosphate buffer or methanol (Chee G L et al, 2012). Taken together, the results suggest that bethoxazin is unlikely to form covalent adducts with biomolecules by reacting with their amino, carboxylic, phenolic, amino oxo, alcoholic, or phosphate functional groups. However, bethoxazin is likely capable of reacting with GSH, peptides and proteins in cells through sulfhydryl conjugation.

The results of chemical stability studies on bethoxazin imply that the electrophilic nature of bethoxazin may be an underlying factor in bethoxazin microbicidal activity and its cytotoxicity in K562 cells. According to bethoxazin's toxicological data, bethoxazin is an irritant to eyes and skin (Bosselaers J et al, 2003). The skin irritation effect of bethoxazin (Bosselaers J et al, 2003) could be linked to its chemical reactivity. The chemical reactivity of some compounds has been proposed to be one of the major causes of allergic contact dermatitis (Chipinda I et al,

2011). As the chemical reactivity studies demonstrated that bethoxazin behaves as an electrophile, the skin irritation effects of bethoxazin could be due to its electrophilicity.

The high chemical reactivity of bethoxazin towards GSH implies that bethoxazin would be susceptible to metabolism by liver enzymes in the human body, through conjugation reactions. If bethoxazin was to be formulated as an oral anticancer agent, it would be susceptible to the first pass effect effect in the liver via conjugation potentially leading to low bioavailabilty in the systemic circulation. The intravenous administration of bethoxazin would still be susceptible to metabolism by the liver through the system circulation. Since bethoxazin has a low water solubility and a Log P o/w value of 2.7 (Bosselaers J, 2003), it may be difficult to formulate as an oral formulation and may also lead to wide non-specific distribution throughout the body.

To explore whether bethoxazin reacts with sulfhydryl groups in cells, a study was undertaken in K562 cells using ThioGlo-1. K562 cells treated with 100 and 33 µM of bethoxazin resulted in a low fluorescence intensity reading. Conversely, K562 cells that were treated with bethoxazin concentration of 11 µM or lower resulted in a high fluorescence intensity reading. The fluorescence intensity reading is directly proportional to the number of free sulfhydryl molecules labelled by ThioGlo-1. Thus, a low fluorescence intensity reading indicates that there are fewer free sulfhydryl molecules to be labeled by ThioGlo-1 implying that bethoxazin inhibited the formation of covalent adducts between ThioGlo-1 and the free sulfhydryl molecules in K562 cells, particularly GSH, (Figure 19). Conversely, low concentration of bethoxazin inhibited the labeling of ThioGlo-1 to a lesser extent, resulting in a higher fluorescence intensity reading.

Notably, a 100 µM bethoxazin treatment resulted in ~90% ThioGlo-1 inhibition relative to control and not 100% (Figure 19, blue bars). This is because bethoxazin incubation time was only 2 hours during the experiment (section 2.3.2.1 A**)**. A 100% ThioGlo-1 labeling indicates that bethoxazin reacts with all the free sulfhydryl molecules in K562 cells, which is not the case. Whereas, 90% ThioGlo-1 labeling means that there are still about 10% free sulfhydryl molecules that did not react with bethoxazin within 2 hours. Since the reaction of bethoxazin with HSA required much more than 5 hours to complete, the reaction of bethoxazin with the cysteine residues of proteins in K562 cells would require more than 2 hours to complete.

The result of the bethoxazin-ThioGlo-1 experiment in normal K562 cells showed that bethoxazin reacts with the biological molecules in K562 cells containing free sulfhydryl groups such as glutathione and the cysteine residues of proteins, resulting in fewer free sulfhydryl molecules to be labeled by ThioGlo-1. Thus, less fluorescence would be emitted resulting in a low fluorescence intensity reading (Figure 19).

Since bethoxazin was capable of depleting sulfhydryl groups such as GSH in cells, a further study using K562 cells with reduced GSH concentration was undertaken to determine whether GSH depletion plays a role in bethoxazin induced cell death. Pre-treatment of K562 cells with 50 µM BSO for 48 hours reduced GSH concentrations by ~ 72 %. A similar result was achieved in the literature where 100 µM of BSO treatment in K562 cells for 48 hours reduced GSH concentration by 71% (Wu X et al, 2008). When the BSO treated K562 cells were treated with bethoxazin at various concentrations for 2 hours, bethoxazin still inhibited the binding of free sulfhydryl molecules to ThioGlo-1 in a similar manner to that which was observed in K562 cells with normal GSH concentrations (Figure 19, red bars). At 100 and 33 µM, bethoxazin strongly inhibited ThioGlo-1 labeling almost by the same % in both BSO treated and normal

cells, indicating that a high concentration of bethoxazin could efficiently inhibit ThioGlo-1 labeling even when the initial GSH concentrations were significantly reduced by BSO. Since ThioGlo-1 is a fluorescent label for any sulfhydryl containing molecule and not specific for GSH, this observation implies that bethoxazin likely reacts with the sulfhydryl moiety of enzymes and proteins (not just GSH) in normal and BSO treated cells and inhibit their labeling by ThioGlo-1. At 11, 3.7, and 1.2 µM of bethoxazin concentration, % ThioGlo-1 labeling was inhibited to a comparatively higher degree in BSO treated cells compared to non-BSO treated implying that there are fewer free sulfhydryl molecules in BSO treated cells compared to non-BSO treated.

Since GSH plays an important role in detoxification of electrophiles (Keterer B, 1988) cells should be more vulnerable to the toxic effects of electrophiles when the concentrations of GSH are low. If bethoxazin was to inhibit the growth of K562 cells by GSH depletion, it would be expected that GSH depleted cells would result in a much lower percentage of viable cells compared to K562 cells with normal GSH concentrations. The cell viability of bethoxazin treated K562 cells was determined in both normal and BSO treated cells by a trypan blue cell viability assay. The cell viability assay revealed a dose response effect of bethoxazin on the viability of the cells (Figure 20). When GSH was depleted in K562 cells by 50 µM treatment of BSO alone for 48 hours GSH was reduced by ~72% and the cell viability was ~92%, which is comparable to that of cells with normal physiological concentrations of GSH. The experimental result indicates that GSH reduction by ~72% did not cause significant cell death in K562 cells.

A comparable IC_{50} of bethoxazin cytotoxicity obtained for GSH depleted and normal cells (after 2 hours of incubation) implies that GSH depleted cells were not significantly more susceptible to bethoxazin compared to normal cells, suggesting that the cytotoxicity of

bethoxazin was not due to its ability to deplete GSH in cells. This observation is consistent with previous observation by Dr. Bharat Bhattarai in our laboratory where the IC_{50} of bethoxazin after 72 hours incubation in BSO-treated cells was comparable to normal K562 cells (0.5 µM and 1 µM, respectively, Dr. Chee's unpublished results). Conversely, a similar experiment reported in the literature for the preservative thiomersal revealed a significant reduction of 16.7 folds in the IC_{50} of BSO treated K562 cell compared to normal K562 cells (2 µM and 0.12 µM, respectively) (Wu X et al, 2008). This observation shows that GSH depletion greatly enhanced the cell growth inhibition effects of thiomersal in K562 cells implying that GSH had a major role in protecting cells from thiomersal. Thus, it is likely that GSH depletion is the major route of cellular death for thiomersal (Wu X et al, 2008), unlike bethoxazin.

Despite the fact that bethoxazin resulted in about 91% of ThioGlo-1 inhibition at 100 µM concentration (Figure 19, blue bars), 50% of the cells were still viable according to the trypan blue assay (Figure 20, blue bars). This indicates that bethoxazin is able to deplete GSH in K562 cells within 2 hours of drug incubation but the GSH depletion over that period of time was not sufficient to cause complete cell death of the population. The IC_{50} of bethoxazin in the ThioGlo-1 bethoxazin experiment (section 3.2.2) after 2 hours was ~100 µM which is 100 fold higher than that determined by the MTS assay after 72 hours of incubation (~1 µM). One explanation of this finding may be the short incubation time in this experiment (2 hours) compared to 72 hours in MTS assay. A longer incubation time is required to allow bethoxazin to react with the free sulfhydryl groups of enzymes and other proteins within the cells since the HSA-bethoxazin reaction required more than 5 hours to complete (section 3.1). A second explanation may be that cellular death involves physiological changes such as

phosphatidylserines translocation from the intracellular to the extracellular side of plasma membrane in the case of apoptosis and that may require`~ 4 hours to take place.

The MTS assay (section 3.3) was used to determine the effect of continuous bethoxazin treatment versus wash out on the $IC_{50.}$ Two sets of K562 cells were initially treated with bethoxazin for 2 hours followed by continuous treatment (set A) or wash out (set B) of bethoxazin. The results of this washing experiment were also consistent with the previous observation that GSH depletion is likely not the major route of cell death. If GSH depletion was the major cause of bethoxazin's toxicity then washing out the compound would have negligible effect on the IC_{50} because 2 hours of drug treatment would be sufficient to deplete GSH by conjugation and inhibit cell growth. Since the reaction of bethoxazin with HSA took more than 5 hours to complete, the reaction of bethoxazin with proteins could be a possible mechanism by which bethoxazin inhibits cellular growth. Therefore, the result suggests that the reaction of bethoxazin with proteins containing critical sulfhydryl groups rather than the depletion of GSH is a more likely mechanism by which bethoxazin induces cell death.

The catalytic activity of DNA topoisomerase IIα enzyme has been shown to be sensitive to sulfhydryl reacting compounds due to the presence of a critical sulfhydryl residue on the enzyme (Hasinoff B et al, 2005; Wu X et al, 2005). Since bethoxazin potently inhibited the catalytic decatenation activity of human topoisomerase IIα enzyme at an IC_{50} of ~1μM (Dr. Chee's unpublished data) and bethoxazin also inhibited the growth of K562 cells at an IC_{50} of ~ 1μM these findings suggest that it is likely that bethoxazin would react with the free sulfhydryl moiety of the cysteine residue of topoisomerase IIα or other peptides / proteins containing free sulfhydryl moiety and this is probably the major route by which bethoxazin exerts its cytotoxicity.

Two key distinct features between necrosis and apoptosis are the cell membrane integrity and energy dependence. Apoptosis is an organized and an energy dependent process in which the cell membrane integrity is maintained. It is characterized by phosphatidylserines translocation from the intracellular to the extracellular side of plasma membrane, shrinkage of cell, nucleus condensation, DNA fragmentation and then disintegration into apoptotic bodies. The apoptotic bodies can be naturally removed by phagocytes. Necrosis is a disordered process characterized by bioenergentic impairment, cellular swelling leading to a disruption of the plasma membrane and cell lysis. It is accompanied by the release of mediators like hepatoma-derived factor to initiate cell growth and tissue repair (Zong W X & Thompson C B, 2006). Cells that die through a necrotic mechanism cannot be naturally removed by phagocytes and may require invasive surgical removal. Necrosis usually results in irreversible cell damage, whereas apoptotic death is reversible.

The mechanism of cell death induced by bethoxazin in K562 cells was analyzed by two-colour flow cytometry, which can distinguish between cell death through apoptosis or necrosis. Apoptotic cells, which have exposed phosphatidylserines can be detected by Annexin V (Kang Y J et al, 2000); while necrotic cells with leaky membranes will have higher affinity to the DNA in nucleus and maybe detected by propidium iodide (Hasinoff B et al. 2006). The flow cytometry analysis (section 3.5) shows that 10 µM bethoxazin treatment for 8 hours did not result in a high Annexin V fluorescent signal indicating that bethoxazin likely does not provoke phosphatidylserines translocation of the cell membrane. However, the fluorescent signal of propidium iodide was significantly higher following 10 µM bethoxazin treatment for 8 hours, indicating that bethoxazin treatment likely results in a leaky cell membrane and allows propidium iodide to stain the nucleus. Therefore, the flow cytometry analysis revealed that

bethoxazin killed the cells through a necrotic rather than an apoptotic mechanism. The fact that bethoxazin killed K562 cells through a necrotic mechanism is consistent with the other experimental results and suggests that bethoxazin likely reacts with peptides and the cysteine residue of proteins containing a free sulfhydryl group in the cytosol and the nucleus leading to cellular swelling and cell membrane damage. Furthermore, bethoxazin is expected to react with the free cysteine residues of the nuclear enzyme topoisomerase IIα through sulfhydryl conjugation since bethoxazin treated cells had high affinity to the nuclear binding dye propidium iodide.

Taken together these results imply that bethoxazin is likely to also be toxic to healthy cells which might result in multiple toxicities. Therefore, bethoxazin is unlikely to be a good anti-cancer drug candidate for further development due to its high cellular reactivity and lack of selectivity. This also explains why bethoxazin is efficient as an industrial microbicidal agent as it is anticipated to cause death in any organisms in its contact. However, it is this lack of selectivity that suggests that bethoxazin would not be a good candidate as a therapeutic anticancer agent.

Many electrophilic drugs are highly reactive towards DNA and proteins, which makes them relatively unsafe due to their ability to induce mutagenesis and potential carcinogenic activity themselves. For example, nitrogen mustards, which are clinically used as anti-cancer agents, can cause mutations and induce the formation of other types of cancers. Likewise, as bethoxazin was shown to behave as an electrophile, it is expected that bethoxazin might induce mutagenesis and be carcinogenic itself. This further suggests that bethoxazin is likely not a good candidate as a therapeutic anti-cancer agent.

The present study provided insight into the chemical reactivity of bethoxazin, the mechanism by which it exerts its growth inhibition effects in human K562 cells as well as micro-organisms such as fungi, algae, and yeast, and the mechanism of cellular death induced by bethoxazin.

Chapter 5: Conclusions and recommendations

5.1. Limitations of the study

The biological effects of bethoxazin were studied in human leukemia K562 cells, which is one of the standard cell lines used in cancer research. The fact that K562 cells are resistant to induction of apoptosis by some anticancer agents more than other cell lines implies that bethoxazin might behave differently in other solid tumour cell lines. For example, the flow cytometry analysis of bethoxazin treated K562 cells, in this study, demonstrated that bethoxazin induces necrosis in K562 cells after 8 hours of treatment. It is possible that bethoxazin induces apoptosis in other types of cancer cell lines that are less resistant to induction of apoptosis.

The use of the human K562 leukemia cell line is a simplified model to study action of drugs in blood born leukemia's of the body. Bethoxazin was directly added to K562 cells, without consideration of other factors such as metabolism and distribution which are important for a drug candidate to be efficiently delivered to the target tissue. As bethoxazin was shown to rapidly react with GSH, it is possible that oral administration of bethoxazin would result in significant hepatic metabolism in the human body through sulfhydryl conjugation leading to low bioavailability within the target site and potentially undesirable toxicities in other tissues upon distribution.

The ThioGlo-1 fluorescence assay was used to determine the sulfhydryl binding of bethoxazin with biological molecules in normal K562 cells and in K562 cells with low GSH levels (reduced by BSO pre-treatment). The measurements of the fluorescence intensity by the microplate reader in the cells with low GSH levels were not optimal as it would be preferable

that the recorded fluorescence intensity readings were of multifold difference from the background.

Bethoxazin has very low water solubility, so stocks of bethoxazin were prepared in DMSO. It would be challenging to find a good solvent for bethoxazin if it was to be formulated as an anti-cancer agent.

5.2. Conclusions

Despite bethoxazin's industrial use as a biocide, its mechanism of action has not been previously reported. The current study focused on exploring the mechanism of action by which bethoxazin exerts its cytotoxic effects. It provided insight into the chemical reactivity of bethoxazin and the possible chemical and biological interactions that can occur in the presence of bethoxazin.

The chemical and biological effects of bethoxazin were investigated in this study. The chemical reactivity of bethoxazin was explored (*in vitro*) using UV-Vis spectroscopy. The chemical reactions of bethoxazin towards a set of compounds with representative functional groups demonstrated that bethoxazin reacts with only sulfhydryl containing molecules such as GSH and HSA by forming covalent adducts. Furthermore, the rate of the reaction of bethoxazin with GSH was much faster than with HSA suggesting that the incubation time required for bethoxazin to react with GSH is much shorter than that required with proteins. These reactions have shown that bethoxazin behaves as an electrophile in chemical reactions and provided insight into its expected biological effects.

It was of interest to explore the biological effects of bethoxazin in K562 cancer cells as bethoxazin was shown to potently inhibit the growth of K562 cells with an IC_{50} of 1 µM. The

current study showed that bethoxazin reacted with the free sulfydryl molecules in K562 cells and inhibited their interaction with the sulfhydryl specific fluorescent label ThioGlo-1. This investigation implies that bethoxazin reacts with sulfydryl containing molecules such as GSH in of K562 cells within 2 hours and requires longer than 2 hours to react with proteins containing free sulfhydryl residues.

The biological effects of bethoxazin in K562 cells were also studied in GSH depleted K562 cells to investigate the effect of depleting GSH on the sulfhydryl binding of bethoxazin to the free sulfhydryl containing molecules within the cells. The results showed that reducing the GSH concentrations in K562 cells did not substantially affect the binding of bethoxazin to the free sulfhydryl biomolecules implying that bethoxazin likely reacts with biomolecules containing free sulfhydryl residues other than GSH.

The trypan blue cell viability assay was performed for the same set of cells that were labeled with ThioGlo-1 to understand if the cytotoxic effects of bethoxazin in K562 cells are related to its interaction with sulfhydryl containing molecules like GSH. The trypan blue assay in normal K562 cells revealed that 2 hours of 100 µM bethoxazin treatment resulted in 50% cell viability while the % ThioGlo-1 inhibition by bethoxazin was ~90% at the same concentration. This observation implies that bethoxazin likely requires more than 2 hours to efficiently kill the cells and that the cellular death involves, at least partially, mechanisms other that GSH depletion. The same cell viability assay was performed in K562 cells with reduced levels of GSH to explore whether or not bethoxazin's cytotoxicity is affected by the initial GSH concentrations in the cells. The results of trypan blue assay suggested that GSH depletion in K562 cells did not sensitize the cells to the cytotoxic effects of bethoxazin.

The MTS cell growth inhibition assay (section 3.3**)**, was performed to further confirm whether or not GSH depletion is the route by which bethoxazin exerts its growth inhibition effects in K562 cells. In this assay, two sets of K562 cells were bethoxazin treated for 2 hours followed by continuous treatment (set A of cells) or wash out (set B) of bethoxazin. The results of the assay revealed that the continuous treatment of bethoxazin enhanced the potency of bethoxazin. This implies that the growth inhibition effects of bethoxazin are likely not due to GSH depletion since both sets of cells were initially bethoxazin treated for 2 hours which is sufficient to deplete GSH in both sets of cells.

The two-colour flow cytometry analysis of bethoxazin treated K562 cells revealed that bethoxazin is likely to induce cell death through a necrotic rather than an apoptotic mechanism. Necrosis is an irregular form of cell death and is characterized by cell membrane damage and release of cellular components. Apoptosis is an organized natural form of cell death, usually involves specific targets, and does not involve membrane damage. The fact that bethoxazin induces cell death through a necrotic mechanism is supported by the earlier findings of bethoxazin in that bethoxazin was shown to react non-selectively with any sulfhydryl containing compounds by forming adducts. Taken together these observations suggest that bethoxazin might react with any free sulfhydryl containing molecules within the cells such as peptides and proteins.

Bethoxazin potently inhibited both the growth of K562 cells with an IC_{50} of 1 μM and also the human topoisomerase IIα enzyme with an IC_{50} of 1 μM (Dr. Chee's unpublished data). Taking into account that the human topoisomerase IIα contains free cysteine residues (Hasinoff B et al, 2005) and the recent investigations, it is likely to suggest that bethoxazin may, at least partially,

exert its cell growth inhibition effects in K562 cells by reacting with the free cysteine residue of topoisomerase IIα.

5.3. Recommendations

Bethoxazin was shown to potently inhibit the catalytic activity of topoisomerase IIα (Dr. Chee's unpublished data). The interaction of bethoxazin with topoisomerase IIα could be confirmed by an experiment similar to that conducted for the anti-cancer agent cisplatin where the formation of cisplatin adducts with the critical cysteine of topoisomerase IIα was determined by ThioGlo-1 (Hasinoff B et al, 2005). If this experiment was conducted with bethoxazin instead of cisplatin, it would be designed to determine the formation of a covalent adduct between topoisomerase IIα and ThioGlo-1 in the presence or absence of bethoxazin. The result would show whether or not bethoxazin actually forms a covalent adduct with the critical cysteine residue of topoisomerase IIα which may further confirm the results of the current study.

The mechanism of cellular death induced by bethoxazin in K562 cells was analyzed by flow cytometry. Other cellular assays maybe conducted in K562 cells to further confirm the necrotic mechanism induced by bethoxazin and rule out apoptosis. In addition, it would be interesting to analyze the cytotoxic effects of bethoxazin on other cancer cell lines to investigate if bethoxazin would exert similar effects to that of K562 cells. Furthermore, exploring the effects of bethoxazin on normal human cells would provide insight into the mechanism by which bethoxazin exerts its undesirable toxicities in normal cells which would be important in the development of this compound into a pharmaceutical product.

Bethoxazin is marketed and commercially available as a racemate. It possesses a chiral sulphoxide moiety consisting of two enantiomers. The importance of chirality has not been investigated and there are no commercially available preparations of pure bethoxazin enantiomers. Enantiomers have similar physical and chemical properties except their ability to rotate polarized light, however enantiomers may have differences in physical, chemical and biological properties compared to the racemate. It is possible that one enantiomer of bethoxazin would be active in inducing cell growth inhibition effects of bethoxazin in K562 cells and the other enantiomer could be inactive. Exploring the chemical and biological effects of the two enantiomers of bethoxazin in the future could provide insight into the active forms of bethoxazin. However, it is probable that the enantiomers of bethoxazin are highly reactive and also toxic.

References

Abbenante, G., & Fairlie, D. P. (2005). Protease inhibitors in the clinic. *Medicinal Chemistry (Shariqah (United Arab Emirates)), 1*(1), 71-104.

Ahluwalia, V. K. C. (2008). *Medical chemistry*. Boca Raton: CRC Press/Taylor & Francis Group.

Baum, E. Z., Ding, W. D., Siegel, M. M., Hulmes, J., Bebernitz, G. A., Sridharan, L., et al. (1996). Flavins inhibit human cytomegalovirus UL80 protease via disulfide bond formation. *Biochemistry, 35*(18), 5847-5855.

Baum, E. Z., Siegel, M. M., Bebernitz, G. A., Hulmes, J. D., Sridharan, L., Sun, L., et al. (1996). Inhibition of human cytomegalovirus UL80 protease by specific intramolecular disulfide bond formation. *Biochemistry, 35*(18), 5838-5846.

Bosselaers, J., Blancquaert, P., Gors, J., Heylen, I., Lauwaerts, A., Nys, J., et al. (2003). A new fungicide and algicide. *Faerg Och Lack Scandinavia, 49*(1), 5-8, 10-11.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

Bruns, R., Jaetsch, T., Kugler, M., & Spetmann, P. (2012). *Fungicidal mixtures for wood preservation* Google Patents.

Casarett, L. J., Doull, J., & Klaassen, C. D. (2008). *Casarett and doull's toxicology : The basic science of poisons*. New York: New York : McGraw-Hill.

Chee, G. L., Bhattarai, B., Daniel Gietz, R., Alrushaid, S., Nitiss, J. L., & Hasinoff, B. B. (2012). Chemical reactivity and microbicidal action of bethoxazin. *Bioorganic & Medicinal Chemistry, 20*(4), 1494-1501.

Chipinda, I., Hettick, J. M., & Siegel, P. D. (2011). Haptenation: Chemical reactivity and protein binding. *Journal of Allergy, 2011*, 839682.

Davis, R. A., Brouwer, W. G., & Valcke, A. R. A. (1998). *Wood preservative oxathiazines* Google Patents.

Denny, W. A. (2001). Prodrug strategies in cancer therapy. *European Journal of Medicinal Chemistry, 36*(7-8), 577-595.

Dragovich, P. S., Prins, T. J., Zhou, R., Brown, E. L., Maldonado, F. C., Fuhrman, S. A., et al. (2002). Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 6. structure-activity studies of orally bioavailable, 2-pyridone-containing peptidomimetics. *Journal of Medicinal Chemistry, 45*(8), 1607-1623.

Dragovich, P. S., Prins, T. J., Zhou, R., Johnson, T. O., Brown, E. L., Maldonado, F. C., et al. (2002). Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. part 7: Structure-activity studies of bicyclic 2-pyridone-containing peptidomimetics. *Bioorganic & Medicinal Chemistry Letters, 12*(5), 733-738.

Dragovich, P. S., Prins, T. J., Zhou, R., Johnson, T. O., Hua, Y., Luu, H. T., et al. (2003). Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 8. pharmacological optimization of orally bioavailable 2-pyridone-containing peptidomimetics. *Journal of Medicinal Chemistry, 46*(21), 4572-4585.

Duan, X., Lu, Q., Xue, P., Zhang, H., Dong, Z., Yang, F., et al. (2008). Proteomic analysis of aqueous humor from patients with myopia. *Molecular Vision, 14*, 370-377.

Ertl, P., Cooper, D., Allen, G., & Slater, M. J. (1999). 2-chloro-3-substituted-1,4-naphthoquinone inactivators of human cytomegalovirus protease. *Bioorganic & Medicinal Chemistry Letters, 9*(19), 2863-2866.

Fadok, V. A., Laszlo, D. J., Noble, P. W., Weinstein, L., Riches, D. W., & Henson, P. M. (1993). Particle digestibility is required for induction of the phosphatidylserine recognition mechanism used by murine macrophages to phagocytose apoptotic cells. *Journal of Immunology (Baltimore, Md.: 1950), 151*(8), 4274-4285.

Foye, W. O., Lemke, T. L., & Williams, D. A. (2008). *Foye's principles of medicinal chemistry*. Philadelphia: Lippincott Williams & Wilkins.

Freshney, R. I. (1987). *Culture of animal cells: A manual of basic technique* (2nd ed.). New York: Liss.

Hagrman, D., Goodisman, J., Dabrowiak, J. C., & Souid, A. K. (2003). Kinetic study on the reaction of cisplatin with metallothionein. *Drug Metabolism and Disposition: The Biological Fate of Chemicals,31*(7), 916-923.

Hagrman, D., Goodisman, J., Dabrowiak, J. C., & Souid, A. K. (2003). Kinetic study on the reaction of cisplatin with metallothionein. *Drug Metabolism and Disposition: The Biological Fate of Chemicals,31*(7), 916-923.

Hasinoff, B. B., Creighton, A. M., Kozlowska, H., Thampatty, P., Allan, W. P., & Yalowich, J. C. (1997). Mitindomide is a catalytic inhibitor of DNA topoisomerase II that acts at the bisdioxopiperazine binding site. *Molecular Pharmacology, 52*(5), 839-845.

Hasinoff, B. B., Wu, X., Krokhin, O. V., Ens, W., Standing, K. G., Nitiss, J. L., et al. (2005). Biochemical and proteomics approaches to characterize topoisomerase IIalpha cysteines and DNA as targets responsible for cisplatin-induced inhibition of topoisomerase IIalpha. *Molecular Pharmacology, 67*(3), 937-947.

Hasinoff, B. B., Wu, X., Yalowich, J. C., Goodfellow, V., Laufer, R. S., Adedayo, O., et al. (2006). Kinamycins A and C, bacterial metabolites that contain an unusual diazo group, as potential new anticancer agents: Antiproliferative and cell cycle effects. *Anti-Cancer Drugs, 17*(7), 825-837.

Hasinoff, B. B., Wu, X., & Yang, Y. (2004). Synthesis and characterization of the biological activity of the cisplatin analogs, cis-PtCl(2)(dexrazoxane) and cis-PtCl(2)(levrazoxane), of the topoisomerase II inhibitors dexrazoxane (ICRF-187) and levrazoxane (ICRF-186). *Journal of Inorganic Biochemistry, 98*(4), 616-624.

Holwerda, B. C. (1997). Herpesvirus proteases: Targets for novel antiviral drugs. *Antiviral Research, 35*(1), 1-21.

Ivanov, A. I., Christodoulou, J., Parkinson, J. A., Barnham, K. J., Tucker, A., Woodrow, J., et al. (1998). Cisplatin binding sites on human albumin. *The Journal of Biological Chemistry, 273*(24), 14721-14730.

Izbicka, E., & Tolcher, A. W. (2004). Development of novel alkylating drugs as anticancer agents. *Current Opinion in Investigational Drugs (London, England : 2000), 5*(6), 587-591.

Jerry, M. (Ed.). (1985). *Advanced organic chemistry reactions, mechanisms and structure* (3 rd Ed. ed.). New York: John Wiley & Sons, inc.

Kang, Y. J., Zhou, Z. X., Wu, H., Wang, G. W., Saari, J. T., & Klein, J. B. (2000). Metallothionein inhibits myocardial apoptosis in copper-deficient mice: Role of atrial natriuretic peptide. *Laboratory Investigation; a Journal of Technical Methods and Pathology, 80*(5), 745-757.

Ketterer, B. (1988). Protective role of glutathione and glutathione transferases in mutagenesis and carcinogenesis. *Mutation Research, 202*(2), 343-361.

Kohn, K. W., Orr, A., O'Connor, P. M., Guziec, L. J., & Guziec, F. S.,Jr. (1994). Synthesis and DNA-sequence selectivity of a series of mono- and difunctional 9-aminoacridine nitrogen mustards. *Journal of Medicinal Chemistry, 37*(1), 67-72.

Langmuir, M., Yang, J., LeCompte, K., & Durand, R. (1996). New thiol active fluorophores for intracellular thiols and glutathione measurement. In J. SlavÃk (Ed.), (pp. 229-233) Springer US.

Leung-Toung, R., Li, W., Tam, T. F., & Karimian, K. (2002). Thiol-dependent enzymes and their inhibitors: A review. *Current Medicinal Chemistry, 9*(9), 979-1002.

Leung-Toung, R., Zhao, Y., Li, W., Tam, T. F., Karimian, K., & Spino, M. (2006). Thiol proteases: Inhibitors and potential therapeutic targets. *Current Medicinal Chemistry, 13*(5), 547-581.

Malich, G., Markovic, B., & Winder, C. (1997). The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. *Toxicology, 124*(3), 179-192.

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., et al. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of bcl-2 and abl. *The Journal of Experimental Medicine, 182*(5), 1545-1556.

Matthews, D. A., Dragovich, P. S., Webber, S. E., Fuhrman, S. A., Patick, A. K., Zalman, L. S., et al. (1999). Structure-assisted design of mechanism-based irreversible inhibitors of human rhinovirus 3C protease with potent antiviral activity against multiple rhinovirus serotypes. *Proceedings of the National Academy of Sciences of the United States of America, 96*(20), 11000-11007.

Medina, J. C., Roche, D., Shan, B., Learned, R. M., Frankmoelle, W. P., Clark, D. L., et al. (1999). Novel halogenated sulfonamides inhibit the growth of multidrug resistant MCF-7/ADR cancer cells.*Bioorganic & Medicinal Chemistry Letters, 9*(13), 1843-1846.

Medina, J. C., Shan, B., Beckmann, H., Farrell, R. P., Clark, D. L., Learned, R. M., et al. (1998). Novel antineoplastic agents with efficacy against multidrug resistant tumor cells. *Bioorganic & Medicinal Chemistry Letters, 8*(19), 2653-2656.

Nichols, D.,. (2004). *Biocides in plastics*. Shrewsbury: Rapra Technology.

NICULESCU-DUVAZ, I., BARACU, I., & BALABAN, A. T. (1989). Alkylating agents. In D. E. V. Wilman (Ed.), *The chemistry of antitumour agents* (pp. 63). London: Blackie.

O'Brien, T., & Linton, S. D. (Eds.). (2009). *Design of caspase inhibitors as potential clinical agents*. United States of America: CRC Press (Taylor & Francis Group).

Palmer, B. D., Lee, H. H., Johnson, P., Baguley, B. C., Wickham, G., Wakelin, L. P., et al. (1990). DNA-directed alkylating agents. 2. synthesis and biological activity of platinum complexes linked to 9-anilinoacridine. *Journal of Medicinal Chemistry, 33*(11), 3008-3014.

Panthananickal, A., Hansch, C., & Leo, A. (1978). Structure-activity relationships in antitumor aniline mustards. *Journal of Medicinal Chemistry, 21*(1), 16-26.

Pastore, A., Federici, G., Bertini, E., & Piemonte, F. (2003). Analysis of glutathione: Implication in redox and detoxification. *Clinica Chimica Acta; International Journal of Clinical Chemistry, 333*(1), 19-39.

Randle, J., Harding, M., Ku, G., SchÃ¶nharting, M., & Kurrle, R. (2001). ICE/Caspase-1 inhibitors as novel anti-inflammatory drugs. *Expert Opinion on Investigational Drugs, 10,* 1207-1209.

Sadowitz, P. D., Hubbard, B. A., Dabrowiak, J. C., Goodisman, J., Tacka, K. A., Aktas, M. K., et al. (2002). Kinetics of cisplatin binding to cellular DNA and modulations by thiol-blocking agents and thiol drugs. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 30*(2), 183-190.

Scozzafava, A., Mastrolorenzo, A., & Supuran, C. T. (2000). Arylsulfonyl-N,N-diethyl-dithiocarbamates: A novel class of antitumor agents. *Bioorganic & Medicinal Chemistry Letters, 10*(16), 1887-1891.

Scozzafava, A., Mastrolorenzo, A., & Supuran, C. T. (2001). Arylsulfonyl-N,N-dialkyl-dithiocarbamates as tumor cell growth inhibitors: Novel agents targeting beta-tubulin? *Journal of Enzyme Inhibition,16*(1), 55-63.

Shan, B., Medina, J. C., Santha, E., Frankmoelle, W. P., Chou, T. C., Learned, R. M., et al. (1999). Selective, covalent modification of beta-tubulin residue cys-239 by T138067, an antitumor agent with in vivo efficacy against multidrug-resistant tumors. *Proceedings of the National Academy of Sciences of the United States of America, 96*(10), 5686-5691.

Shen, X. C., Tao, L., Li, W. K., Zhang, Y. Y., Luo, H., & Xia, Y. Y. (2012). Evidence-based antioxidant activity of the essential oil from fructus A. zerumbet on cultured human umbilical vein endothelial cells' injury induced by ox-LDL. *BMC Complementary and Alternative Medicine, 12*, 174-6882-12-174.

Stamatiou, R., Paraskeva, E., Gourgoulianis, K., Molyvdas, P. A., & Hatziefthimiou, A. (2012). Cytokines and growth factors promote airway smooth muscle cell proliferation. *ISRN Inflammation, 2012*, 731472.

Tyagarajan, K., Pretzer, E., & Wiktorowicz, J. E. (2003). Thiol-reactive dyes for fluorescence labeling of proteomic samples. *Electrophoresis, 24*, 2348-2358.

Van, D. F. M. A. J. (2003). *Preservative formulations comprising an oxathiazine and amine oxides* Google Patents.

Vanden Berghe, T., Vanlangenakker, N., Parthoens, E., Deckers, W., Devos, M., Festjens, N., et al. (2010). Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. *Cell Death and Differentiation, 17*(6), 922-930.

Voiculetz, N., & Niculescu-DuvÄƒz, I. (1990). General mechanisms of carcinogenesis and anticarcinogenesis. *Archives Roumaines De Pathologie Experimentales Et De Microbiologie, 49*, 105-117.

Wallace, D. F., & Dickinson, D. J. (2004). *16S rRNA analysis of the bacteria associated with biocide degradation*. Slovenia: The International Research Group on Wood Preservation 35th Annual Meeting.

Wang, J., Fan, T., Yao, X., Wu, Z., Guo, L., Lei, X., et al. (2011). Crystal structures of enterovirus 71 3C protease complexed with rupintrivir reveal the roles of catalytically important residues. *Journal of Virology, 85*(19), 10021-10030.

Wang, Z., Watt, W., Brooks, N. A., Harris, M. S., Urban, J., Boatman, D., et al. (2010). Kinetic and structural characterization of caspase-3 and caspase-8 inhibition by a novel class of irreversible inhibitors. *Biochimica Et Biophysica Acta, 1804*(9), 1817-1831.

Williams, G., & Bacon, M. (2006). *Wood preservative formulations* Google Patents.

Wu, X., & Hasinoff, B. B. (2005). The antitumor anthracyclines doxorubicin and daunorubicin do not inhibit cell growth through the formation of iron-mediated reactive oxygen species. *Anti-Cancer Drugs, 16*(1), 93-99.

Wu, X., Liang, H., O'Hara, K. A., Yalowich, J. C., & Hasinoff, B. B. (2008). Thiol-modulated mechanisms of the cytotoxicity of thimerosal and inhibition of DNA topoisomerase II alpha. *Chemical Research in Toxicology, 21*(2), 483-493.

Yang, F., Gu, N., Chen, D., Xi, X., Zhang, D., Li, Y., et al. (2008). Experimental study on cell self-sealing during sonoporation. *Journal of Controlled Release : Official Journal of the Controlled Release Society, 131*(3), 205-210.

Zong, W. X., & Thompson, C. B. (2006). Necrotic death as a cell fate. *Genes & Development, 20*(1), 1-15.