INVESTIGATION OF SOME MOLECULAR MECHANISMS OF CYTOTOXIC 1,5-DIARYL-3-OXO-1,4-PENTADIENES

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in Partial Fulfillment of the Requirements
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بسم الله الرحمن الرحيم

"In the Name of Allah, the Most Beneficent, the Most Merciful"

ABSTRACT

Glutathione S-transferase GSTπ has been one of the significant targets for cancer treatment in the past several years. The reason behind that is 1) its overexpression in some cancer cells compared to normal ones 2) its ability to cause resistance against cancer chemotherapeutics and 3) its protective role against reactive oxygen species (ROS). We have synthesized a large number of compounds which have strong potency against different cancer cell lines. These compounds possess a 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. In the present study some investigations as to the way in which cytotoxicity is mediated was undertaken. Our results have demonstrated that the analogs **NC 2067** and **NC 2081** behaved as substrates for GSTπ and reduced the level of GSH. This was apparent by the decrease in the concentrations of both compounds after the addition of GSTπ and GSH. In addition, both agents caused about 3-7 folds increase in ROS levels. The dichlorodihydrofluorescein dye was used for this purpose due to its fluorescence characteristic after being oxidized by ROS. High levels of these species cause a drop in the mitochondrial membrane potential. This phenomenon was detected when the monomeric form of JC-1 levels were increased after treatment. The reduction of 2-3 folds was seen when the cells were treated with the IC_{50} values of both compounds. In addition, both agents inhibited oxygen consumption implicating their ability to inhibit oxidative phosphorylation. We also evaluated the effect of both agents on mitochondrial swelling. **NC 2081** caused swelling using concentrations of 10 μM and 50 μM. This was apparent when the absorption of an isolated rat liver mitochondrial solution decreased after the addition of the compound. The addition of the higher concentration caused about 2 fold greater effect than the lower one. On the other hand, compound **NC 2067** produced minimal swelling only at a concentration of 50 μM.

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Dedicated
to
My Parents and Family

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LIST OF ABBREVIATIONS

μL	Microliter
μM	Micromole
5-FU	5-Fluorouracil
ANT	Adenine nucleotide translocator
AT	Adenosine-tyrosine
ATP	Adenosine triphosphate
CCAC	Canadian Council of Animal Care
CCCP	Carbonylcyanide 3-chlorophenylhydrazone
CDNB	1-Chloro-2,4-dinitrobenzene
COX2	Cyclooxygenase 2
CRL-1790	Normal colon epithelial cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER+	Estrogen receptor positive
FDA	Food and Drug Administration
GC	Guanine-cytosine
GSH	Glutathione
GSTπ	Glutathione S-transferase π
HIF-1	Hypoxia-inducible factor-1
HL-60	Human promyelocytic leukemia cells
Hr	Hour
HSC-2, HSC-4	human oral squamous cell carcinoma cell lines

IL	Interleukin
JNK	c-Jun N-terminal kinase
LOX	Lipoxygenase
min	Minute
MOPS	3-(N-morpholino)propanesulfonic acid
MRP-1	Multidrug resistance associated protein-1
NC 2067	1-[4-[2-Dimethylaminoethoxy]phenylcarbonyl]-3,5-bis[phenylmethylene]-4-piperidone hydrochloride
NC 2081	1-[4-{2-[4-Morpholinyl]ethoxy}phenylcarbonyl]-3,5-bis[phenylmethylene]-4-piperidone hydrochloride hemihydrate
nm	Nanometer
p53	Tumor protein 53
PTP	Permeabilty transition pore
ROS	Reactive oxygen species
sec	Second
SRB	Sulforhodamine B
TLK199	γ-Glutamyl-benzylcysteinylphenylglycyl diethyl ester
TNF	Tumor necrosis factor
UV	Ultraviolet
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

Cancer is recognized as one of the main causes of death. Numerous efforts have been taken to study the biochemical differences between cancer and normal cells in order to design agents that would have the ability to treat this disease. In the past decades, these efforts resulted in new agents finding their place into clinical practice. Both pharmacological and non-pharmacological approaches are used in cancer treatment, each associated with significant limitations. One major drawback of the currently available pharmacological approaches is the serious adverse effects accompanying the use of anticancer drugs, due mainly to their potential to harm normal cells and not just malignant ones. Consequently, there is an urgent need to identify new agents that have the ability to target cancer cells and have minimal or no effects on normal healthy cells. The established differences in the biochemical behavior of both cell types have made such options achievable.

In this thesis work I investigated two novel cytotoxic agents, namely, 1-[4-[2-dimethylaminoethoxy]phenylcarbonyl]-3,5-bis[phenylmethylene]-4-piperidone hydrochloride (**NC 2067**) and 1-[4-{2-[4-morpholinyl]ethoxy}phenylcarbonyl]-3,5-bis[phenylmethylene]-4-piperidone hydrochloride hemihydrate (**NC 2081**) for their ability to selectively inhibit the growth of cancer cells and to understand their mechanisms of action.

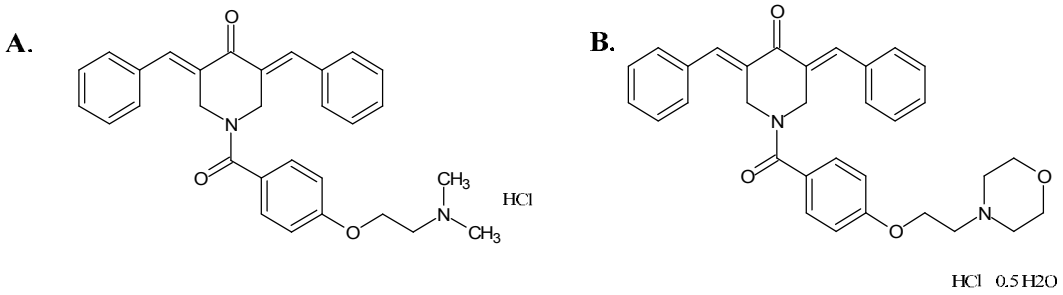


Figure 1.1: A. The structure of **NC 206**7, B. The structure of **NC 2081**

2. BACKGROUND

2.1 Cancer biology

Cancer is characterized by the abnormal growth of cells that tend to proliferate continuously without control. (Kleinsmith, 2006). In the United States it is estimated that one out of four deaths is due to cancer (Jemal et al., 2007). Cancer is now recognized as the leading cause of death in Canada according to the Canadian Cancer Society (Canadian cancer statistics, 2011)

Cancers are formed from cells that have a defective DNA structure, which leads to abnormality in the function(s) and reproduction of cells. It is generally accepted that a single abnormality in one of these events is insufficient to cause cancer (Croce, 2008) since cancer is a multi-step process. Genetic abnormalities may occur in proto-oncogenes, which regulate cell growth, or tumor suppressor genes which normally favor growth inhibition. Mutations that lead to an induction in the activity of proto-oncogenes, or inhibition of the tumor suppressor genes, are initial steps for cancer development. Examples of oncogenes include transcription factors, growth factors, growth factor receptors and apoptosis regulators. Tumor suppressor genes function alongside proto-oncogenes but with an opposite effect. Such genes tend to suppress cell division when DNA is damaged until it is repaired. If repair is not possible, tumor suppressor genes such as p53 induce apoptosis (Croce, 2008). Cancer cells can also be classified into malignant or benign forms. Malignant cancers have the ability to invade and destroy other cells and even undergo metastasis to other parts of the

body, whereas benign tumors grow only in specific locations and have a lower possibility to invade other tissues.

2.2 Cancer treatment methods

In clinical practice, physicians use regimens that combine two or more methods/drugs for cancer treatment. Different approaches are usually needed in order to give maximal potency against cancer cells while minimizing drug resistance and adverse effects. The usual treatment approaches for cancer include surgery, radiotherapy, hormonal therapy, immunotherapy and chemotherapy.

2.2.1 Hormonal therapy

Certain types of cancer cells require specific hormones to grow. Estrogen for example is involved in the pathogenesis of estrogen receptor positive (ER+) breast cancer. Treatment options for such cancers involve blocking estrogen receptors by tamoxifen (Zilli et al., 2009), which has been a mainstay therapy for post-menopausal women having breast cancer for many years (Zilli et al., 2009). Another type of hormone-dependent cancer is prostate cancer which depends on androgens for its development. For this reason, androgenic blockers are used as a treatment method for prostate cancer. It is evident that blocking these receptors by agents such as flutamide can be used as a first line therapy for prostate cancer which is effective by regulating its growth (Beekman and Hussain, 2008).

2.2.2 Anti-Angiogenesis therapy

Angiogenesis is the process of new blood vessel development. This phenomenon normally occurs normally during wound healing processes or in the placenta. Cancer cells have the ability to stimulate the formation of new vessels towards the tumor in order to supply itself with nutrients. The idea of anti-angiogenesis as cancer therapy was proposed several decades ago (Folkman, 1971). The approval of bevacizumab (Avastin) for cancer treatment has encouraged researchers to start focusing on angiogenesis as a new treatment. Nowadays, it is becoming accepted as a treatment for cancer (Ferrara et al., 2005).

2.2.3 Immunotherapy

Immunotherapy is the use of the immune system to fight cancer. For the past few decades, the term immunotherapy has been widely popularized. It was thought earlier that the immune system is limited to fighting infections. Recently several important discoveries have shown its role in perturbing cancer progression (Keibel et al, 2009).

These observations and theories have encouraged scientists to develop this relatively new strategy for fighting cancer commonly termed as either active or passive immunotherapy. Active immunotherapy can be subdivided into specific and non-specific therapy. Specific therapy involves designing active immunotherapy agents to induce certain immune cells for attacking various antigens on cancer cells (Lesterhuis et al., 2011). On the other hand, non-specific active immunotherapy utilizes the broad activation of the immune system cells by certain agents. These

agents include cytokines such as interferon and interleukins (IL-2, IL-12). They regulate both adaptive and passive immune systems. Interferon and interleukin have been approved by the FDA for certain cancer types, such as the approval of IL-2 for renal cell carcinoma and leukemia (Hurley and Chapman, 2005; Yang et al., 2003; Schuster et al., 2006).

2.2.4 Chemotherapeutic agents

Most of the previously mentioned treatments can be used only for local tumors. Once metastasis occurs, a systemic treatment needs to be undertaken which uses chemotherapeutics. These compounds are used to kill cells or stop their growth in order to treat cancer. Chemotherapy is widely used in clinical practice. Unfortunately, drugs have dose limiting toxicity particularly on cells that divide rapidly such as hemopoietic and mucosal cells on the gastrointestinal lining. Of particular concern is the effect of cytotoxic chemotherapeutic agents on stem cells.

Agents with cytotoxic activity function in several ways with DNA being the favorite target. Some agents are able to attack DNA and inhibit its replication while others can prevent DNA synthesis. However, attacking the mitochondria and mitotic spindle are two other mechanisms in which some agents exert their activity (Lind, 2011).

Attacking DNA directly is the principal approach amongst most chemotherapeutic agents which tend to cause DNA damage and cell death. Agents can achieve this by alkylation of key atoms located on the DNA structure thereby forming strong

covalent bonds. This mode of action is known to cause potent cytotoxicity in general and operates in such drugs as mitomycin, cyclophosphamide, and cisplatin (Lind, 2011). Other agents perturb the DNA structure through electrostatic forces (Lind, 2011), intercalation with DNA or binding to DNA grooves. A number of chemotherapeutic compounds act by inhibiting the synthesis of DNA such as antimetabolites which include methotrexate, 6-mercaptopurine and 6-thioguanine (Lind, 2011).

2.2.4.1 DNA Groove binders

Apart from alkylation, chemotherapeutic agents can also target DNA via noncovalent bonding which can occur at the minor groove region of DNA or in between DNA nucleotides. Non-covalent binding to DNA involves electrostatic (e.g., hydrogen bonding) and/or van der Waals' interactions which are the two major forces that govern ligand-DNA binding activity.

Sequence specificity is an important criterion when targeting DNA as this will reduce the potential side effects caused by chemotherapeutic compounds. A ligand needs to cover a minimum of 16 to18 nucleotide binding sites in order to have good selectivity at the genomic level (Neidle, 2001). This requirement may present a challenge as the larger the molecule the less stable it becomes and the likelihood of its contact surfaces to become "out of phase" tends to increase causing a decrease in the binding affinity. Sequence selective agents prefer specific DNA sequences compared to others. Examples of such agents include pyrrolo[1,4]benzodiazepinone

antibiotics (Hurley et al., 1988), netropsin and distamycin (Zimmer and Wahnert, 1986) and bleomycin (Stubbe and Kozarich, 1987). These agents were found to target specific DNA sequences. Netropsin and distamycin prefer AT rich sequences, while bleomycin prefers GCs sequences (Povirk et al., 1989).

2.2.4.2 Intercalation

Intercalating agents bind non-covalently and reversibly with nucleic acids. A number of intercalating chemotherapeutic agents are now employed clinically for ovarian cancer, breast cancers and acute leukemia, while others are still in clinical trials (Brana et al., 2001). Ethidium bromide, doxorubicin, daunomycin and thalidomide are examples of DNA intercalators.

Intercalation occurs when a ligand aligns itself between the DNA adjacent bases. Usually the compound has a flat planar aromatic structure (Sinha et al., 2006) which allows it to slide easily within the narrow space that exists between adjacent DNA bases. These agents can cause structural changes to the DNA topology and cause genotoxicity (Mortelmans and Zeiger, 2000).

2.2.4.3 Mitotic/spindle and topoisomerase inhibitors

These agents have the ability to either stabilize the microtubules once they are formed or inhibit their formation. Either mechanisms cause a limited flexibility in these organelles which results in retardation in chromosomal alignments and

inhibition of cell growth. Examples of these agents are paclitaxel, vincristine and vinblastine (Lind, 2011).

Proteins, such as topoisomerases, which are important in cell division, are also targets for anti-cancer agents. Proteins which play a role in the last stage before cell division can be attacked by cytotoxic drugs to inhibit cell division (Lind, 2011).

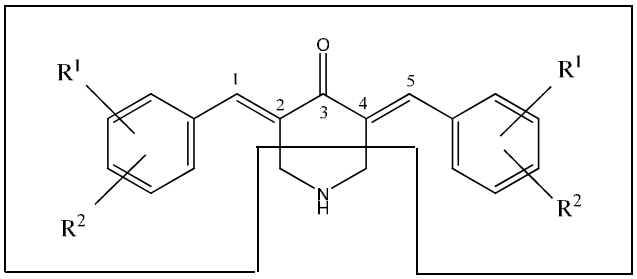
2.3 Thiol alkylators

2.3.1 Overview

As explained above, most treatment options involving chemotherapy have serious side effects and limitations. Therefore, there is an urgent need to develop novel anticancer agents which display high selective toxicities towards neoplasms rather than affecting the normal cells.

Over the last several years we have focused on developing a number of cyclic and acyclic conjugated unsaturated dienone derivatives possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore as tumor selective cytotoxins, Figure 2.1. Generally these agents show high selectivity towards cancer cells. The pharmacophore is known to interact with cellular thiols (Mutus et al., 1989; Das et al., 2009). Since thiol groups are not present in DNA, these agents may lack the ability to interact with DNA thereby limiting or eliminating genotoxicity. This property is a significant benefit over other chemotherapeutic agents since attacking DNA may result in potent cytotoxicity but it may also induce mutations that might be passed to daughter cells.

Therefore, having a strategy which would achieve high potency without genotoxicity is desirable.



1,5-diaryl-3-oxo-1,4-pentadienyl group

Figure 2.1: Structure of cytotoxic 3,5-bis(benzylidene)-4-piperidones

Designing agents that exert their cytotoxicity in different pathways have shown great benefit over agents acting on a single pathway. This can be due to different reasons. Targeting different mechanisms would enhance the potency and minimize the chance of drug resistance. This is used for various serious illnesses to minimize drug resistance development and increase effectiveness (Espinoza-Fonseca, 2006). For example, when treating certain bacterial infections, a multi-drug regimen is usually used with each agent having its own mechanism of action. The same principle is used for cancer treatment. Instead of administering several drugs with different targets, having a single drug with different targets might be more convenient and yet effective.

2.3.2 Curcumin

Curcumin, (Figure 2.2) is extracted from Curcuma longa Linn roots. The reason behind our interest in curcumin is it having similar structural features as our compounds. Curcumin contains two 3-aryl-2-propenoyl groups. Figure 2.2 shows the pharmacophore of interest contained in the structure. However, the main difference found between its structure and our agents is the presence of the piperidine ring in our compounds rather than being an acyclic moiety. This heterocyclic scaffold was introduced to minimize the flexibility of the acyclic molecules. In addition, the presence of nitrogen allows the addition of other side chains to be placed on the piperidyl nitrogen atom.

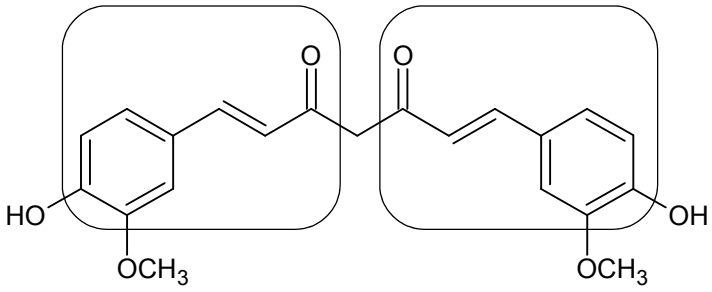


Figure 2.2: Structure of curcumin

Curcumin has been used for various medical conditions and has an established safety profile (Aggarwal et al., 2003). Curcumin is used for such conditions as rheumatoid arthritis, liver disease and urinary tract infections (Adams et al., 2004). It is also known for its anti-inflammatory and antioxidant properties. Some antiviral, antibacterial and antifungal activities were also observed for curcumin (Aggarwal et al., 2007).

Curcumin has also showed some anticancer properties (Sa and Das, 2008) and it's *in vitro* antiproliferative (Mehta et al., 1997) and apoptotic induction behavior has been investigated (Kuo et al., 1996; Liao et al., 2008). In addition, curcumin has showed some antiangiogenic effects when tested *in vivo* and *in vitro* (Lin et al., 2007). Due to these factors, curcumin is of major interest in the battle against cancer. It was previously shown that curcumin can exert its biological activity by down regulating the expression of several proteins such as NF-kappa B, lipoxygenase (LOX), cyclooxygenase (COX2), tumor necrosis factor (TNF) and others. It also inhibits the activity of the protein tyrosine kinases c-Jun N-terminal kinase (JNK) and serine/threonine kinases (Aggarwal et al., 2003).

However, its high lipophilic nature has limited its use for cancer treatment. It has a high first pass effect and therefore low bioavailability (Shoba et al., 1998). Therefore, it is important to synthesize analogs of curcumin that have the safety profile of curcumin but with an improved pharmacokinetic behavior.

2.3.3 Acyclic Mannich bases

The Mannich bases initially prepared in our laboratory have a conjugated styryl group attached to a keto function. These compounds had only one conjugated unsaturated keto group, Figure 2.3. A large number of compounds derived from these bases were synthesized with different functional groups. When the acyclic Mannich compounds were evaluated for biological activity, they showed high cytotoxicity against cancer cells (Dimmock and Taylor, 1975; Das et al., 2009). As anticipated, they also showed high rates of reactions with a model thioalkyl compound

(Dimmock et al, 1980). However, when tested *in vivo* they had high murine toxicity (Dimmock et al., 1991) which led to alternate strategies being implemented.

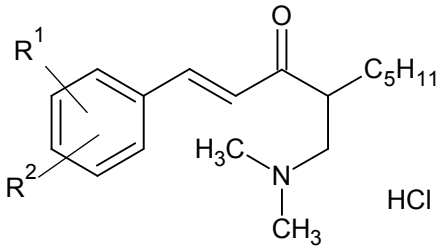


Figure 2.3: Structure of acyclic Mannich bases

2.3.4 Cyclic Mannich bases

The incorporation of the acyclic component of acyclic Mannich bases into a piperidine ring limited the flexibility possible with the acyclic Mannich compounds. Such a limitation was hoped to reduce the toxicity probably caused by certain conformations of the acyclic agents. Based on such a hypothesis, cyclic Mannich bases were synthesized. In addition to the limited flexibility, cyclic Mannich bases with two instead of one conjugated styryl keto groups were prepared, vide infra (Figure 2.4). Although less cytotoxicity was observed for the cyclic Mannich compounds compared to acyclic compounds (Figure 2.4), they still retained appreciable cytotoxic activity (Dimmock et al., 1990).

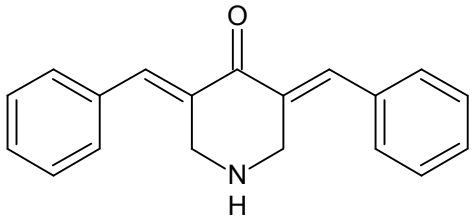


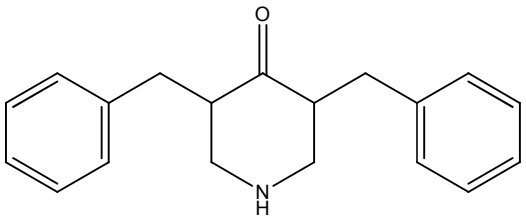
Figure 2.4: Structure of a cyclic Mannich base (compound **1**)

In a detailed study showing the activity of a wide range of compounds, the following conclusions were drawn. Compared to compound **1**, the lack of flexibility in the cyclic analogs lowered the cytotoxic potency; however it also reduced *in vivo* toxicity. Cyclic or acyclic compounds having a quaternary ammonium group resulted in high potency. However, a disadvantage was their DNA binding capability. This could result in mutagenicity (Dimmock et al., 1990)**.**

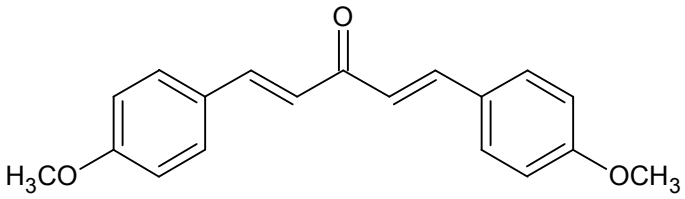
2.4 Some structure-activity relationships

2.4.1 The central ring

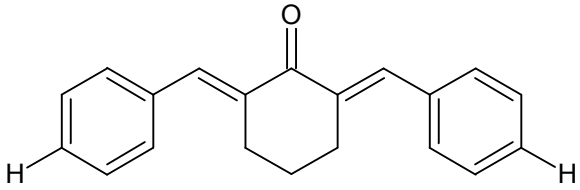
The cytotoxic potencies of some acyclic Mannich bases were higher than curcumin itself. Two factors might have affected the potency. The distance between the two aryl rings in curcumin is longer than the distance between same aryl rings in compound **1** (Adams et al., 2004). In addition, the different substitutions on the aryl ring likely have some influence on the activity.



Compound **1**



Compound **2**



Compound **3**

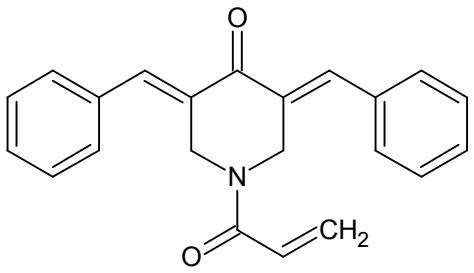
The replacement of the secondary amino group in compound **1** by a methylene group in **3** caused a significant decrease in cytotoxic potency. However, **3** was still appreciably potent. Compound **1** showed limited activity *in vivo* but it did not cause any animal mortalities. This is probably due to the reduced hydrophilicity compared to the acyclic Mannich bases and the accumulation of the agent in adipose tissue (Dimmock et al., 1990).

2.4.2 N-Acyl groups

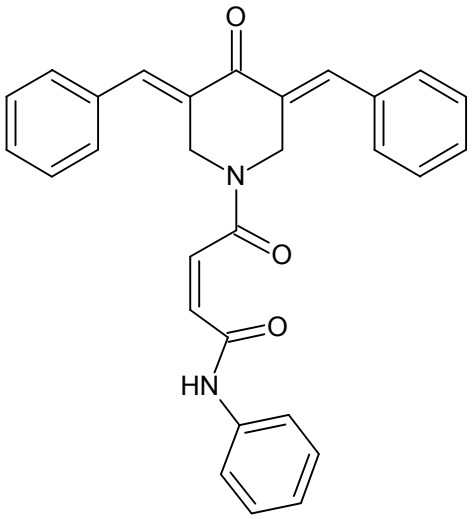
After establishing the activity of the 3,5-bis(benzylidene)-4-piperidones, some side chains were proposed to be placed on the piperidyl nitrogen atom. As explained above, the high lipophilicity was likely a major reason for the limited *in vivo* activity. Therefore, introducing a hydrophilic group at this site might increase the activity *in vivo* (Lipinski et al., 2001). In addition, it was proposed that these substitutions may

interact at an auxiliary binding site. Although this latter hypothesis was not proven, such side chains had a significant effect on the potency (Dimmock et al., 1992). For example, compound **1** with no N-acyl group and **4** with an added N-acyl group, differed significantly in their cytotoxicity against L1210 leukemia cells. Generally, N-acyl substitution caused a significant increase in potency on a wide range of cell lines. This is probably due to the introduction of an additional conjugated system capable of reacting with cellular thiols (Dimmock et al., 2001).

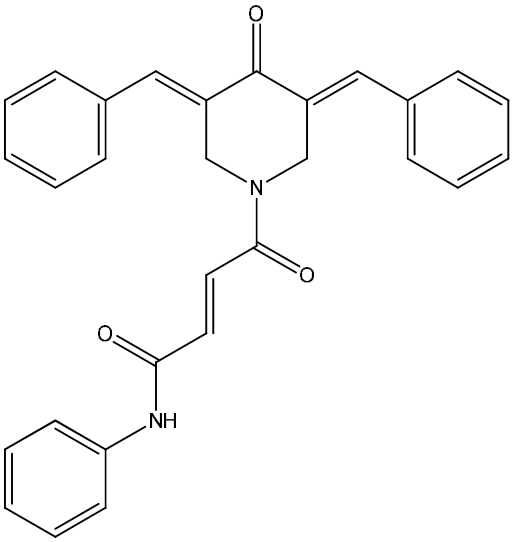
In addition, it was also proposed that the torsion angles (θ) between the aryl ring and the adjacent olefinic group has may some have impact on cytotoxic potency. In the case of compound **4**, the introduction of an acyl group caused about a 24% increase in the torsion angle compared to the non-acylated analog (Dimmock et al., 2001).



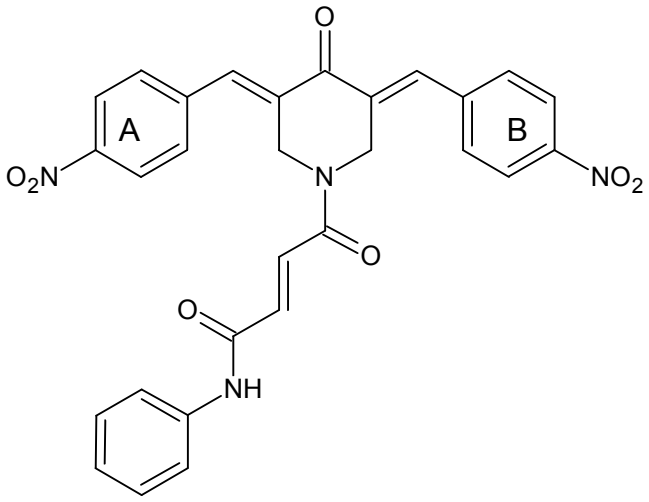
Compound **4**



Compound **5** (Z conformation)



Compound **6** (E conformation)



Compound **7**

The difference in potencies between compound **5** and **6** (Jha et al., 2007) are as follows. When evaluated against human Molt 4/C8 and CEM T-lymphocytes and murine L1210 leukemia cells, the results have shown the greater potency of compounds possessing the Z conformation in compound **5** rather than **6** in which the double bond in the N-acyl group adopts the E conformation (Jha et al., 2007). This behavior was generally observed in all related compounds. This implies that the side chain is in a preferred location in relation to the substituted piperidine ring.

The introduction of the side chain in compound **6** led to a great increase in potency compared to compound **1** (Jha et al., 2007). Introducing a nitro group on rings A and B also caused a greater increase in potency for compound **7** compared to compound **6** (Jha et al., 2007). This might be due to two reasons. Introducing an electron-withdrawing group would result in decreasing the electronegativity of the olefinic groups adjacent to the aryl rings which might cause a stronger electrophilic attack

with thiols. In addition, having a nitro group at such positions might cause some hydrogen bond formations with a binding site.

2.4.3 Substitutions on the aryl rings

Some derivatives of compound **1** were evaluated. All analogs had the same core structure except for the substitutions on the aryl rings. The insertion of substituents on the aryl ring caused different effects depending on the group added. The chloro and the methoxy substitutions caused a great decrease in potency when screened against HSC-2, HSC-4 and HL-60 cell lines. Other substitutions such as nitro caused an increase in potency (Pati et al., 2008).

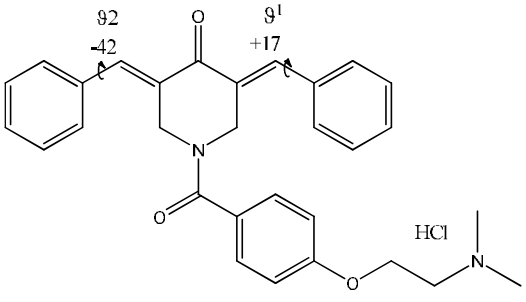
2.5 NC 2067 and NC 2081

After showing that compounds having the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore are potent agents targeting cancer cells, we were interested in studying the mechanisms by which these agents exert their biological activity. In this regard we chose two representative compounds designated **NC 2067** and **NC 2081**. A brief description of their biological and chemical characteristics is presented here.

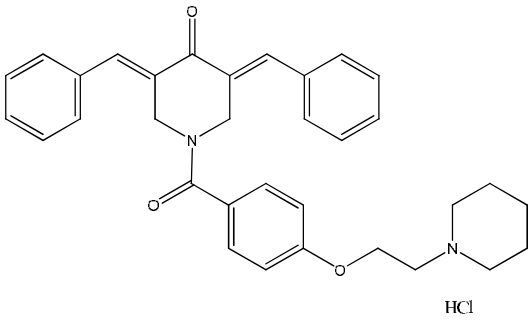
As explained above, a wide range of compounds were evaluated and relatively clear ideas of the structures required for optimal activity were drawn. Therefore, these observations lead to the synthesis of compounds **NC 2067** and **NC 2081**. **NC 2067** and **NC 2081** were evaluated here as two representative agents. Other compounds with similar structures might have the same or better cytotoxic potencies. However,

NC 2067 and **NC 2081** already have established biological activities. In addition, using ChemBioDraw program (version 12), the calculated logP (clogP) value for both compounds ranged between 4 and 5 and this is suitable for the biological activity (Lipinski et al., 2001). The logP value is used to determine the lipophilicity (or hydrophilicity) of a compound. Instead of measuring logP experimentally, clogP is determined using some programs. Both agents had no substitutions on the aryl rings. We wanted to establish the activity of agents lacking aryl substitutions as a first step. Later, other agents with substitutions can be evaluated. Both compounds have a basic group at their side chain, namely dimethylamino and 4-morpholinyl substituents. In addition, the torsion angle (□) between the enone group and the aryl rings varied significantly. The effects of these differences were studied.

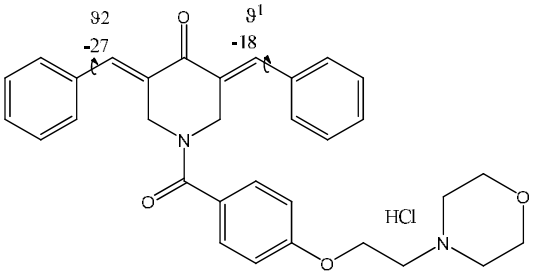
A detailed study using both compounds and other analogs revealed some interesting structure-activity relationships. When comparing the activity of **NC 2067** with **1**, the activity of **NC 2067** was either double or similar potency compared with **1**, depending on the cell line. These results encouraged the synthesis of other derivatives such as **NC 2081, 8 and 9** with different substitutions. The activities of these compounds were increased when compared to compound **1** (Das et al., 2007).



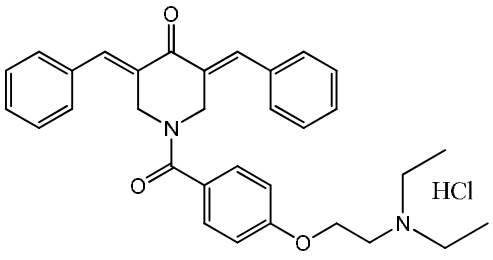
NC 2067



Compound **8**



NC 2081



Compound **9**

It is noticed that the 4-nitro substitution on the arylidene aryl rings caused a remarkable increase in potency in the analogs of **NC 2067, 8, 9** and **NC 2081** compounds. These latter compounds had similar IC_{50} values against Molt 4/C8 and CEM cell lines despite the difference in their side chains.

In general, potency was favored when a strong basic group was found at the side chain and methyl or nitro substituents were placed on the aryl rings.

Using different colon cell lines, namely COLO205, HCT-166, HCT-15, KM12, SW-620 and HCC-2998 the activity of the same compounds was also measured. Compound **NC 2081** generally showed high potency against some of these cell lines and the average IC_{50} values were lower than that of other compounds including the parent compound **1**. Unfortunately, compound **NC 2067** was not tested on these cell lines (Das et al., 2007).

2.6 Mannich–chalcones as a potential prodrugs

Using a different approach, Mannich bases were combined with a known cytotoxic group namely chalcones. The basis for this is the possibility of these agents to liberate 3,5-bis(benzylidene)-4-piperidones and a chalcone (Figure 2.5). Both of these groups of compounds are known to have cytotoxic properties (Ducki, 2007). The complexes formed had only limited potency which could have been due to the large bulkiness of the molecules or difficulty in penetrating the cell membrane. In addition, the smaller side chains in these compounds did not have strong potency either (Dimmock et al., 2002).

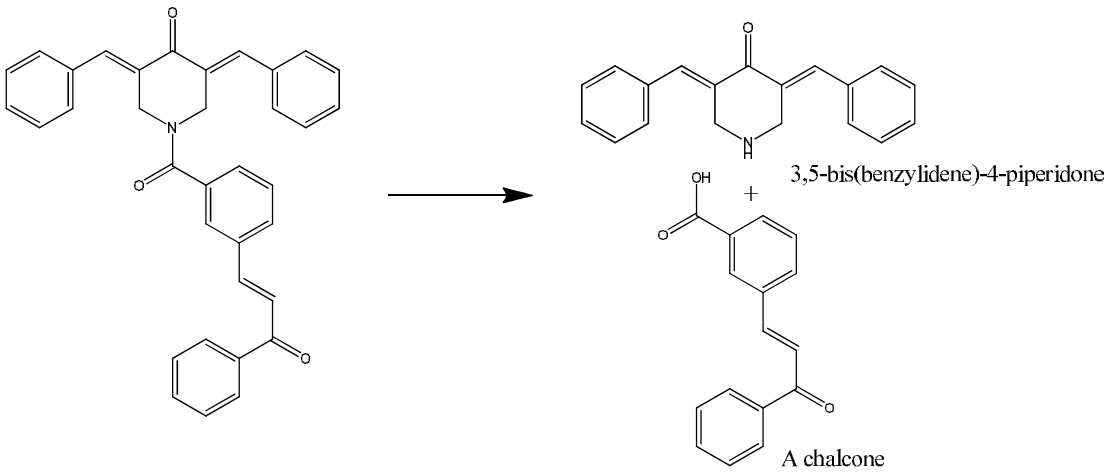


Figure 2.5: The proposed dissociation of a Mannich-chalcone compound to a cytotoxic Mannich base and chalcone intracellularly.

2.7 Mitochondria

Recently, interest in mitochondrial targeted therapy has been shown. Targeting mitochondria can be accomplished via different pathways. For example, targeting the mitochondrial DNA, mitochondrial metabolic pathways, mitochondrial membrane has been suggested and reveals promising results (Armstrong, 2006). In this work we are interested in studying the effect of some compounds on mitochondria in a detailed manner. Previously, some work was performed to evaluate some unsaturated compounds synthesized in Dimmock's lab on mitochondrial functions (Dimmock et al., 1976). In concert with this, we intend here to further study the effect of unsaturated compounds on the mitochondria. Therefore, in this section, a complete

description of the mitochondrial function and importance to cancer cells will be attempted. This will explain our rationale in targeting this organelle.

2.7.1 Anatomy and physiology of mitochondria

The mitochondrion is a spherical shaped organelle distributed in the cytosol. It is composed of three main parts. First the external mitochondrial membrane encloses the mitochondrion large pores that would allow molecules having molecular weights of 5000 Dalton or less to cross freely from cytoplasm to the inner space. Proteins and phospholipids are the main components found in the outer membrane. Second, the inner mitochondrial membrane is located beneath the outer membrane. The inner membrane is folded to increase the area in which oxidative phosphorylation occurs. Third, there is space between both membranes which is called the inter membrane space.

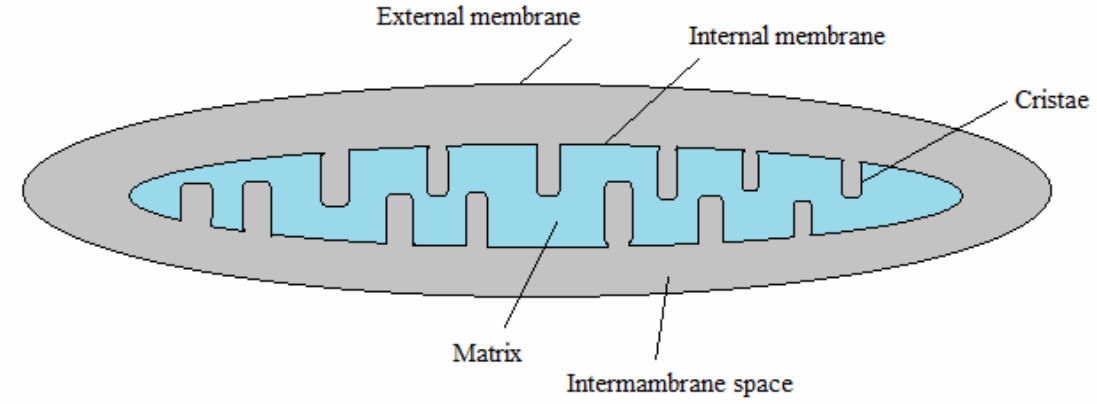


Figure 2.6: Structure of mitochondria

The electron transport chain is one of the most important biochemical cascades occurring in cells. The protein complexes involved in this chain are located in the

mitochondrial inner membrane (Rouslin, 1983) A proton concentration gradient across the inner membrane is generated while electrons from NADH and succinate are shuttled from complexes I and II through complex III and finally complex IV. Complex V (ATP synthase) uses the difference in proton concentrations to generate ATP (Ide et al., 2001; Aloysius et al., 2010).

2.7.2 Mitochondria and apoptosis

Apoptosis is a process of programmed cell death in which the cells commit suicide. This mechanism is necessary for healing, growth, embryo development and other physiological events (Elmore, 2007; Greenhalgh, 1998). Abnormal apoptosis may result in various diseases including cancer, neurodegenerative and cardiovascular diseases and other health problems (Lowe and Lin, 2000; Mattson, 2000). In the past decade, researchers have been able to identify the mechanism of apoptosis in detail (John, 2000). It has been observed that the mitochondrion has a substantial role in this process.

Apoptosis mainly depends on caspases. They are involved in a cascade of events which happen in a sequential manner so that once activated the cell usually dies. The activation of these proteins can occur due to several factors such as DNA damage, extrinsic signals, and mitochondrial membrane opening which are all different mechanisms for initiating apoptosis. Each one of these processes has the ability to stimulate the cascade (Figure 2.6).

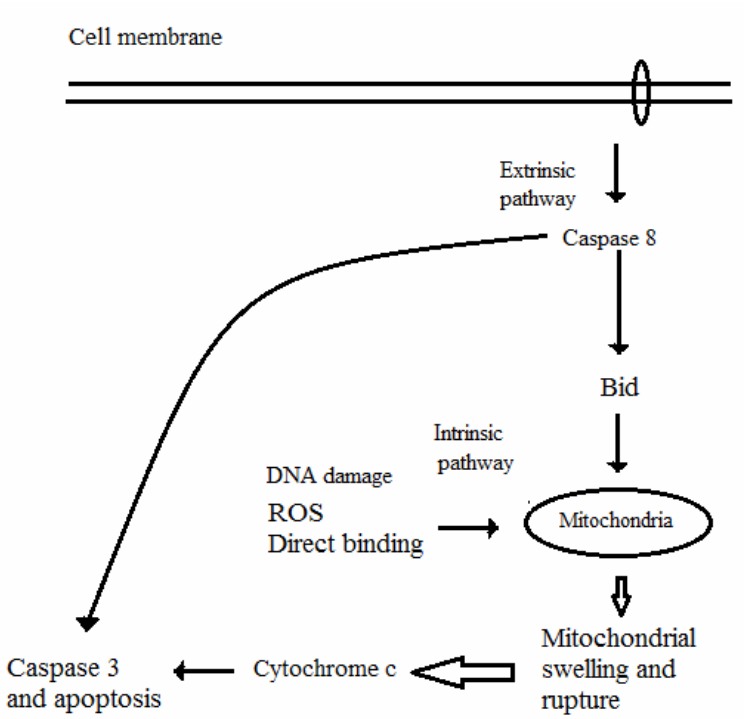


Figure 2.7: Initiation of apoptosis. The critical involvement of mitochondria is presented here. Apoptosis can be triggered by an intrinsic or extrinsic pathway. The extrinsic pathway is activated when ligands bind to Fas receptors causing an initiation of a cascade of caspases which will lead to apoptosis. The extrinsic pathway may or may not involve the mitochondria. The intrinsic pathway may be triggered when damage occurs to an important compartment of the cell. DNA damage, high ROS levels, mitochondrial damage by direct insult can all lead to intrinsic apoptosis initiation (Green and Reed, 1998).

Mitochondria play a very significant role in apoptosis. That is DNA damage, oxidative stress, Ca^{+2} overload and ATP depletion (Brookes et al., 2004; Roy et al., 2008) can all lead to a mitochondrial effect that leads to apoptosis.. These incidents cause an increase in the mitochondrial membrane permeability (Crompton, 1999) which results in the swelling of the mitochondrial matrix causing the opening of pores through the outer membrane and release of cytochrome c (Green and Kroemer, 2004). This will result in the activation of all caspases and induces apoptosis. These

processes can occur due to covalent binding of compounds with some critical thiols. These thiol groups are found in important cysteine residues in proteins regulating the permeability of the mitochondrial membrane. For example, alkylation occurring at the thiol group in Cys 56 in the adenine nucleotide transporter (ANT) protein results in the opening of the pores which lead to the entrance of solute (water) causing rupture of the mitochondria (Costantini, et al 2000).

The permeability of the mitochondrial membrane is regulated by the permeability transition pore (PT) located on the outer membrane of the mitochondria. This pore is composed of three main proteins: cyclophilin D (cyp D) found in the matrix, ANT found in the inner membrane, and the voltage dependent anion channel (VDAC) found in the outer membrane (Green and Kroemer, 2004). Figure 2.7 shows the structure of the pore.

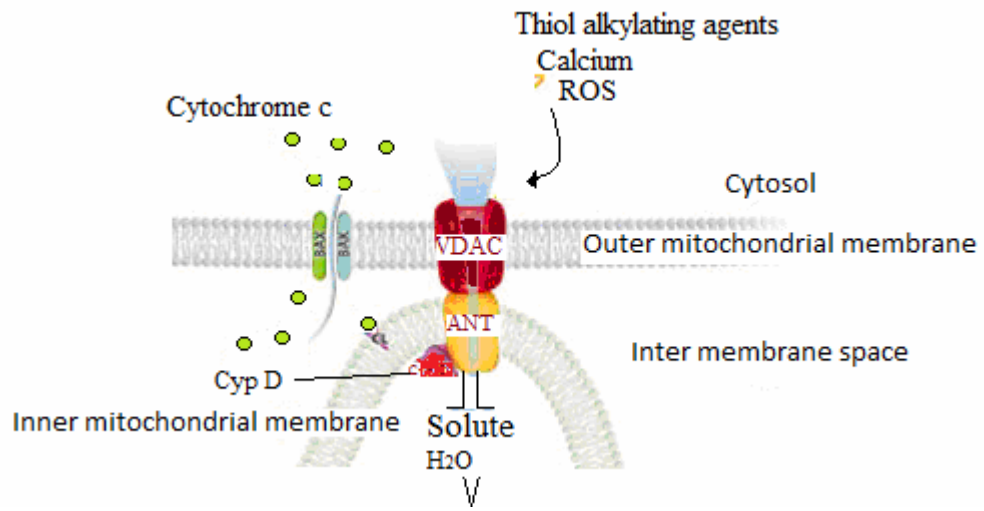


Figure 2.8: The structure of the permeability transition pore and the mitochondrial membranes. The structure includes VDAC, ANT and cyp D. Thiol alkylating agents, calcium overload and reactive oxygen species (ROS) are three main agents that affect the VDAC function (Green and Kroemer, 2004).

2.7.3 Differences between mitochondria in cancer and non-malignant cells

In order to design agents that have the ability to target cancer cells only, it is important to study the differences between both malignant and non-malignant cell types. After studying the biochemistry of cancer and normal cells, a large number of differences were noted such as differences in the proteins expressed on the cell membrane, inside the nucleus and in mitochondria. Here, I present the most significant ones related to mitochondria.

Cancer cells are able to survive in hypoxic conditions. Several pathways have been proposed to accomplish this, for example by activation of the hypoxia-inducible factor 1 (HIF-1). These proteins are overexpressed when the cells are in a hypoxic environment (Ke and Costa 2006). The proteins cause an increase in the glycolytic pathway flux which implies less dependence on oxidative phosphorylation (Ke and Costa 2006). Hypoxic conditions also induce the expression of the vascular endothelial growth factor (VEGF) (Ke and Costa 2006). This protein causes the growth of blood vessels towards the cancer cells. The enzyme hexokinase II also has an important role in cancer cells. This overexpressed enzyme catalyzes the rate limiting step in glycolysis which phosphorylates glucose. As a result, glycolysis is over activated in tumors (Chen et al., 2009). In cancer cells, these enzymes are associated with the mitochondrial membrane and use the ATP synthesized in the mitochondria to produce glucose-6-phosphate. The actions are preferred because

cancer cells tend to inhibit the oxidative phosphorylation pathway and induce glycolysis (Ralph and Neuzil, 2009).

Protons are pumped across the inner membrane to the inter membrane space. These positively charged particles cause a difference in potential across the membrane called the mitochondrial membrane potential (Chen, 1988). This potential is the reason for the accumulation of positively charged lipophilic molecules in the mitochondrion. Interestingly, the potential across the membrane in cancer cells is higher than normal cells by up to 60mV (Chen, 1988; Modica-Napolitano and Aprille, 1987). This would cause the attraction of positively charged lipophilic agents more towards cancer cells than normal ones. The exact mechanism for such an increase in cancer cells is unknown. Some proposed explanations include the variations in the respiratory complexes, ATP synthases or even proton conduction.

Lactic acid is the end product of the glycolysis process in the absence of oxygen. Cancer cells tend to increase the production of lactic acid by various means (Yamagata et al., 1998) which are required to kill normal cells and facilitate metastases (Gatenby and Gillies, 2004). However, in order to survive in such conditions, they overexpress proton pumps which keep protons outside the cells. Targeting these pumps can caused great damage to cancer cells (Morimura et al., 2008; De Milito and Fais, 2005).

The lower dependence on the electron transport chain causes oxygen accumulation. This can lead to superoxide $O_2^{\cdot -}$ production. Although reactive oxygen species cause

a great deal of damage to cellular DNA and proteins, cancer cells intentionally keep these compounds in high concentrations by the continuous inhibition of oxidative phosphorylation. This state is called oxidative stress (Pelicano et al., 2004). Reactive oxygen species (ROS) are important in cancer cells as they cause further DNA mutations whether in the nucleus or in the mitochondria. In addition, ROS functions as an intracellular signal (Waris and Ahsan, 2006). ROS should always remain under certain threshold concentrations. Exceeding these levels, by for example, inhibiting the electron transport chain, will cause cell death by inducing apoptotic proteins. It was recognized that agents which cause an increase in the ROS levels induce cancer cells to die. Examples of these agents include betulinic acid and menadione (Sasaki et al., 2008). These agents can target one of the respiratory complexes, the mitochondrial inner membrane or DNA polymerase γ in mitochondria (Ralph and Neuzil, 2009).

A fifth difference between normal and cancer cells is the overexpression of antioxidant enzymes (Pelicano et al., 2004; Trachootham et al., 2009) and GST (Laborde, 2010) in cancer cells. As explained in the section outlining some of the differences between cancer and normal mitochondria, cancer cells are in a state of continuous oxidative stress (Schumacker, 2006). Cancer cells manage to adapt to such an increased stress by overexpressing antioxidant proteins and peptides such as GSH (Pelicano et al., 2004; Trachootham et al., 2009) as well as GST (Laborde, 2010; Wang et al., 2009; Wang et al., 1997). Their overexpression is at the levels required to scavenge the high levels of ROS (Pelicano et al., 2004; Trachootham et

al., 2009). The enzyme GST can also help to decrease oxidative stress by reducing electrophiles such as aldehydic lipid peroxidation products (Hayes and Strange, 1995) and by influencing cellular redox homeostasis (Tew, 2007). On the other hand, non-malignant cells have normal antioxidant enzyme levels that keep cells in normal stress conditions with minimal ROS accumulation (Pelicano, 2004). A slight inhibition of antioxidant enzymes will lead to a greater increase in ROS in cancer cells (Pelicano, 2004). Unlike cancer cells, a slight inhibition of antioxidant enzymes in normal cells means that these cells still retain antioxidant enzyme levels significantly higher than is needed to scavenge the ROS . Hence inhibition of antioxidant enzymes and GST emerges as a potential therapeutic strategy for designing anticancer agents (Pelicano, 2004).

The GST enzyme family is present in at least 8 classes (Ricci et al., 2005; Trute et al., 2007). The GST enzyme has two main domains. The G-site, which is the hydrophilic N-terminal that binds glutathione and the H-site, which is the hydrophobic C-terminal side that binds to the substrate (Tew and Gate, 2001). A tyrosine amino acid is very important for the activity since it accepts a proton released by the thiol glutathione. Replacing this amino acid by phenylalanine, for example, greatly reduced the activity of the enzyme (Tew and Gate, 2001). GST classes differ in their amino acid sequences at the H-site, causing a variation in substrate specificities (Tew and Gate, 2001). These differences might be used to design compounds that target a specific isozyme.

Chemotherapy agents were found to have high efficiency but their use is compromised by certain shortcomings. One of the main issues regarding the available chemotherapeutic agents is the development of resistance. Cancer cells can employ different mechanisms to accomplish this. Increasing the expression of the targeted protein is one mechanism. A second approach is changing the conformation of the protein. The third approach which is the most commonly employed approach is the overexpression of the transporters, called ATP-binding-cassettes (ABC), which would efflux chemotherapeutics from the cell (Gottesman, 2002).

Despite these issues, chemotherapy remains the most important approach. Therefore, restoring the chemosensitivity of tumor cells to chemotherapeutics might be one approach to enable these agents to restore their effectiveness. Depending on the mechanism of the resistance developed, different approaches have been employed. For example, increasing the dose administered may help overcome the problem of highly expressed proteins (Basholt, et al 1996)**.** However, this is limited by the toxicity seen when high doses are used. Changing the conformation of the protein targeted can only be solved by employing different agents that target different proteins. This would explain the need for various agents for the treatment of cancer (Golan and Tashjan, 2012). ABC overexpression can theoretically be overcome by inhibiting these transporters. In order for ABC transporters to efflux a compound, a conjugation should occur between the toxin and GSH via GST. Therefore, most cancer cell lines overexpress GSTπ compared to normal cells (Laborde, 2010; Mannervik et al., 1987). GST plays an important role in drug resistance (Cui et al.,

2008; Townsend and Tew, 2003; van Bladeren and van Ommen, 1991). GST catalyzes the conjugation of GSH with a cytotoxic agent and subsequently the GSH conjugate can be effluxed from the cells (Cui et al., 2008). The role of GST in drug resistance makes it an attractive chemotherapeutic target (Tew and Gate, 2001; Townsend and Tew, 2003; Laborde, 2010), and inhibition of GSTπ has induced cell death selectively in cancer cells (Cui et al., 2008).

In addition, it has been noticed that the inhibition of the GST enzyme has been shown to reduce resistance to chemotherapeutic agents (Balendiran et al., 2004). Attempts to inhibit these proteins have received great success *in vitro* (Balendiran et al., 2004; Cui et al., 2008; Garcia-Rui. et al., 2000; O'Dwyer et al., 1991).

Among other sources, ROS is produced in the electron chain transport which is located in mitochondria. Therefore, it can be assumed that an increase in mitochondrial ROS levels will have its first effect on the mitochondrial membrane. GST works in conjunction with other ROS scavengers to reduce the electrophiles (including ROS), Figure 2.8.

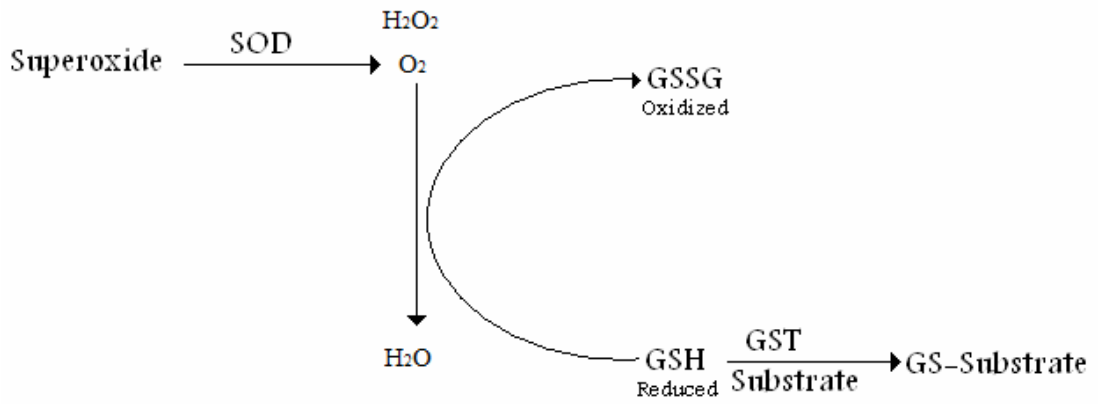


Figure 2.9: A general scheme showing the role of GST in detoxification mechanisms.

2.8 GST inhibition

Most tumor cells have higher GST than the corresponding normal cells. Therefore, GST inhibition may be more detrimental to tumors than normal cells. One example of agents having the potential ability to inhibit GST is those with the α,β-unsaturated keto group.

Our limited knowledge of the active site of GST has made it difficult to design agents that selectively and strongly bind to a specific class (Mahajan and Atkins, 2005). The electrophilic nature of GST substrates is a common phenomenon. This electrophilic group attacks the electron rich sulfur atom in GSH. Although GST can facilitate this reaction, it might also occur in the absence of the enzyme. As an example, chlorambucil forms an ion in cells. This is quenched by GSH which attaches to the electron deficient carbonium ion of the drug to prevent it from reacting with DNA. The reaction is a typical electrophilic-nucleophilic reaction, Figure 2.9 (Tew and Gate, 2001).

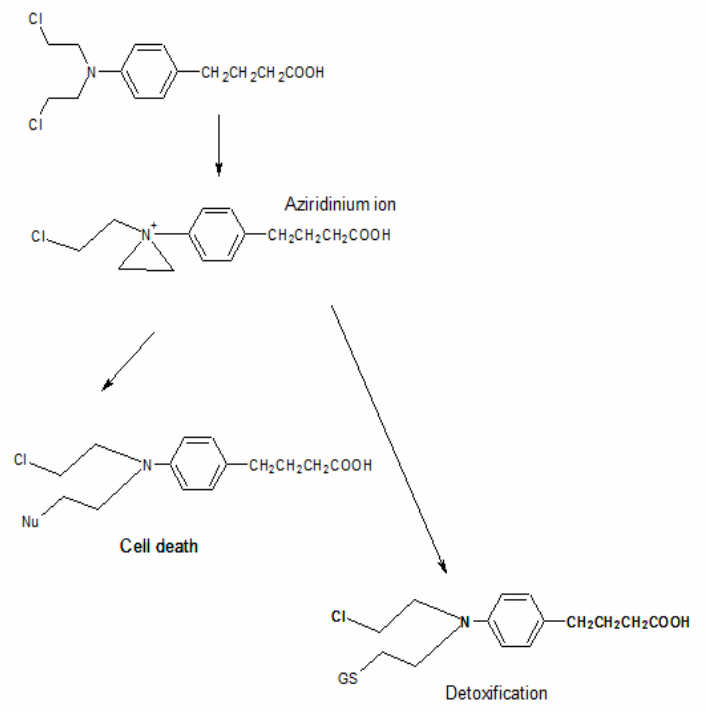
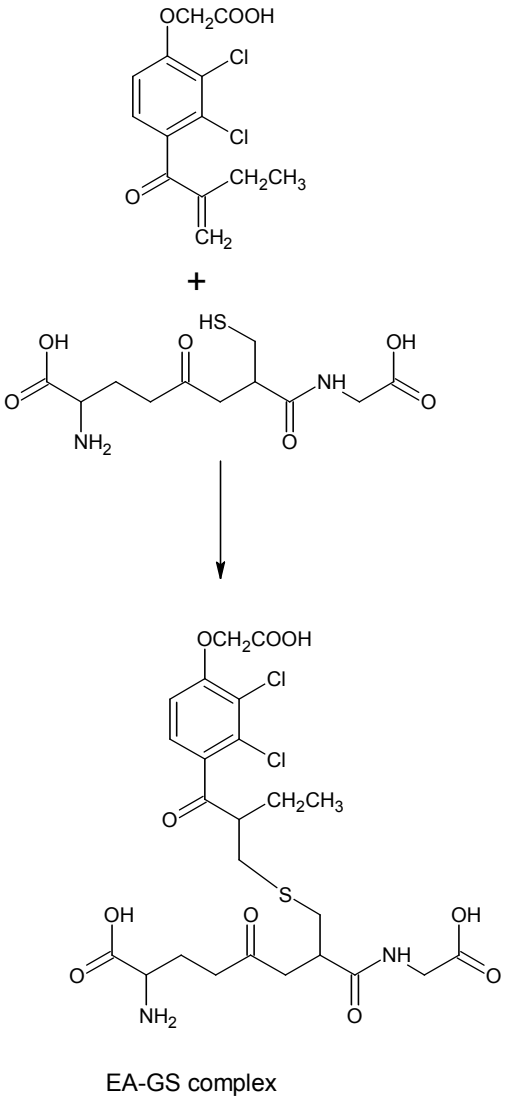


Figure 2.10: Mechanism of chlorambucil binding with GSH

Ethacrynic acid, a loop diuretic (Goldberg, 1966), was found to have GST inhibitory activity (Ploemen et al., 1993). It has been shown to inhibit GST –α, -π and -μ. Ethacrynic acid can exert such inhibition by binding to the active site, competitively and non-competitively. Non-competitive binding occurs when an electrophilic attack takes place (Ploemen et al., 1990; Tew et al., 1997; Zhao and Wang, 2006; Tew and Gate, 2001). It is also known for its depletion of GSH by conjugating its alkene group to the thiol group via a Michael addition, Figure 2.10. Such conjugation may

occur spontaneously or mainly with GST catalytic activity (Esterbauer et al., 1991; Lagisetty et al., 2010; Zhao and Wang, 2006).



EA-GS complex

Figure 2.11: Formation of the complex between GSH and ethacrynic acid (EA)

In order to study the importance of structural features, the binding of ethacrynic acid and its derivatives to GSTπ has been undertaken. It was noticed that the replacement of

the chloro atom by methyl, bromo or fluoro substituents at position 3 of the aryl ring did not have any effect on the GSTπ inhibition (Zhao and Wang. 2006).

Other agents which inhibit GST are known as glutathione analogs. γ-Glutamyl-benzylcysteinylphenylglycyl diethyl ester (TLK199) inhibits GST and particularly GSTπ. TLK199 can also inhibit the multidrug resistance-associated protein-1 (MRP-1) (Zhao and Wang, 2006).

Studies of the binding of a series of chalcone derivatives to GSTπ revealed differences in their binding ability depending on the chemical structure. The majority of the compounds inhibited GSTπ. Interestingly, these chalcone derivatives have a carboxylic acid group attached at the α position of the unsaturated ketone. A Michael addition reaction is expected to occur between the olefinic group and the thiol moiety at the H-site of the enzyme (Wang et al., 2009). From studying the compound-GST interactions, it is clear that strong interactions are occurring with the carboxylic acid. Moderate interactions are noted at the α,β- unsaturated carbonyl group and the hydrophobic fields (Wang et al., 2009). Methyl and methoxy groups found at the aryl ring (R1) seem to have minimal effect on the activity (Figure 2.11).

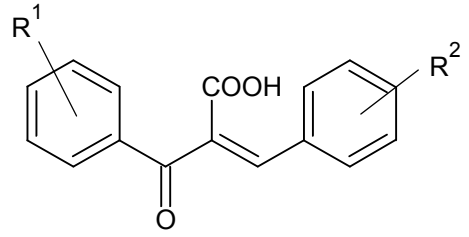
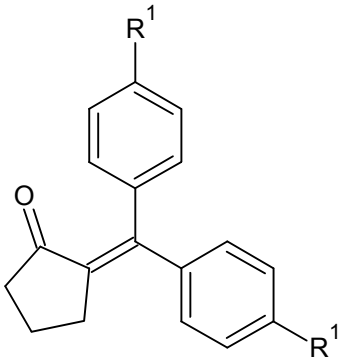
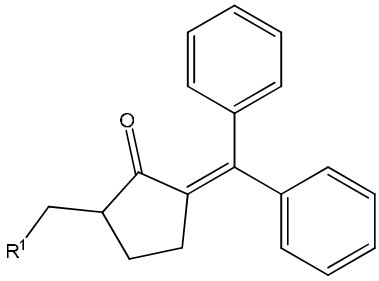


Figure 2.12: Structure of chalcones

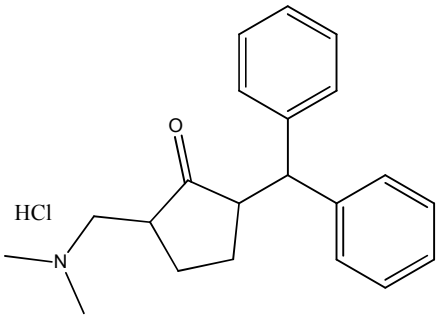
Various different Mannich compounds were synthesized and evaluated for their activity against GSTπ. Compounds **10a** and **10b** did not show any significant effect. These agents have no 2-alkylaminomethyl group on the cyclopentanone ring (Wang et al., 2005). Compounds which have a dimethylaminomethyl and 4-morpholinylmethyl groups at position 2 of the cyclopentyl ring (**11a** and **11b**, respectively) had greater ability to inhibit GSTπ than **10a**. This would imply the importance of the aminomethyl group on the cyclopentyl ring (Wang et al., 2005).



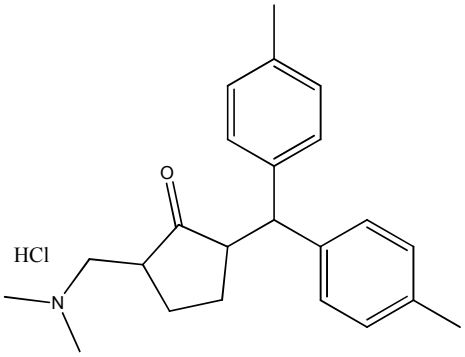
Compound **10a**: R^1= H **10b**: R^1=CH_3



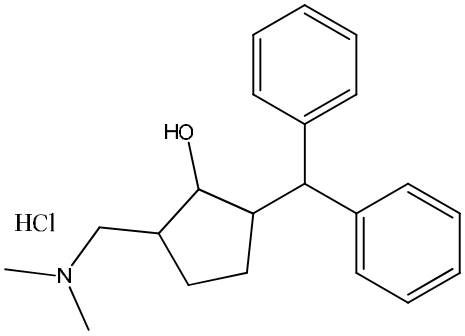
Compounds **11a**: R^1= dimethylamino **11b**: R^2= 4-morpholinyl **11c**: R^3= 1-piperidyl **11d**: R^4= 1 –pyrrolidinyl



Compound **12a**



Compound **12b**



Compound **13**

Compound **11a** with the dimethylaminomethyl group had the highest potency in reducing GSH levels compared to derivatives **11b, 11c** and **11d** which contained 4-morpholino, pyrrolidino, or piperidino-methyl groups (Wang et al., 2005). The reduction of the ketone group in compound **12a** to compound **13** reduced the inhibitory activity (Wang et al., 2005). It is noticed that compounds **10a** and **13** have limited ability in inhibiting GSH. This reveals the importance of the 2-alkylamino structure and the cyclopentanone group in these compounds (Wang et al., 2005). Comparing the activities of compounds **11** and **12** reveal that the α,β-

unsaturated bonds are not crucial for the activity since both derivatives have the same GST inhibiting ability (Wang et al., 2005).

2.9. Rationale

The presence of an electrophilic group on the unsaturated compounds presents them as a potential target for the thiol group in GST enzyme and GSH peptide (Shiraki, et al, 2005). Both are elevated in most cancer cells compared to normal cells. The inhibition of GST or the depletion of GSH will indirectly increase ROS levels. In addition, the production of ROS may increase by inhibiting one of the electron transport chain complexes. These complexes contain some thiol groups (Cortes-Rojo et al., 2011) which can be alkylated by the unsaturated ketones. Based on this rationale, we established our hypothesis and objectives.

3. HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

a. **NC 2067** and **NC 2081** inhibit GST by acting as a competitive substrate, which in turn, depletes cellular GSH content and increases ROS levels.
b. **NC 2067** and **NC 2081** exhibit selective cytotoxicity to colon cell lines.
c. **NC 2067** and **NC 2081** interfere with the electron transport chain to affect mitochondrial membrane function leading to cellular death.

3.2 Objectives

Objective 1: To establish the activity of two agents, namely, [4-[2-dimethylaminoethoxy]phenylcarbonyl]-3,5-bis[phenylmethylene]-4-piperidone hydrochloride (**NC 2067**) and 1-[4-{2-[4-morpholinyl]ethoxy}phenylcarbonyl]-3,5-bis[phenylmethylene]-4-piperidone hydrochloride hemihydrate (**NC 2081**) against one colon cancer cell line (HCT-116). This was followed by an evaluation of their activity against one normal cell line (CRL-1790).

Objective 2: To evaluate the effect of both agents on the activity of GSTπ.

Objective 3: To investigate the effects of these agents on different mitochondrial processes including membrane potential, electron transport chain and mitochondrial swelling.

4. MATERIALS AND METHODS

HCT-116, CRL-1790 cell lines, McCoy's and Minimum Essential Media were all purchased from ATCC (Burlington, Ontario). 5-Fluorouracil, carbonylcyanide 3-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol, sulforhodamine B, JC-1, 1-chloro-2,4-dinitrobenzene (CDNB) and DMSO were all purchased from Sigma-Aldrich, (Oakville, Ontario).

4.1 Anti-proliferation assay

The purpose of this experiment was to determine the potency of both agents on HCT-116 and CRL-1790 cells. HCT-116 cells used for the cytotoxicity evaluation were between passages 42 to 44. Passages for CRL-1790 were between 10 and 12. The cells were cultured in their respective growth medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The colon HCT-116 cells were cultured in McCoy's medium while CRL-1790 cells were grown in Minimum Essential Media.

The method of the National Cancer Institute, USA for determining the IC_{50} values was used (Voigt, 2005). Cells were plated at $5x10^4$ cell/ml concentration in 100 µL

and left for 24h to attach. Then the agents were added using concentrations ranging between 0.0095-20 μM for the HCT-116 neoplasm and 0.06-50 μM for CRL-1790 cells and incubated for 48h. The experiment was conducted in triplicate on three separate occasions and standard deviations were less than 10%. The concentration of DMSO was 1% when HCT-116 was used and 0.5% for CRL-1790. 5-Fluorouracil (5-FU) was used as a positive control. Concentrations used for 5-FU ranged from 0.001 to 100 μM.

After 48h the cells were fixed on a plate in each well using 50 μL (50% w/v) trichloroacetic acid. Cells were incubated for 1 h at 4^{o}C. Then the cells were washed and left to dry. Sulforhodamine B (SRB) was used to monitor the change in cell viability. It was prepared first in 1% acetic acid at a concentration of 1mg/ml as a stock solution. Addition of 100 μL of this solution to cells in each well was performed and incubated for 10 min at room temperature. Cells were washed using 1% acetic acid and left to air dry. The remaining dye was dissolved in 200μL of 10 mM Trizma base. A Biotek spectrometer was used to measure the absorption at 515 nm. On day one when the drugs were added, one extra plate was treated with trichloroacetic acid and sulforhodamine B as indicated previously. This plate will be referred to as the Tz plate. It represents the number of cells on the day when the drugs were added. Cell growth was then calculated using the following formula:

$$\%\text{Cell growth} = \frac{OD_{sample} - OD_{Tz}}{OD_{control} - OD_{Tz}}$$

Three different cell batches were used and for each batch the experiment was conducted in triplicate.

4.2 Evaluation of ROS levels in cancer cells

The main intention behind performing this assay was to evaluate the effect of both agents on ROS levels. Dichlorodihydrofluorescein diacetate dye was used to determine such levels. The HCT-116 cells were cultured in 96-well plates. The number of cells seeded was $5x10^3$ and left for 24h to attach. Compounds were prepared in DMSO at 5 mM concentration. Dilutions were made using media to a concentration of 10 μM. Then, 94 μL media were added to 5 μL of the previous drug/media solution. By the addition of 1 μL DMSO, the required concentration will results in 1% DMSO. Some volumes adjustments were required for each compound. Then, the cells were incubated with IC_{50} concentrations of **NC 2067** and **NC 2081** (0.571 μM for **NC 2067** and 0.503 μM for **NC 2081**) for 48h. 5-Fluorouracil was used as the positive control (Hwang et al., 2007) using its IC_{50} value of 3.5 μM. Subsequently, dichlorodihydrofluorescein diacetate was added and incubated with the cells for 30 min at a final concentration of 5 μM. Once inside cells, it is hydrolyzed by esterases to produce 2',7'-dichlorodihydofluorescein which is then oxidized by ROS to produce a fluorescent product (2',7'-dichlorofluorecein) which is excited at 483 nm to emit light at a wavelength of 530 nm. Higher intensities indicate high ROS levels. The media was removed and the cells were washed twice with 100 μL phosphate buffered saline (PBS). This was performed to remove extracellular

probe, and to minimize interference from phenol red. PBS (100 μL) was finally added and the fluorescence was measured [excitation at 483nm, emission at 530nm] using a Biotek fluorometer. Three different cell batches were used and for each batch the experiment was conducted in triplicate.

4.3 Mitochondrial membrane potential

Our main intention behind this assay is to measure the effect of both agents on the mitochondrial membrane potential. For this purpose we used the JC-1 dye. The idea behind using this dye is that it forms monomers in cells with low mitochondrial membrane potential (unhealthy cells). It forms J-aggregates in the mitochondria of healthy cells which have a high mitochondrial membrane potential. Therefore, agents expected to have an effect of MMP will cause an increase in the monomeric form and reduce the J-aggregates. However, the formation of monomers is usually easier to detect compared to J-aggregates especially when the decrease in membrane potential is relatively minimal (Di Lisa et al., 1995). Three different cell batches were used and for each batch the experiment was conducted in triplicate.

The experiment was conducted based on previous publications (Salvioli et al., 1997; Cayman Chemical Company, 2011). HCT-116 cells $5X10^3$ cell/ml were cultured in 96-well black plates. The lower side was transparent. After letting the cells attach for 24 h the cells were treated with the IC_{50} concentrations of **NC 2067** and **NC 2081**. As explained above for compound preparation, the same steps were followed here.

Compounds were prepared in DMSO at 5 mM concentration. Dilutions were made using media to a concentration of 10 μM. Then, 94 μL media were added to 5 μL of the previous drug/media solution. By the addition of 1 μL DMSO, the required concentration results in 1% DMSO. Some volume adjustments were required for each compound. The concentration of CCCP and 2,4-dinitrophenol was 1 μM. They were used as positive controls. The JC-1 dye was prepared in the media as a stock solution at concentration 20 μg/ml. After 48 h, 10μL of JC-1 were added to each well and mixed. Cells were incubated for 30 min at 37^{o}C and 5% CO_2. The cells were centrifuged at 400x g and the media was removed. Phosphate buffered saline (PBS) (100 μL) was added and again centrifuged. This process was performed twice until there was complete removal of the excess dye. 100 μL PBS was added to each well. Fluorescence was measured [Ex 485 and Em: 535 for monomeric JC-1 detection] using a Biotek spectrometer.

4.4 GSTπ inhibitory activity

We intended to study to binding ability of both compounds to behave as substrates for GSTπ. Our preliminary experiments were performed based on the published method (Appiah-Opong et al., 2009). Pure GSTπ was from the Vaccine and Infectious Disease Organization-International Vaccine Centre, Saskatoon, Saskatchewan (VIDO-InterVac). GSH and CDNB were used to monitor its activity. A binding between GSH and CDNB in the presence of GST would result in the conjugate form that absorbs UV light at 340 nm. Unfortunately, both **NC**

2067 and **NC 2081** have maximum absorptions close to a wavelength of 340 nm. Therefore, we decided to employ a different approach. First, the absorption of 25 μM of **NC 2067** and 250 μM of GSH was determined. This results in a GSH:compound ratio of 10. Then GST was added to the solution making its final amount 1.2 μg. This amount was used based on previous data which showed the optimal rate of absorption decrease. The addition of GST was found to increase the volume by 2% and it had a minimal effect on the volume increase. The reduction in absorption over time was determined for both agents. After the addition of GST, absorption readings were taken after 0, 2, 4, 6 and 10 min until no change in absorption was seen. The same steps were followed for **NC 2081** except that the concentrations used were different. The absorption of 30 μM **NC 2081** and 300 μM GSH was determined. This still gave the same GSH:compound ratio of 10.

Solutions of GST and GSH were prepared in phosphate buffer pH = 7. Temperature was kept at room temperature. The drugs were first prepared in DMSO and the total DMSO in the mixture was 1%. The experiment was conducted in triplicate.

4.5 Oxygen consumption assay

We intend to study the effect of both agents on the rate of oxygen consumption. This can be measured by determining the change in oxygen concentration with time. After detaching all cells from a flask, $4x10^6$ cells/ml were prepared. The oxygen concentration was determined by instilling 1 ml cells in the oxymeter chamber to measure the oxygen concentration. The measurement was taken for 2 min. CCCP

was then added at concentration 5 μM. This was performed by preparing CCCP in DMSO at concentration 1 mM, then 5 μL was added to cells to obtain the right concentration. After observing the oxygen level changes for 5 min, one of the compounds was added. This was performed by preparing a stock solution of 1 mM in DMSO, then 10 μL to obtain the concentration of 10 μM which was followed by another 40 μL of the same stock as explained below. The effect of the agents was observed by indicating a change in the oxygen consumption rate. The initial concentration of **NC 2067** and **NC 2081** was 10 μM, subsequently an additional 40 μM was added. Three different cell batches were used and for each batch the experiment was conducted in triplicate.

4.6 Mitochondrial swelling assay

Our main rationale behind performing this assay was to establish the effect of both agents on mitochondrial swelling. This assay depends on the fact that once mitochondria swell more UV light passes through. After removing the liver from a rat, mitochondria were isolated from the liver using conventional differential centrifugation (Kowaltowski et al., 1996). Wistar male rats between 250 and 350g weight were used. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adherence to the Canadian Council on Animal Care guidelines fr humane animal use. Prior to the absorbance measurement, isolated mitochondria were diluted in swelling buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 5 mM potassium phosphate, 1 mM MgCl, pH 7.2, with 5 mM

succinate. In order to measure the amount of swelling in the mitochondria, the light scattered was determined at a wavelength of 520 nm. Mitochondria were suspended to an initial absorbance close to 1.5. A kinetic mode sampling the absorbance every 10 sec was performed to monitor changes in absorption. Once a steady absorbance line was noticed, Ca^{++} was added to induce swelling in the mitochondria at concentration of 50 μM. This resulted in a significant drop in the UV absorbance. After confirming the responsiveness of the isolated mitochondria, the effect of our compound on mitochondrial swelling was examined. 20μL of the suspension in 1 ml resulted in about 1.5 absorbance value. **NC 2067** and **NC 2081** were added separately at concentrations of 10μM and 50μM. The kinetic mode absorbance was recorded for 10 min while observing the change in its values. Two rats were sacrificed; the experiment was conducted in triplicate for each liver sample.

4.7 Statistics

One way ANOVA with Dunnett's multiple comparison post-hoc analysis was used to determine the significance of the effects between treatment groups and control. A p value below 0.05 was considered significant.

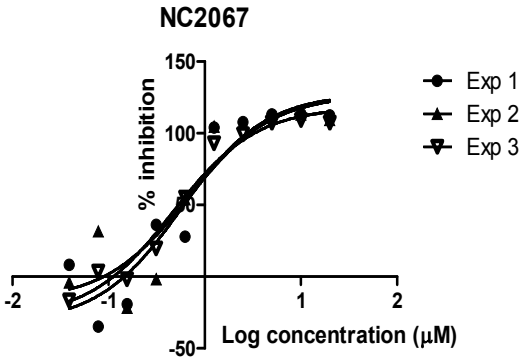
5. RESULTS

5.1 Anti-proliferative assay

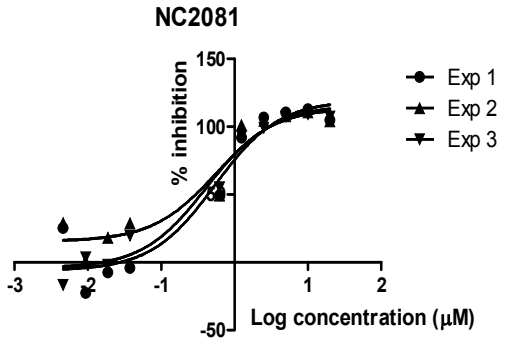
After conducting the antiproliferative assay we managed to plot a graph describing the effect of different concentrations on the proliferation of HCT-116 and CRL-1790. Anti-proliferative assay was performed using the SRB dye. Log concentrations were plotted against the percentage of inhibition for the IC_{50} calculations. A sigmoidal graph was observed. IC_{50} was determined at the concentration at which the effect is half between the maximum (100% inhibition) and the minimum (0% inhibition).

This would determine the concentration at which number of cells was reduced by half after the incubation period compared to control.

The anti-proliferative assay reveals the high cytotoxic potencies of both **NC 2067** and **NC 2081** against the human HCT-116 colon cancer cell line (Figure 5.1). The IC_{50} values were near 0.5 μM, and displayed more than 5-fold greater potencies than 5-FU (Table 5.1). On the other hand, both compounds showed weak growth inhibition against normal CRL-1790 cells (Figure 5.2 and Table 5.1). (The cell growth inhibition was 31% and 16% at concentrations 25 μM for **NC 2067** and **NC 2081**, respectively). IC_{50} for 5-FU against CRL-1790 could not be determined because a maximum inhibition of 100% was not reached. These IC_{50} values were used for ROS and membrane potential assays.



b.



c.

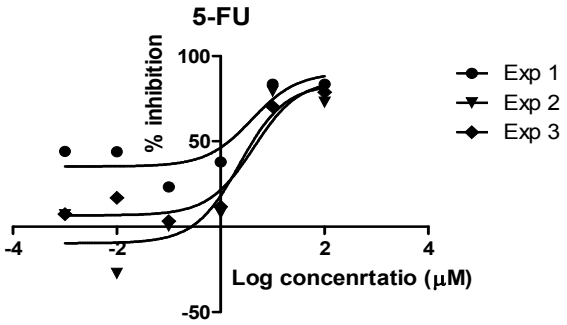


Figure 5.1: a: The anti-proliferative activity of **NC 2067** against HCT-166 cells b. The anti-proliferative activity of **NC 2081** against HCT-166 cells c. The anti-proliferative activity of 5-FU against HCT-166 cells. IC_{50} could not be determined form experiment 1. Only Experiments 2 and 3 were used to calculate the average.

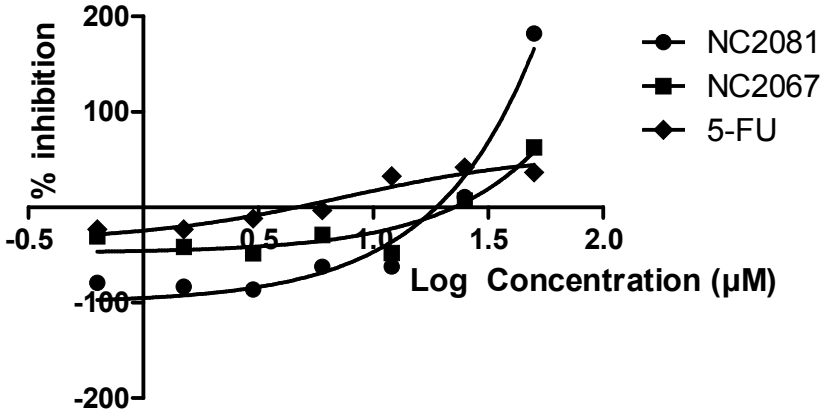


Figure 5.2: CRL-1790 cell growth inhibition related to concentration. Incubation was performed for 48h after which sulforhodamine B SRB was added Concentrations

higher than 50μM were not soluble in media and 0.5% DMSO. The experiment was performed three times, in each experiment each concentration was made in triplicate

Table 5.1. Anti-proliferative activities of **NC 2067** and **NC 2081**. IC_{50} of both agents on HCT-116 cells ranged from 0.5 to 0.6 μM. IC_{50} of both agents on CRL-1790 was above 25μM.

Compound	*IC_{50} μM±SD**	
	HCT-116 cell line	CRL-1790 cell line
NC2067	0.571±0.105	> 25
NC2081	0.503 ±0.081	> 25
5-FU	3.558±1.06	>25

* SD= Standard deviation

5.2 ROS evaluation

The increase in ROS levels was apparent after cells were treated to both agents. This was concluded after observing an increase in fluorescence intensities of Dichlorodihydrofluorescein. The average increase of fluorescence intensities represents an increase in ROS. One may conclude from Table 5.2 that both agents caused a 3-7 fold increase in fluorescence intensity when HCT-116 cells were treated with the IC_{50} concentrations of **NC 2081** and **NC 2067** for 48 h. The difference in ROS induction between each compound and DMSO was significant with a p value less than 0.05 (Appendix 1). It should also be noted that the number of cells was reduced by half after the incubation, therefore, the intensities (fluorescence units) were doubled for all three agents to be comparable with the number of cells in the DMSO condition.

Table 5.2. Effect of cytotoxic agents on ROS levels in HCT-116 cells. Both agents, and 5-FU were able to induce ROS about 3-7 fold. The average increase of fluorescence intensities represents an increase in ROS. 5-FU was used as a positive control. The value of p was less than 0.05 when the effect of each agent was compared with DMSO. Each experiment was conducted in triplicate.

Compound	*Average intensities±SD**
NC 2067	9946±252
NC 2081	5036±994
5-FU	7298±560
DMSO	1387±55

* SD= Standard deviation

5.3 Mitochondrial membrane potential

After performing this assay we were able to observe the effect of agents on MMP. Table 5.3 reveals the ability of both the agents to increase the levels of the monomeric form of JC-1 in HCT-116 cells about 2 fold which indicates that **NC 2067** and **NC 2081** reduce the mitochondrial membrane potential in these cancer cells. The p value was found to be less than 0.05 for all agents when compared with DMSO. The monomeric form was measured at Ex of 485 nm and Em of 535 nm. The monomeric form tends to accumulate in unhealthy cells with lower mitochondrial membrane potential. For **NC 2067** and **NC 2081**, the number of cells were reduced by half after 48 h. Therefore, the intensities were doubled for both agents to be comparable with the number of cells in the DMSO condition.

Table 5.3. Evaluation of the mitochondrial membrane potential of HCT-116 cells after treatment with **NC 2067**, **NC 2081** and positive controls CCCP and 2,4-dinitrophenol.. JC-1 was added after incubation with the test compound for 48 h. The p value when comparing between both agents and DMSO was less than 0.05. Each experiment was conducted in triplicate.

Compound	*Average±SD**
NC 2067	6074±456
NC 2081	6376±509
2,4-dinitrophenol	7541±769
CCCP	7277±519
DMSO	3069±20

*SD= Standard deviation

5.4 GST activity

After conducting the explained experiment it was revealed that both compounds **NC 2067** and **NC 2081** behave as substrates for GSTπ. The top curve indicates the absorption of one compound alone. The addition of GSH and GSTπ caused a great drop in absorption. Decrease in absorption continued when it was measured every 2 min. This reduction was stopped after 10 min indicating that agent's concentration is steady. The decrease seen at the beginning implied a decrease in drug concentration caused by the removal of agents or binding molecules with other compounds (GSH). Such binding will cause a change in chemical structure and therefore change in λmax. The concentration of both compounds was not affected by the addition of GSH nor GST individually, and in these conditions the compounds were stable during the period of the experiment.

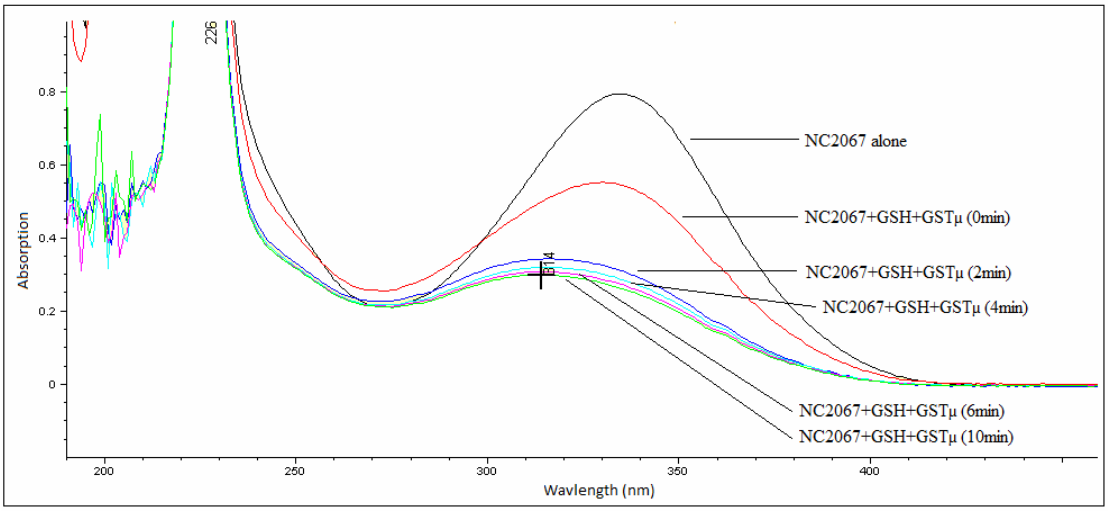


Figure 5.3: Absorbance for **NC2067** with time after the addition of GSH and GST. The decrease in absorbance at ~314 nm implies a decrease in concentration which is the result of the attachment of the agent with GSH. GSTπ was required for this attachment. A decrease in absorbance was measured with time until about 10 min.

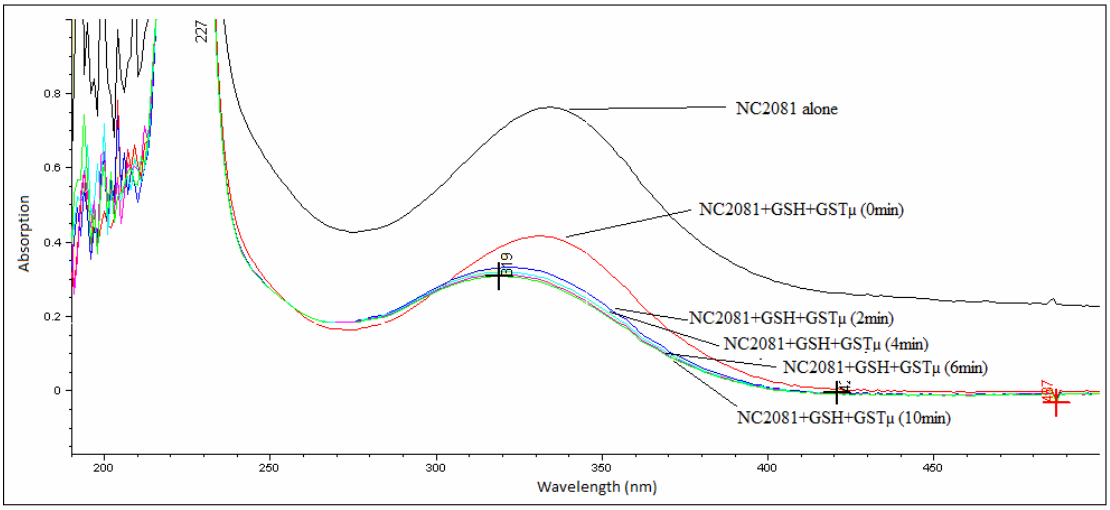


Figure 5.4: Absorbance for **NC2081** with time after the addition of GSH and GST. The decrease in absorbance at ~319 nm implies a decrease in concentration which is the result of the attachment of the agent with GSH. GST was required for this attachment. A decrease in absorbance was measured with time until about 10 min.

5.5 Oxygen consumption assay

Measuring the oxidative phosphorylation activity can be conducted by determining the changes in oxygen concentration over a period of time. The measurement of oxygen concentration over time determines the rate of concentration change. This value is represented by the slope produced when the oxygen concentration is plotted against time. Treating the cells with 5 μM CCCP caused an increase in the oxygen consumption rate from 2 to 7 μM/min (Figure 5.5 and Table 5.4). However, adding **NC 2067** or **NC 2081** caused a significant inhibition in the oxygen consumption rate. The addition of both agents was conducted in two phases. The first phase involved the addition of 10 μM. This caused about a 50% decrease in the respiratory rate for both agents. When 40 μM were added, the rate decreased by about 50% again for both agents. The oxygen consumption of untreated cells was measured for about 10 min. The rate was steady for that period and equaled 2 μM/min. The addition of CCCP caused about a 4 fold increase in the consumption rate.

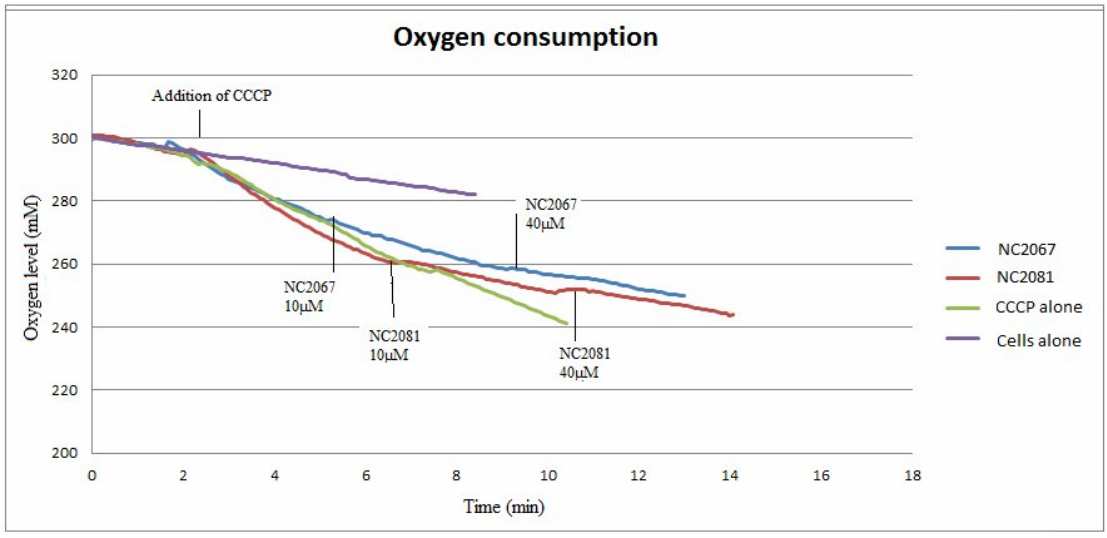


Figure 5.5: Oxygen consumption with the addition of CCCP, **NC 2067** and **NC 2081**. The purple line represents the cells alone respiration. CCCP was added at 5 μM at

the beginning as indicated in the figure. Both agents were added 2 min after the addition of CCCP at concentrations of 10 μM as indicated in the figure. After recording the oxygen consuption for 2-3 min, another 40 μM of each agent was added making the final concentration 50 μM.

Table 5.4: Oxygen consumption in response to the addition of compounds **NC 2067** and **NC 2081**. The inhibition or stimulation percentage compared to the column before the addition. The addition of CCCP caused an increase in oxygen consumption rate while the addition of the compounds caused a decrease in the rate. Percentage of inhibition after CCCP was added was related to the rate when nothing was added.

	Addition of CCCP (5 μM) (% stimulation ±S.D.)	**Addition of NC2067 (10 μM)** (% inhibition ±S.D.)	**Addition of NC2067 (50 μM)** (% inhibition ±S.D.)
Percentage change	69.4±12.6	51.54±8.52	27.65±3.04

	Addition of CCCP (5 μM) (% stimulation ±S.D.)	**Addition of NC2081 (10 μM)** (% inhibition ±S.D.)	**Addition of NC2081 (50 μM)** (% inhibition ±S.D.)
Percentage change	55.93 ± 2.69	59.52±6.68	31.56±13.88

5.6 Mitochondrial swelling assay

Compound **NC 2081** produced some swelling of mitochondria when treated with 10 and 50 μM of the compound (Figure 5.6). This was represented by a decrease in absorption with time. The decrease in absorbance was measured until a steady line was seen. The absorbance was taken every 10 sec for about 10 min. The addition of 10 and 50 μM of **NC 2081** caused swelling as shown in the figure. The addition of the higher concentration caused about 2 fold more than the lower one. A decrease in absorbance from 1.5 to 1.3 for the higher concentration was noted. A decrease of 0.1

absorbance unit was observed when 10 µM was added. On the other hand, compound **NC 2067** produced swelling only at a concentration of 50 µM (Figure 5.7). The absorbance decreased from 1.5 to 1.37 over the time period measured. No change in absorbance over this time period was seen using 10 µM **NC 2067**. This would imply that **NC 2067** has minimal effect on mitochondrial swelling. A solution of Ca^{+2} ions was used as a positive control at 50µM concentration (Figures 5.6 and 5.7). This caused a significant decrease in absorbance implying that a rapid and substantial amount of swelling was induced.

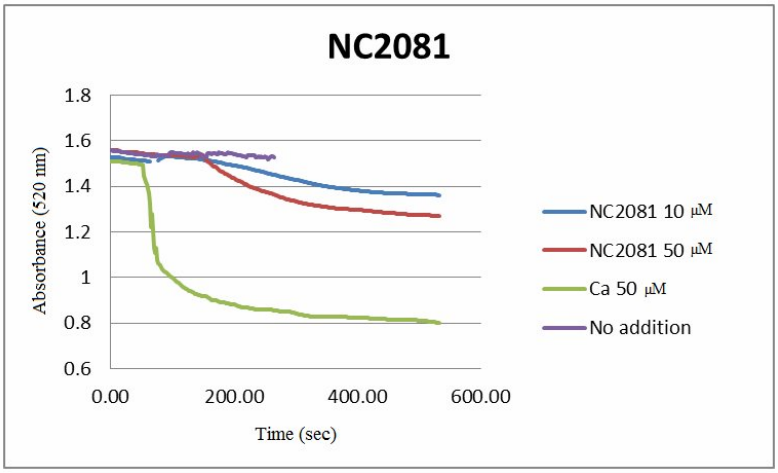


Figure 5.6: Mitochondrial swelling after the addition of **NC 2081** and Ca^{++}. The compound was added after measuring the steady absorbance of mitochondria for about 20 to 40 sec.

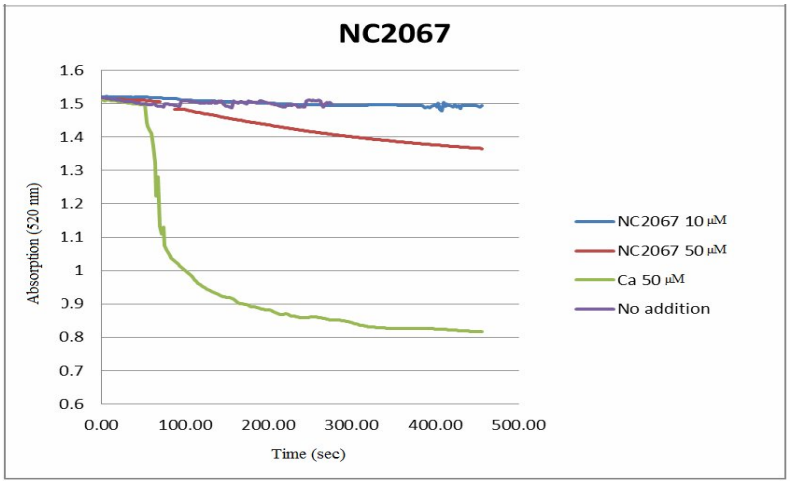


Figure 5.7.: Mitochondrial swelling after the addition of **NC 2067** and Ca^{++}. The compound was added after measuring the steady absorbance of mitochondria for about 20 to 40 sec.

6. DISCUSSION

The main objective of our laboratory is to synthesize anticancer agents that interact with thiols rather than having DNA as the main target as is the case with many contemporary antineoplastic drugs. The compounds of major interest in our laboratory contain the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore which are believed to have a high potential for targeting thiols which may include GST and GSH (Das et al., 2007). Therefore, we conducted a series of experiments to determine some of the modes of action of **NC 2067** and **NC 2081**. Both compounds

have the pharmacophore of interest with different N-acyl side chains. We chose these compounds because they possess potent cytotoxic properties towards a number of cancer cell lines (Das et al., 2007).

Cancer cells tend to shift their energy dependency to glycolysis (Van der Heiden et al., 2009). This change in metabolism reduces the efficiency of respiratory oxidation which in turn results in ROS accumulation (Hileman et al., 2004). Malignant cells have managed to adapt to such an increase in elevating ROS concentrations by overexpressing GST (Mannervik et al., 1987; Laborde, 2010), GSH and antioxidative enzymes (Estrela et al, 2006). These proteins act in conjunction with other antioxidants as electrophilic and reactive oxygen species scavengers (Figure 2.6). However, to maintain the state of oxidative stress in cancer cells, antioxidative enzymes are found in certain levels which cause the ROS concentrations to remain relatively high. High ROS levels are required for cell proliferation and signaling in tumors. However, extremely high concentrations of ROS can initiate apoptosis (Trachootham et al., 2009;Toyokuni et al., 1995). An increase in ROS levels is also expected to have an immediate effect on the mitochondrial membrane.

In general, we have evaluated the effect of both agents on cell proliferation, ROS levels, mitochondrial membrane potential and GST. In addition, we also determined the effect of both agents on cellular oxygen consumption and mitochondrial swelling. The anti-proliferative results were anticipated because previous reports have established the high potency against cancer cells for **NC 2067** and **NC 2081** and some related compounds (Das et al., 2007).

This antineoplastic effect might be the result of targeting GST. As explained above, high GST and GSH concentrations compared to ROS levels in normal cells allow a slight reduction in GSH's levels without affecting ROS concentrations. This is presumably because normal cells have low oxidative stress conditions (Schumacker, 2006). On the other hand, cancer cells remaining in a continuous oxidative stress have sufficient GSH and antioxidative enzyme concentrations to scavenge ROS. Therefore, a slight reduction in GSH would render the ROS levels higher than the cancer cells can accomodate. As a result, the mitochondrial membrane potential in tumors will be reduced to levels at which apoptosis can be initiated.

6.1 Anti-proliferative determinations

As indicated previously, both agents cause potent inhibition of the growth of HCT-116 cancer cells. They also had a much less cytotoxic effect on normal cells (CRL-1790) with an IC_{50} value above 25 μM. The exact IC_{50} figures for the normal cell line were not achievable since a plateau at high concentrations was not observed. That is, in order to obtain an IC_{50} a complete sigmoidal curve should be present which would have a steady mimimum at inhibition and a steady maximum inhibition. As shown in Figure 5.1 at high concentrations the effect of the compounds was not maximized. Higher concentrations would be necessary to in order to establish such relationship. With the normal cell line, the maximum concentration used was 50 μM. Higher concentrations of solutions were not possible when a maximum of 0.5 % DMSO was used. This concentration of DMSO was used after optimization experiments

conducted with different DMSO concentrations revealed that 0.5% DMSO did not affect the viability of the cells.

Although the SRB assay is widely used it has some disadvantages. The SRB dye is used to measure the proteins in cells. Thus, the exact number of healthy cells cannot be determined using this dye. For example, proteins in cells going through apoptosis might be detected and show a falsely high number of living cells.

6.2 GST and GSH

Agents with high potency on cancer cells exert their action by various mechanisms. Our first intention was to provide more evidence to our hypothesis in which such specificity is mainly caused by inhibiting key mechanisms critical in cancer cells and not normal ones. Therefore, we first showed the ability of agents to act as substrates for GST activity and thereby decrease GSH levels.

In order to evaluate our hypothesis, we determined the effect of both agents on GSTπ, a class that is usually overexpressed in cancer cells (Mannervik et al., 1987; Laborde, 2010). The results revealed the ability of GST in conjugating both agents to GSH. This observation implies that both compounds behave as substrates for GSTπ. Also, as substrates of GST these compounds would deplete GSH and render the cells less protected from reactive species. This effect will contribute to an increase in ROS levels leading to mitochondrial membrane potential reduction and cell death.

The results show a reduction in the absorbance of the compounds until a certain absorbance is reached. Not reaching an absorbance of zero, despite the use of 1:10

ratio of GSH:compound, would imply the formation of a GS-compound complex, which might inhibit the enzyme by product inhibition. This result was also observed for other compounds such as ethacrynic acid (Awasthi et al., 1993). To confirm this behavior, the GS-compound complex should be synthesized and evaluated against GST activity. The synthesis of the GS-compound complex may reveal that the compound is likely to bind to the enzyme's H-site.

One of the main disadvantages of the method used here was the lack of the ability to determine the exact amounts of the products formed. Determining these data would enable us to quantify the binding strength and obtain other pharmacokinetic data.

Two agents were tested in my work which are representative of several series of 1-acyl-3,5-bis(benzylidene)-4-piperidones. It is conceivable that other members of this group of compounds will also react with GSH and GST in the same way. However, other factors such as the bulkiness and accessibility of the 1,5-diaryl-3-oxo-1,4-pentadienes to interact with the binding site will also influence the rate and extent of thiol depletion.

Both agents did not bind with GSH without the presence of GST. This would imply that GST is required to activate GSH or the compound in order to link both together. GSH is known to be activated by deprotonating the thiol group in the portion of GSH leaving a negatively charged sulfur atom. The electrons in α,β-unsaturated ketones are polarized as shown in Figure 6.1 and hence nucleophilic attack occurs at the beta carbon atoms of the cytotoxin.

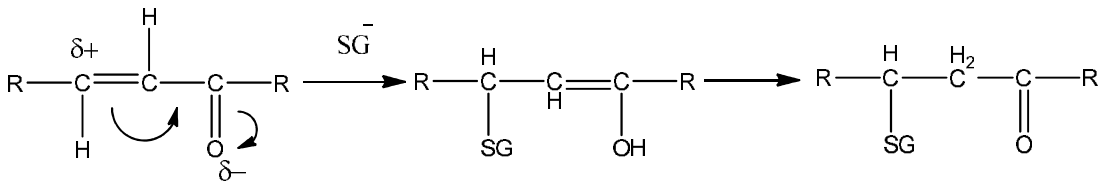


Figure 6.1: The reaction of thiols with α,β-unsaturated ketones

The main limitation to this technique as explained previously is the overlapping of the absorption peaks with each other. One other issue is the presence of all of the compounds (reactants and products) in the same vessel. Therefore, the exact effect of each compound is not achievable. A solution to this problem might be using HPLC after the reaction had reached equilibrium. Each separated fraction can then be analyzed.

In addition, the possibility of the binding between GST and the compounds was also studied. When GST and the compounds were both incubated for 20 min, no decrease in the absorption of the compounds was seen. This observation confirms that the compounds do not bind with GST covalently. Non-covalent interactions do occur because the enzyme is able to combine the compounds with GSH.

In summary, we have shown the conjugation of the compounds was obtained only after the addition of both GSTπ and GSH which may be explained as the catalytic ability of GST in binding GSH with the compounds. Therefore, the use of LC-MS is recommended for the exact detection of the products.

6.3 Reactive oxygen species

About a 3-7 fold increase in ROS was measured after the treatment of HCT-116 cells with both agents. One reason for this increase in ROS levels may be due to the depletion of GSH when acting as a substrate of GST. However, another factor which may lead to ROS induction is the inhibition of the oxidative phosphorylation pathway (Mattiazzi, el at, 2004). Therefore, the possible effect of both agents on these pathways is explained in the next sections.

ROS elevation is linked to several consequences. These molecules have the ability to react with macromolecules in cells which would damage proteins, DNA and induce some apoptotic signals (Benhar et al., 2001; Benhar et al., 2002). Because ROS is mainly produced by respiration processes, it is reasonable to assume their effect will initially be exerted on mitochondria. In the present case, we used the dye dichlorodihydrofluorescein diacetate to detect ROS.

The IC_{50} values of both compounds against HCT-116 were used throughout the mode of action investigations, unless indicated otherwise. After 48 h the number of cells was reduced to half in the treated wells compared to DMSO treated. Therefore, after measuring the fluorescence in each well, the value was multiplied by 2 for the treated wells to compensate for the drop in cell number.

One major limitation of this technique is the inability to distinguish between the different types of ROS (H_2O_2, O-, OH.) although it is claimed that the 2'.7'-dichlorodihydrofluorescein assay has some specificity towards H_2O_2 (Rhee et al,

2010) . In our case, we were interested in knowing the effect of the agents on ROS in general.

6.4 Mitochondrial membrane potential

The MMP is the result of the difference between the proton concentrations across the inner mitochondrial membrane. Protons are highly concentrated in the inter membrane space compared to the matrix. This difference is caused by pumping protons into that space by respiratory complexes (I-IV). This potential is used by complex V (ATP synthase) to synthesize ATP when using the energy derived by a downhill flow of protons (Chen, 1988). This potential is critical for cellular survival for several reasons. As explained previously, the synthesis of ATP directly depends on the MMP. Reducing the MMP might cause a reduction in ATP production and be the reason why bringing MMP to zero will induce apoptosis. On the other hand, affecting the electron flow by attacking one of the complexes directly will cause a blockage of flow to complex IV leading to oxygen accumulation which causes ROS production (Ricci et al, 2003; Chen et al, 2007).

The MMP is known to be affected by various causes. ROS leads to direct damaging effects on membranes, opening of the MPT pore and collapse of the MMP (Vercesi et al., 1997). ROS can also cause mutations in mitochondrial DNA which would cause structural deformities in membranes (Ehlers et al., 1999).

After showing that both agents were able to induce ROS, we wanted to determine if such increases would cause any effect on the MMP. Our results have shown some ability for both agents to reduce the MMP. Because our results suggest that the

agents can directly deplete GSH, it can then be proposed that the consequent increase in ROS caused the effect on mitochondrial membrane potential.

JC-1 was used as a dye to measure the membrane potential. Healthy cells have a high MMP and in such cells, JC-1 forms J-aggregates that are excited and emit light at wavelengths of 560 nm and 595 nm, respectively. In unhealthy cells with altered MMP, JC-1 forms monomers that are excited and emit light at wavelengths of 485 nm and 535 nm, respectively.

Other dyes such as DiOC6(3) and rhodamine 123 can be used for the same purpose. However, JC-1 seems to be more selective towards changes in the MMP rather than the cellular membrane potential (Salvioli et al., 1997). Despite this advantage, JC-1 had low solubility in media which causes difficulty in dissolving it while it is incubated with cells. One other issue is the low sensitivity of the monomeric forms. In other words, in order to form the J-aggregates, MMP should be greatly decreased (Di Lisa et al., 1995). In our results, the J-aggregates form was not altered perhaps because of the lack of any large drop of the MMP.

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol were both used as positive controls. Both agents are uncouplers that cause a decrease in the difference in the proton concentrations. This is achieved by the attachment of the agents to a proton from the intermembrane space and bringing it across the inner membrane to the matrix (Korde et al., 2005). Performing this would bypass the ATP synthase producing heat rather than ATP.

6.5 Oxygen consumption

After evaluating the effects of the agents on cell proliferation, ROS and MMP, we wanted to determine if there was any effect of these compounds on oxygen consumption. In mitochondria, ROS are mainly produced by the inhibition of oxidative phosphorylation. Hence the effect of the agents on oxidative phosphorylation was a reasonable approach.

Using an oxymeter, the levels of oxygen in solution were measured. The oxygen consumption rate was increased by the addition of a known uncoupler (CCCP). The subsequent addition of agents caused an inhibition in oxygen consumption. This was determined by the decrease in the oxygen consumption rate. The range of reduction in phosphorylation is 2 to 3 fold, depending on the agents and concentrations.

These results imply that both agents inhibit the electron transport chain. The exact protein(s) inhibited is/are yet to be identified.

Complex I is believed to be the rate limiting step in the electron transport chain which can be inhibited by rotenone. It reduces NADH to NAD+ in the first step in the chain. When inhibited by rotenone (Li et al, 2002) the entire chain is not completely blocked. This complex, in addition to complex III, is believed to be the main generators for ROS (Turrens, 1997). Complex II is another electron supplier which also delivers an electron to complex III. The reduction of succinate to fumarate accompanies this process.

Complex III is oxidized when it receives electrons from the previous complexes (I and II) and delivers these electrons to complex IV via cytochrome c. Antimycin and cyanide are two agents that inhibit complex III and IV, respectively (Huang et al., 2005; Ricci et al., 2003) Inhibiting the oxygen consumption causes the cessation of ATP production and cell death. In addition, increases in ROS levels can be seen when some complexes are inhibited, Figure 6.2 shows these processes.

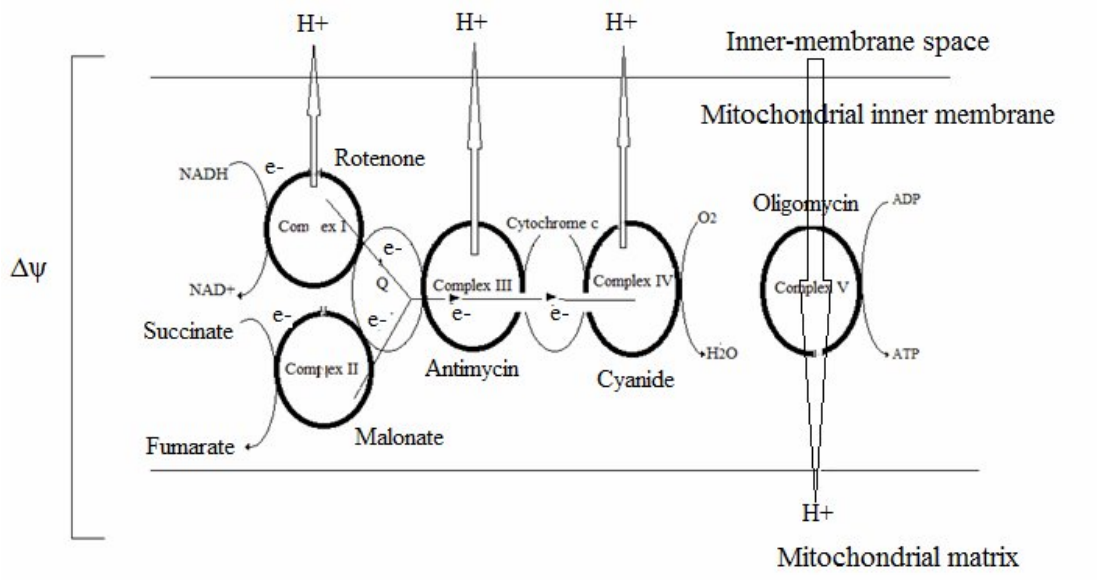


Figure 6.2: Structure of the electron transport chain in the mitochondria. Rotenone, malonate, antimycin, cyanide and oligomycin are inhibitors and are shown near the protein they block. Δψ: mitochondrial membrane potential (Ricci et al., 2003).

Using this method we will not be able to determine which complex is inhibited. Other techniques such as measuring specific complex activities are needed for such a purpose.

6.6 Mitochondrial swelling assay

Inducing mitochondrial swelling can be achieved by the alkylation of the sulfhydryl groups on the mitochondrial membrane (Costantini et al., 2000). In addition, swelling can occur as a result of GSH depletion and ROS elevation (Galindo et al., 2003). Since both agents have the ability to deplete GSH and elevate ROS, we wished to determine the effect of these agents on mitochondrial swelling. As explained previously, **NC 2081** had some mitochondrial swelling induction ability at concentrations of 10 μM and 50 μM. **NC 2067** has an effect on mitochondrial swelling when 50 μM concentration was used but no effect at a concentration of 10 μM. The concentrations used were quite high since the incubation time is only 10 min. The ability in causing swelling was also observed previously for related compounds (Das et al., 2008).

Apparently, inducing mitochondrial swelling by depleting GSH gives only a weak response. On the other hand, alkylation of the mitochondrial membrane pore leads to a opening which causes significant swelling. Alkylation occurs at the thiol group in Cys 56 in the ANT protein. Examples of such thiol cross-linking agents are dithiodipyridine, bismaleimidohexane and others. Although some of these agents lack the unsaturated keto group, they have the ability to oxidize the critical amino acid (Cys 56) in ANT.

Ca^{+2} is known as a mitochondrial swelling inducer (Chappell and Crofts, 1965). Its role in mitochondria has been established for the past two decades. Transporters to

organize the entry to and from the mitochondria include Ca^{+2} uniporter, Na^{+}/Ca^{+2} carrier, Na^{+}/H^{+} antiporter and Na^{+}-independent efflux of Ca^{+2}. High Ca^{+2} concentrations can induce the opening of the permeability transition pore (PTP) (Picard et al., 2008).

The mechanism in which the PTP is opened is not fully understood. Cyclophilin D is believed to bind with the pore protein in the ANT matrix side which would open the pore protein (Woodfield et al, 1998). Ca^{+2} is one substance that induces cyclophilin D to bind to the PTP. Opening these pores allows water to flow in and cause mitochondrial swelling which, depending on how severe, will cause rupture of the mitochondrial outer membrane.

Both cytotoxins inhibited the respiratory chain. As previously indicated, neither agent showed the ability to bind to the sulfhydryl group on GSH without GST as the catalyst. GSH and the sulfhydryl groups in the mitochondrial membrane might have different characteristics. However, it is still reasonable to suggest the ability of the agents to bind with the mitochondrial membrane.

The difference in activity between both compounds can be attributed to the following reasons. Both compounds contain a nitrogen base in their side chains. However, the basicity of the nitrogen atom in **NC 2067** is more than 100 fold greater then the basicity of the morpholine group of **NC 2081.** As shown in the figures presenting the structures of **NC 2067** and **NC 2081**, the torsion angels between the aryl rings and the olefinic carbon are different in each compound. That is □1 and □2 in compound **NC 2067** are +17 and -42 respectively. However, in **NC 2081**, □1 and □2 when

measured simply by using Polymol program are-18 and -27. Such differences might also have some effect of the binding of agents to certain proteins affecting the membrane permeability. However, these differences in structure did not cause major differences in activity in the other assays reported here.

6.7 Future work

For future work, it would be interesting to establish whether a wider range of analogs act by the same mechanisms. This may suggest that not only these two compounds are able to cause these effects, but all compounds possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore would act in the same manner. In addition, a strong structure-activity relationship may be established. The GST and GSH results should encourage us to study the kinetic studies in depth. The use of HPLC and mass spectrometry are two techniques proposed to isolate all compounds and then determine the quantity of each.

These results showed that these agents have some effect on mitochondria; however, the exact mechanism of involvement is yet to be addressed. Different pathways can be affected and result in cell death. For example, affecting an electron respiratory complex is one mechanism for cell death. The exact complex inhibited should be determined. In addition, the influence of other mechanisms may involve the alkylation of ANT, damaging mitochondrial DNA and affecting the Krebs cycle. The effect of these compounds on these mechanisms has not been evaluated yet.

After the evaluation of the effect of both compounds, **NC 2067** and **NC 2081**, other compounds, possessing the same pharmacophore, will be evaluated against the same mechanisms. This will reveal if these pathways are affected by compounds having the same structures.

Later, after confirming the activity of these compounds *in vitro* we would like to evaluate their activity *in vivo.* A previously evaluated compound possessing the same pharmacophore was evaluated and was unsuccessful *in vivo* (Dimmock et al., 1990). However, compounds **NC 2067** and **NC 2081** have not been evaluated *in vivo*. These compounds have different physicochemical properties which would encourage the evaluation in mice. For example, they have lower logP than the previously evaluated compounds. This would improve the biodistribution rather than residing in the adipose tissues. In addition, both compounds lack the quaternary ammonium ion which is probably the main reason for the neurotoxicity seen in previous evaluations.

7. CONCLUSIONS

NC 2067 and **NC 2081** possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore inhibit the proliferation of HCT-116 cancer cells while exerting minimal effect on normal ones. The IC_{50} values for both agents ranged from 0.5 - 1 μM and above 25 μM for cancer and normal cells, respectively. One proposed mechanism of action is their being substrates of GST and thus depleting GSH levels. This will cause two main actions. The first is the increase in ROS levels and the second is the lowering of the cancer cell's ability in developing resistance to certain chemotherapeutics. Both agents were able to increase ROS levels by 3-7 fold.

A second mechanism which might also lead to the induction of ROS levels is the inhibition of the electron transport chain which was determined as the inhibition of oxygen consumption. Both agents inhibit oxygen consumption implying that this might be another mechanism for ROS induction.

The increase in ROS levels caused by both agents is one mechanism to decrease the potential around the mitochondrial inner membrane. As explained, these agents seem

to increase ROS by two mechanisms, the first is the decrease in GSH and the second is the blockage of oxygen consumption. These mechanisms were proved experimentally and seem to be the most likely pathway for ROS increase. In addition, it is reasonably assumed covalent interactions are not happening between the agents and the mitochondrial membrane.

NC 2081 had mitochondrial swelling induction ability at a concentrations of 10 μM and 50 μM. **NC 2067** has an effect on mitochondrial swelling when 50 μM is used but no effect at a concentration of 10 μM. The increase in ROS levels might cause some mitochondrial swelling but in this case the increase was not severe enough to produce a large effect.

APPENDIX

Appendix 1: Table showing post-hoc results when comparing the effect of each compound and the effect of DMSO on ROS levels. Significant effect was seen when these comparisons were made. GraphPad Prizm program was used to preform one way ANOVA -Dunnett's multiple comparison test.

Dunnett's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	95% CI of diff
DMSO vs NC2067	8559	Yes	9684 to 7434
DMSO vs NC2081	3500	Yes	4625 to 2375
DMSO vs 5-FU	5911	Yes	7036 to 4786

Appendix 2: Table showing post-hoc results when comparing the effect of each compound and the effect of DMSO on MMP. Significant effect was seen when these comparisons were made. GraphPad Prizm program was used to preform one way ANOVA -Dunnett's multiple comparison test.

Dunnett's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	95% CI of diff
DMSO vs NC2067	-3005	Yes	-4221 to -1789
DMSO vs NC2081	-3307	Yes	-4523 to -2091
DMSO vs 2,4-Dinitrophenol	-4472	Yes	-5688 to -3256
DMSO vs CCCP	-4208	Yes	-5424 to -2992

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