SYNTHESIS, STRUCTURE-ACTIVITY RELATIONSHIP STUDY, AND MODE
OF ACTION STUDY OF 1,4-NAPHTHOQUINONE BASED
ANTICANCER AND ANTIMICROBIAL AGENTS

by

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ABSTRACT

Synthesis, Structure-Activity Relationship Study, and Mode of Action Study of 1,4-Naphthoquinone Based Anticancer and Antimicrobial Agents

by

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Utah State University, 2016

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Synthesizing bioactive small molecules by structural modification of 1,4-naphthoquinone was the primary goal of this research. Several bioactive compounds with anticancer, antifungal, and antibacterial activities were synthesized. All the synthetic protocols were optimized in such ways that do not require cumbersome purification.

First, a new protocol for the synthesis of NQM111 was developed. NQM111 is a highly potent anticancer agent developed in our laboratory, but the old protocol does not provide enough quantity for \textit{in vivo} study. Therefore, a new safe and improved method was developed which provides enough quantity for \textit{in vivo} study.

The second project involves the synthesis of 1,4-naphthoquinone conjugated with an aromatic group. These compounds are a highly potent anticancer agent with \textasciitilde 8-fold selectivity towards cancer cell lines than the non-cancer cell line. A mode of action study of this compound was identified, and it was observed that these compounds generate reactive oxygen species,
which triggers apoptosis.

The final project involves the synthesis of 1,4-naphthoquinone based antifungal, and antibacterial compounds. These compounds are multi-cationic in nature with a hydrophobic tail. Six different analogs with varying hydrophobic tails were synthesized and tested for their antibacterial and antifungal activity. These compounds showed excellent activity against wide range of fungi including resistant strains.

(246 Pages)
Synthesis, Structure-Activity Relationship Study, and Mode of Action Study of 1,4-Naphthoquinone Based Anticancer and Antimicrobial Agents

by

Jaya P. Shrestha, Doctor of Philosophy
Utah State University, 2016

The first three projects involve the synthesis and the mode of action study of 1,4-naphthoquinone based anticancer agents. These anticancer agents are highly potent against a wide range of cancer cell lines. These compounds showed ~8-fold selectivity towards the lung cancer cell line than the normal cell line. We also studied the mode of action and observed that generation of reactive oxygen species is the primary mode of action.

The final project involves the synthesis of multi-cationic antimicrobial agents. These compounds are active against both bacteria and fungi. These compounds are very easy to synthesize and can be scaled up if large quantities are needed.
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ΔΨm: Mitochondrial membrane potential
APCI: Atmospheric pressure chemical ionization
ATP: Adenosine triphosphate
CAA: Cationic anthraquinone analog
CAT: Catalase
CIB: Center for integrated biosystems
CNS: Central nervous system
DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride
DCFDA-AM: 2′,7′-dichlorofluorescein diacetate
DMF: Dimethyl foramide
DMEM: Dulbecco’s Modified Eagle’s medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DTP: Developmental therapeutic program
EDTA: Ethylenediaminetetraacetic acid
ESI: Electron spray ionization
ETC: Electron transport chain
EU: European Union
FACS: Fluorescence activated cell sorting
FBS: Fetal bovine serum
FDA: Food and drug administration
FBS: Fetal bovine serum
G⁺: Gram positive
G⁻: Gram negative
GI₅₀: Growth inhibition by 50%
GPX: Glutathione peroxidase
GSH: Glutathione
IARC: International agency for research on cancer
LC₅₀: Lethal concentration for 50%
MCAA: Multi-cationic anthraquinone analog
MCB: Monochlorobimane
MIC: Minimum inhibitory concentration
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI: National cancer institute
NMR: Nuclear magnetic resonance
NQ1: Quinone oxidoreductase 1
PBS: Phosphate-buffered saline
PI: Propidium Iodide
PS: Phosphatidylserine
Q10: Ubiquinone
QAC: Quaternary ammonium complex
ROS: Reactive oxygen species
SAR: Structure activity relationship TB: Tuberculosis
SOD: Superoxide dismutase
SQR: Semiquinone radical
TB: Tuberculosis
TBAF: Tetrabutylammonium hexafluorophosphate

TLC: Thin layer chromatography
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CHAPTER 1

GENERAL INTRODUCTION

The development of therapeutic agents for the treatment, and prevention of diseases, and to relieve pain has played a critical role to pave the pathway for the practice of modern medicine. Over many centuries, medicines have been discovered and used in the form of crude extracts or dried parts of plants and animal products.¹ People in different civilizations used plants such as poppy, henbane, mandrake, camphor, ginseng, jimson weed, rhubarb, and aloe, for over 4000 years for folk medicine.² Some of these plants are still being used as a traditional medicine. A systematic discovery and development (use of active chemical ingredient as a medicine rather than plant extracts), took place only after the discovery of prontosil by Gerhard Domagk in 1930s, and penicillin by Alexander Fleming in 1928.³ Before the discovery of antibiotics, infectious diseases were one of the biggest blights on the human history. In 1900, one-third of all deaths in the United States was caused by either pneumonia, tuberculosis, or diarrhea.⁴ Nowadays, out of these three diseases, pneumonia is the only top ten cause of death in the United States. The major deaths are resulting from other conditions such as cardiovascular disease and cancer.⁴ Since 1999, cancer has been the leading cause of death in the United States for individuals less than 85 years of age.⁵

Even though the mortality rate caused by infectious diseases is decreasing, the rate of infections caused by those pathogens and the
discovery of new infectious diseases are not decreasing. Between 1940 and 2004, a staggering 335 new infectious diseases were discovered, and it is estimated that three new diseases are being identified every couple of years. 

**Figure 1.** Top 10 causes of death in the United States during 1900 (top) and 1997 (bottom) 

Worldwide 13.7 million people are infected with active tuberculosis (TB), and 2 billion people are infected with an inactive form of TB. 247 million people are infected with malaria every year. Similarly, according to the International Agency for Research on Cancer (IARC), there were 14.1 million new cancer cases and 8.2 million cancer related deaths worldwide in 2012. So far, there are more than 200 types of known cancers. The rise of drug-resistant microbes and cancers, which used to be curable in the past, complicated the situation even more. Through mutation or genetic exchange, infectious agents can develop resistance to available drugs and evolve into
new deadly strains. Therefore, there is an imminent need for the development of new antimicrobial agents.

Extraction of active components from natural products and chemical synthesis of therapeutic agents are the two major sources of drug discovery for anticancer and antimicrobial agents. Plants and animals produce many therapeutic agents as a physiological byproduct or as a defense mechanism. Since most of the naturally occurring compounds have been in nature for a long time and been exposed to several living things, chances are very high that some of the species have already developed resistance or will develop resistance soon. In this situation, development of antimicrobial and anticancer agents by chemical synthesis comes in as a very effective tool. The chemically synthesized drug candidates are newly exposed to the environment, and it will take much longer to develop resistance when compared to naturally occurring drugs. Eventually, most of the antimicrobial compounds will have some resistance. It is estimated that the annual resistant infections cost about USD 20 billion in excess health care cost in USA\(^9\) and €1.5 billion in the European Union (EU).\(^{10}\) There is an ongoing and never-ending battle between drug development and drug resistance by microbes, and hence, we need to develop new antimicrobial agents constantly. We can achieve this goal only by chemical modifications to already known scaffolds since the discovery of new bioactive lead compounds is very rare.
1. Quinones

Quinones are one of the most popular scaffolds for the synthetic chemist to use as a core structure. They are a vast class of compounds, widely distributed in nature, and occur abundantly in plants, animals, and microbes. They are often used as pharmaceutical drugs (anticancer, anti-inflammatory, antibacterial, antifungal, antiplatelet, neuroprotective agents, and laxative agents), daily nutrition, dyes, chemical reagents, and as a charge carrier in a flow cell battery. Quinone motifs are responsible for some of the key role in the biological system. Ubiquinone (Q10), a 1,4-benzoquinone derivative with a 50 carbon isoprenoid side chain, plays an essential role in aerobic respiration to generate adenosine triphosphate (ATP) (Figure 1.1). Q10 is a part of the electron transport chain (ETC), where it acts as a mobile electron carrier, transferring electrons from complex I to complex III, or from complex II to complex III (Figure 1.1).

![Figure 1.1. Coenzyme Q10 (left) and Electron Transport Chain (right)](image)
Quinone moieties have not only attracted enormous research interests; they have also resulted into several FDA approved commercial drugs. Some of the quinone based FDA approved drugs with different bioactivities are listed in Figure 1.2.

1.1. Naphthoquinone

Naphthoquinone is one of the most important subclasses of quinone. They are organic molecules derived from naphthalene. They are planar in geometry and contain aromatic ring fused to quinone. 1,2-naphthoquinone and 1,4-napthoquinone are the two types of naphthoquinone core present in nature (Figure 1.3). The prefix 1,2 or 1,4 represents the position of the ketone functional groups.

![Figure 1.3. 1,4-napthoquinone (A); 1,2-napthoquinone (B)](image)

They are present in various parts of plants such as leaves, flowers, roots, barks, and woods. Aside from being used as antimicrobial agents and anticancer agents, molecules bearing naphthoquinone scaffolds have also been employed as inhibitors against vitamin K-dependent carboxylase, protein kinase, coenzyme Q, as laxative, and as a growth stimulator for bifidobacteria.
Figure 1.2. Commercial drugs containing quinone motif.
Vitamin K, a representative class of 1,4-naphthoquinone which includes vitamin K1, vitamin K2, and vitamin K3 play important roles in the biological system. Vitamin K1 is a naturally occurring molecule and also known as phylloquinone (Figure 1.4). It is required for blood coagulation. Vitamin K2 or menaquinone (Figure 1.4) is of vital importance for bone health. Vitamin K3 or menadione is a synthetic form and used as a quinone model for in vivo studies. In combination with ascorbic acid, it is under clinical trial as an anticancer agent.

![Figure 1.4. Vitamin K series](image)

The naphthoquinone core structure is fascinating for medicinal research because even a small change of substitution can drastically change its biological activities (Figure 1.5).

Our laboratory is dedicated to synthesizing small bioactive molecules by structural modification of 1,4-naphthoquinone. We use the classical
approach of drug design and development, where we screen several newly synthesized

![Chemical structures of Psychorubrin, Naphthazarin, Phygon, Pradimycin, and 1,4-naphthoquinone analogs](image)

Figure 1.5. 1,4-naphthoquinone analogs showing diverse bioactivities

compounds for their biological activities. This approach of drug design is one of the most successful methods to develop lead compounds, and has led to several new FDA approved drugs. Several bioactive analogs of 1,4-naphthoquinonewere already synthesized via azide chemistry.\textsuperscript{11c, 11d, 31} By using a simple [3+2] cycloaddition with azide and oxidation, followed by methylation gives the product in very high yield. In all the synthetic steps, these compounds do not require any cumbersome purification process via column chromatography. High overall yields and no requirement for column chromatography make them excellent candidates for commercialization. Once we characterize compounds, we test them against cancer cell lines and
several pathogenic microbes. Once we hit an active lead compound, we carry out structure-activity relationships (SAR) to synthesize analogs that are even more potent. Several patented bioactive compounds are synthesized in our laboratory from this pathway (Figure 1.6).

Figure 1.6. Patented antibacterial agent from our lab (WO2012021409 A30).

Benzoquinone and anthraquinone are another very similar quinone motifs present in nature. Benzoquinone lacks aromatic benzene ring attached to 1,4-naphthoquinone while anthraquinone has an extra aromatic ring attached to 2,4 position. Even though we use 1,4-naphthoquinone core as the starting material, our final products contain an aromatic triazole ring attached to the 2,4 positions, resulting in a structure very similar to anthraquinone. Therefore, we called these analogs anthraquinone analogs. These anthraquinone analogs are alkylated to form cationic salts, hence abbreviated as cationic anthraquinone analog (CAA).
1.2. Understanding cancer and naphthoquinone-based anticancer drugs

1.2.1. Oxidative stress

Oxidative stress is defined as the imbalance between reactive oxygen species (ROS) and antioxidant defenses. The concentration of ROS is highly regulated in cells. The imbalance is usually caused by the overproduction of ROS, which overcomes the antioxidant defenses, and accumulates inside cells. Once accumulated, they can damage and alter different cellular components such as proteins, lipids, DNA, and RNA. Even though the higher concentration of ROS is harmful; a lower concentration is required for a subcellular process such as enzyme activation, gene transduction, disulfide bond formation.

1.2.2. Reactive oxygen species (ROS)

ROS is a group of highly reactive free radical derivatives of oxygen. It includes superoxide radical anion, peroxides, hydroxyl radical and singlet
Superoxide radicals are the product of one-electron reduction of molecular oxygen, which rapidly converts to hydrogen peroxide.\textsuperscript{34} Hydrogen peroxides are the least reactive among other ROS, so its lifetime in cells is much longer than any other ROS.\textsuperscript{34}

![Structure of molecular oxygen and common ROS](image)

**Figure 1.8.** Structure of molecular oxygen and common ROS

1.2.3. ROS Sources and modulation inside the cell

ROS generation is a natural process, and they are by-products of the oxidative phosphorylation in the mitochondria.\textsuperscript{35} In ETC (Figure 1.1), some of the electrons can escape while transporting from ETC complex I to III or from complex II to III via coenzyme Q10. The escaped electrons reduce molecular oxygen forming highly reactive superoxide radical. It is estimated that up to 2\% of oxygen consumed during ATP synthesis are reduced into ROS via this pathway.\textsuperscript{36} Complex I is the most ROS-generating site as the highest number of premature electrons leak from this complex. Enzymes such as cytochrome P45s are also responsible for some of the ROS generated.
Antioxidant defenses modulate ROS in cells. These radical scavengers mostly involve enzymes and small molecules. Glutathione, superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), vitamin E, ascorbic acid, beta-carotene, and vitamin A are some of the examples. Glutathione and thioredoxin scavenge ROS by the oxidation of dithiols; the oxidized thiols are recycled back to reduced form by the corresponding reductase (Figure 1.9).

![Figure 1.9. Reduction of ROS by glutathione](image)

The second method of ROS modulation is through an enzyme called SOD, which rapidly catalyzes the formation of hydrogen peroxide from superoxide radical. The hydrogen peroxide is then converted to neutral oxygen and water by catalase. A representative diagram (Figure 1.10) below shows a mitochondrial schematic of different functions associated with ROS generation.
1.2.4. Level of ROS in cancer cell versus normal cell

Cancer cells have an elevated concentration of endogenous ROS when compared to normal cells. They are rapidly dividing and metabolically very active.

The elevated rate of metabolic activity in cancer cells requires more ATP than normal cell lines. The extra metabolic burden on ETC leads to the formation of elevated ROS level. It is also proposed that ROS can damage mitochondrial DNA, which results in mutation. The mutation causes more electrons to escape from ETC forming more ROS.

1.2.5. Mechanism of cell death involving quinone

The toxicities of quinones are mostly based on their ability to generate ROS and to function as a Michael acceptor. The carbonyl groups on quinones allow...
acceptance of one or two electrons and protons forming semiquinone which in turn reduces molecular oxygen, forming ROS. The strength that enzymes are affected by naphthoquinone depends on the redox potential, molecular structure, pH, quinone/enzyme ratio and the presence of oxygen. Some studies have also shown that naphthoquinone may act as both an arylator and a redox cycler.

1.2.5.1 Quinone Redox mechanism
1.2.5.1.1. Enzyme catalyzed redox process

Quinones undergo reductive activation by two major enzymes such as cytochrome P450s and cytochrome P450 reductase. The bioreductive activation can go with by 1 electron reduction process or by 2 electron process. The 1 electron reduction of quinone forms semi-quinone, while 2 electron reduction forms dihydroquinone (Figure 1.12). The semiquinone or
hydroquinone can be recycled back to the quinone under normal oxygen level forming superoxide.

![Figure 1.12. General scheme for quinone oxidation and reduction showing the formation of semiquinone/hydroquinone and ROS.](image)

Cytochrome P450 reductases are the main enzymes that catalyze quinone reduction via the 1 electron process. Cytochrome P450 reductases lack a metal center, so they can only reduce quinones by 1 electron at a time. Cytochrome P450 enzymes contain an iron core, which activates quinone by 2 electron process using NADPH. Another enzyme called NAD(P)H: quinone oxidoreductase 1 (NQ1), also reduces quinone by a 2-electron process forming hydroquinone. This process is facilitated by cofactors such as FAD and NAD(P)H. NQ1 activates Pro-drugs such as geldanamycin, mitomycin, β-lapachone, and streptonigrin.

1.2.5.1.2. Non-enzymatic redox process

Quinones can also be activated non-enzymatically. An oxidant such as ascorbate can oxidize quinone, forming semiquinone (Figure 1.13). Menadione, a naphthoquinone derivate with ascorbate, is under clinical
investigation for anticancer studies. Several studies have shown that
anthraquinones such as alizarin, dantron, mitoxantrone, and daunorubicin are
not activated by ascorbate. Therefore, it is possible that only a few quinones
are activated by ascorbate and may not be the principal mechanism of
quinone activation.

Figure 1.13. Ascorbate mediated quinone oxidation and reduction showing
the formation of semiquinone/hydroquinone and ROS.  

1.2.5.2. Quinone as a Michael acceptor

Quinone derivatives with open α, β-unsaturated ketones are vulnerable
to nucleophiles via Michael addition. It is already reported that menadione
covalently binds to β-93 thiol groups of human hemoglobin via Michael
addition forming menadione thioester. The process of thioester formation is
related to menadione-induced hemolysis of erythrocytes. Doxorubicin and
daunorubicin can also alkylate DNA and other nucleophilic macromolecules
via Michael addition. These compounds do not have an α, β-unsaturated
site, but forms after bio-activation. They undergo reductive cleavage of sugar moiety forming an α, β-unsaturated site at ring D.\textsuperscript{40}

1.2.6. Quinone based drugs in cancer treatment

1.2.6.1. Anthracyclines

Anthracyclines are antibiotics used in cancer chemotherapy, and they are one of the most successful cancer treatments ever developed. They are natural products and derived from \textit{Streptomyces}. They are active against leukemias, lymphomas, breast, stomach, uterine, ovarian, bladder, and lung cancers. The dose-dependent cardiotoxicity often limits their usefulness and are often given with dexrazoxane, an iron chelator as a cardioprotective agent. Daunorubicin, idarubicin, doxorubicin, epirubicin, and valrubicin are the FDA approved anthracyclines for chemotherapy (Figure 1.3).

1.2.6.2. Anthracenediones

Anthracenediones are synthetic analogs of anthracyclines. They lack a ring D in anthraccline and a sugar moiety. These analogs of anthracyclines were developed as a less cardiotoxic form of anthracycline. Pixantrone and mitoxantrone are an example of two anthracenediones, currently used in the market. Mitoxantrone is mostly used to treat metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma. Mitoxantrone intercalates into DNA and instigates DNA crosslinking and DNA strand break. It is also known to inhibit topoisomerase II.
1.2.6.3. Mitomycin C and its analogs

Mitomycins are derived from \textit{Streptomyces} species. Mitomycin C is one of the major compounds from this class, and used as both antibiotic and anticancer. Mitomycin C is derived from \textit{Streptomyces caespitosus}. Mitomycin C is a bioreductive alkylating agent, which generates electrophile upon reduction, and bind covalently to cellular macromolecules. A bioreductive activation process of mitomycin C by enzyme NQ01 and DNA crosslinking is shown in Figure 1.16.

![Structure of Mitomycin C](image)

**Figure 1.14.** Anthracenedione analogs

**Figure 1.15.** Structure of Mitomycin C
Figure 1.16. Mechanism of mitomycin C activation and DNA alkylation

1.2.6.4. Streptonigrin and related compounds

Streptonigrin is a metal binding antibiotic antitumor agent isolated from *Streptomyces flocculus*. It shows a broad range of anticancer activity including breast, lung, lymphoma, melanoma, as well as head and neck cancer. It inhibits DNA and RNA synthesis, breaks DNA strands, induces unscheduled DNA synthesis, forms DNA adducts, and also inhibits topoisomerase II. Streptonigrin produces DNA damaging effect by autooxidation of the quinone moiety to semiquinone in the presence of NADPH, which reduces molecular oxygen to free radical. Quinone moiety is essential in Streptonigrin as antitumor activity was lost without quinone moiety.
1.2.6.5 Cribostation

Cribostatins are isoquinoline quinones isolated from a sea sponge.\textsuperscript{46} So far, seven cribostatins have been isolated.\textsuperscript{47} All of the members were isolated from \textit{Cribrochalina} except Cribostation 7, which was isolated from \textit{Petrosia} sponge (Figure 1.18). All seven compounds possess antineoplastic activity and IC\textsubscript{50} ranges from 0.045-25 µM against murine leukemia cell line.\textsuperscript{47} These compounds also possess antibacterial and antifungal activities.

\textbf{Figure 1.17.} Streptonigrin and related compounds
1.3. 1,4-Naphthoquinone derivatives as antimicrobial agent

Various 1,4-naphthoquinone derivatives with antibacterial activity are already known (Figure 1.19), e.g. plumagin, juglone, and lawsone, which are naturally occurring naphthoquinone of plant origin. Alkannin, shikonin, and their derivatives are other natural naphthoquinone products whose antimicrobial activity has been widely investigated.

Figure 1.18. Seven known members of the cribostatin family

- Cribostatin 1
- Cribostatin 2
- Cribostatin 3
- Cribosatin 4
- Cribosatin 5
- Cribosatin 6
- Cribosatin 7
Figure 1.19. Naturally occurring antibacterial 1,4-napthoquinone analogs

Our laboratory has extensively published 1,4-napthoquinone based antibacterial agents. These compounds are highly effective against *S. aureus* with MIC value of 0.032 µg/mL. These compounds are shown to inhibit redox processes at MIC level and disrupt membrane at the higher concentration. By varying chain length at R and R’ position (Figure 1.20 C), several new analogs were synthesized and studied their antibacterial effect on *S. aureus* and *E. Coli*. The effect of alkyl chain length on MIC value for both *S. aureus* and *E. Coli* can be observed in Figure 1.20.
Figure 1.20. MIC values against (a) *S. aureus*, (b) *E. coli*, and (c) Compound structure.
1.4. Advantages of naphthoquinone derivatives via azide chemistry

[3+2] cycloaddition of azide with 1,4-naphthoquinone provides an easy one step method to attach any functional group on naphthoquinone. The protocol for [3+2] cycloaddition is well established, and several approaches are already published to attach alkyl or aromatic groups. The reaction is thermodynamically controlled; and depending on temperature or addition of Lewis acid, several other biologically important heterocyclic molecules can be synthesized (Figure 1.21), including indanedione, anthraquinone, benzazepine, and aziridine. Even though a mixture of several products is obtained from this method, our group has specialized into driving those reactions to obtain one major product. It is done either by addition of Lewis acid or using thermodynamic control. Figure 1.21 shows possible degradation pathways of the intermediate to give different heterocyclic molecules.

Water solubility is another issue with many biologically active molecules. For example, the anthraquinone motif has two benzene rings on either side of quinone, and many anthraquinone drugs have poor solubility in water. NQ component synthesized from the cycloaddition can be methylated at N1 to form a cationic salt (NQM component), which enhances its water solubility.
Figure 1.21. Fragmentation of intermediate to form different heterocycles
1.5. Brief Introduction to the biological techniques used in this dissertation

1.5.1. Flow cytometry

Flow cytometry provides rapid physical and chemical characteristics of a single cell as they pass through a narrow channel in a fluid stream through a laser beam. The laser beam excites the fluorescently labeled cell and a detector measures the fluorescence intensity. Multiple lasers can be used to determine different cellular characteristics at the same time. It is a very rapid technique, which can scan ~10,000 cells in less than a minute. The technique is widely used in research with more than one-third of papers in the *Journal of Immunology* include flow cytometric data.\(^{50}\)

1.5.2. MTT assay

Use of MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), is a very well-known assay for determination of cell viability.\(^{51}\) It is a colorimetric assay for accessing cell metabolic activity. Under the defined condition, NAD(P)H-dependent cellular oxidoreductase enzymes reduce slightly yellow tetrazolium MTT dye into purple insoluble formazone.\(^{51}\) The absorption measurement of formazone compared to control directly reflects the cell viability.

![Reaction Scheme for MTT Assay](image)

**Figure 1.22.** Reduction of tetrazolium salt to formazone during MTT assay
1.5.3. Cell cycle analysis

The cell cycle is defined as the period between the successive division of a cell into two daughter cells. Eukaryotic cell division is divided into four different phases (G1, S, G2, and M). During G1, cells prepare for DNA replication and contain 2n chromosomes. During S phase, replication of DNA occurs, and there is a broad distribution of DNA content. By the end of S phase, cells should contain 4n chromosomes. Now they enter G2 phase, where they start to grow again for cell division. Once ready, they enter mitosis and divide into two daughter cells. At different phases of cell cycle, various cellular enzymes and machinery are specifically active. Cell cycle analysis helps to identify cell cycle arrest. The analysis is based on DNA labeling with propidium iodide (PI), a fluorescent dye. Flow cytometric analysis of cells containing PI-labeled DNA generates a cell population histogram in respect to PI fluorescent intensity. The amount of fluorescence is directly correlated to DNA content in the cell or different phase of the cell.

![Cell cycle](image)

**Figure 1.23.** Cell cycles (Left) and layout of cell cycle analysis plot from flow cytometry (Right)
1.5.4. FITC Annexin-V/PI Apoptosis assay

The process of apoptosis is a programmed cell death.\(^{53}\) It is a normal physiologic process for the removal of unwanted cells. During this process, translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the outer surface takes place.\(^{54}\) Annexin-V, a Ca\(^{2+}\)-dependent phospholipid-binding protein, has a high affinity for PS, and fluorochrome-labeled Annexin-V can be used for the detection of exposed PS using flow cytometry (A\(^{+}\)).\(^{55}\) The assay is a double staining process where propidium iodide (PI), a membrane impermeable dye is also used to stain cell. Since there is no compromise in membrane integrity, no PI can get into the cell, thus no fluorescence from PI (PI\(^{-}\)). In later stages of apoptosis, the integrity of cell membrane is compromised. As a result, both Annexin-V and PI fluorescence becomes positive.

\[\text{Figure 1.24. Different stages of Apoptosis: (Left) normal cell, PS is still located inside the plasma membrane; (Center) early apoptotic cell with Annexin V-FITC on exposed PS, (Right) Late apoptosis with ruptured plasma membrane, PI bound to DNA and Annexin V-FITC bound on PS}\]
1.5.5. ROS detection assay

The level of ROS in the cell can be detected using 2',7'-dichlorofluorescein diacetate (H2DCFDA-AM), which is a non-fluorescent dye.\(^{56}\) H2DCFDA-HA is non-toxic and can penetrate cell membrane easily. Once they get into the cell, cellular esterase cleaves this compound into H2DCF dye, which is also a non-fluorescent. If any ROS species are generated by the cell, these ROS can oxidize non-fluorescent dye into a fluorescent DCF dye. Flow cytometry or fluorescent microscopy tool can be used to detect the fluorescence intensity, which reflects the level of ROS generated in that period.

![Diagram of ROS detection by DCFDA dye](image)

**Figure 1.25.** Mechanism of ROS detection by DCFDA dye

1.5.6. Glutathione depletion assay

Glutathione plays a critical role in the cellular defense mechanism against oxidative and nitrosative stress in the mammalian cell.\(^{57}\) In early
stages of apoptosis, glutathione is diminished. The level of glutathione is
determined by reacting monochlorobimane (MCB), a thiol reactive
fluorescence dye (excitation/emission 380/461 nm). By incubating cell lysate
with MCB, the level of fluorescence intensity reflects the amount of
glutathione present.

![Glutathione-S-transferase reaction](image)

**Figure 1.26.** Mechanism of detection of glutathione depletion

1.5.7. Mitochondrial membrane potential measurement

In response to the electrical potential across the inner mitochondrial
membrane, several cationic dyes distribute into the mitochondria
electrophoretically. Rhodamine 123, Mito Tracker Red, and Mito Tracker
Green are some examples of those dyes. Upon incubation of these dyes
with cells, the fluorescence intensity is directly proportional to the membrane
potential. The fluorescence intensity can be detected via fluorescence
microscopy or flow cytometry.
Figure 1.27. The structure of rhodamine 123 (Left) and mitochondrial membrane showing membrane potential and site of distribution of rhodamine 123 (Right).

1.5.8. Caspase-3 activity assay

Caspases are released during the early stages of apoptosis either by the extrinsic or intrinsic pathways. The intrinsic pathway involves cellular stress and changes to mitochondria that release cytochrome c. The caspase cascade activation leads to the activation of activator caspase -3 and -7, which in turn cleave many cellular death target proteins. In this assay, the caspase-3 enzyme cleaves the substrate and converts it to fluorescence 7-amino-4-methyl coumarin (Figure 1.28). The fluorescence intensity is proportional to the activity of caspase-3.

Figure 1.28. Mechanism of detection of caspase activity
1.6. References


(3) Bentley, R., Different roads to discovery; Prontosil (hence sulfa drugs) and penicillin (hence beta-lactams). *J Ind Microbiol Biotechnol* 2009, 36 (6), 775-86.


CHAPTER 2

SAFE AND EASY ROUTE FOR THE SYNTHESIS OF 1,3-DIMETHYL-1,2,3-TRIAZOLIUM SALT AND INVESTIGATION OF ITS ANTICANCER ACTIVITIES

2.1. Abstract

We have developed a new safe and easy route for the synthesis of 1,3-dimethyl-1,2,3-triazolium derivatives. We have previously reported the synthesis of 4,9-dioxo-1,3-dimethylnaphtho[2,3-\(d\)][1,2,3]triazol-3-ium chloride from methylation of 1-methyl-1\(H\)-naphtho[2,3-\(d\)][1,2,3]triazole-4,9-dione. The synthesis of 1-methyl-1\(H\)-naphtho[2,3-\(d\)][1,2,3]triazole-4,9-dione is inefficient as a significant amount of by-product is formed that is difficult to separate and also unsafe as it requires the use of hazardous methyl azide as a starting material. It is, however, important to develop an improved method for the synthesis of 4,9-dioxo-1,3-dimethylnaphtho[2,3-\(d\)][1,2,3]triazol-3-ium salt due to its significant anticancer activities. Herein, we report a safe and convenient route for the synthesis of this compound, which lead to more detailed exploration of its profound anticancer activities. The compound synthesized in this new method shows significant anticancer activities against melanoma, colon cancer, non-small cell lung cancer and central nervous system (CNS) cancer with GI\(_{50}\) values ranging from low \(\mu\)M to nM.

\[^a\]Adapted with permission from [Shrestha, J. P.; Chang, C. W., *Bioorg Med Chem Lett* 2013, 23 (21), 5909-11.] Copyright 2013. Elsevier B.V.
2.2. Introduction

1,3-Dipolar cycloaddition of azides has attracted considerable interest due to its potential applications in various areas.\textsuperscript{1} This reaction is typically carried out by reacting alkynes with azides leading to the formation of 1,2,3-triazoles.\textsuperscript{2} In a similar fashion, we have reported the synthesis of 1-alkyl-1\textit{H}-naphtho[2,3-\textit{d}][1,2,3]triazole-4,9-diones, 4 via a tandem cycloaddition/oxidation of 1,4-naphthoquinone, 1 and alkyl azides, 3 (Scheme 2.1).\textsuperscript{3} Further alkylation of the cycloaddition/oxidation adducts using alkyl triflate, 5 forms the 4,9-dioxo-1,3-dialkynaphtho[2,3-\textit{d}][1,2,3]triazol-3-ium salts, 6 which can be viewed as cationic anthraquinone analogs.\textsuperscript{4}

\begin{center}
\textbf{Scheme 2.1.} Two-step synthesis of compound 4
\end{center}

Several of the cationic anthraquinone analogs exert interesting biological activities ranging from antibacterial, antifungal to anticancer. Except methyl azide and ethyl azide, the alkyl azides employed for this synthesis were prepared from a nucleophilic substitution of sodium azide and the corresponding alkyl halides, 2. Since small organic azides are known to be
explosive, we used methyl and ethyl 1-bromoacetate as the surrogate of methyl or ethyl halides.\textsuperscript{5} This method is, however, inefficient which offers a mixture of desired product and by-product. This mixture is hard to separate and not possible to scale-up.

2.3. Design and Synthesis of 1,3-dimethyl-1,2,3-triazolium salt via new route

In an attempt to resolve this problem, we have previously developed an alternative one-pot approach for the synthesis of the same cationic compounds by generating alkyl azides \textit{in situ} (Scheme 2.2).\textsuperscript{5-6} This method has the shortcoming of generating by-product 7, which is hard to be separated from the desired product, 4. Also from this study, a particular cationic anthraquinone analog, 4,9-dioxo-1,3-dimethylnaphtho[2,3-d][1,2,3]triazol-3-ium (R=R'=Me) was noted to manifest strong anticancer activities, in particular against melanoma, colon cancer, non-small cell lung cancer and central nervous system (CNS) cancer. Thus, there is an imminent need for an efficient and safer synthetic approach for the synthesis of this particular compound.

Alkyl azides that have the ratio of the sum of carbon and oxygen atoms to nitrogen atoms lower than three are considered too reactive or explosive and cannot be safely isolated.\textsuperscript{7} Therefore, 1,2,3-triazole substituted with shorter alkyl chains, such as methyl or ethyl, cannot be safely synthesized by using methyl azide or ethyl azide. In an effort to expand the library of cationic anthraquinone
analogs, we were surprised to find out that, upon methylation of a triazole adduct,

Scheme 2.2. One-pot synthesis of compound 4

1-p-methoxybenzyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione, 4a’, an unexpected product, NQM111 was obtained in good yield (Scheme 2.3). However, same methylation of 1-benzyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione, 4b’,5 yielded the expected product, 9 rather than compound NQM111. Therefore, we proposed that the electron-donating effect of p-methoxy render the p-methoxybenzyl group a good leaving group during methylation (Scheme 2.4). The resulted 1-methyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione, 10,5 can then be further methylated in the presence of excess MeOTf to form NQM111. Although no attempt has been made to isolate compound 10 during this process, we have previously demonstrated that NQM111 can be synthesized by methylation of compound 10 using MeOTf.4a

The relationship between electron-donating effect and leaving group capability was re-exemplified in another compound 4c’ containing a 2-
methoxyethoxymethyl group at the N-1 position (Scheme 2.5). Upon methylation using methyl triflate, \textbf{NQM111} was also obtained in good yield. This reaction proceeded, presumably, via similar mechanism and intermediate. In both syntheses, the initial cycloaddition/oxidation starts by using \(p\)-methoxybenzyl azide or 2-methoxyethoxymethyl azide rather than hazardous methyl azide and thus, is superior in providing \textbf{NQM111}. In fact, both of these syntheses can provide 1–2 g of \textbf{NQM111} in one batch. When necessary, the counterion, triflate can be exchanged for chloride using Dowex 1X (Cl\(^-\)) resin.

2.4. Anticancer Activity of \textbf{NQM111}

Following the success in the safe and scale-up synthesis of compound \textbf{NQM111}, more detailed investigation of the anticancer activities of \textbf{NQM111} was conducted through the Developmental Therapeutic Program (DTP) of National Cancer Institute (NCI). Compound \textbf{NQM111} is particularly active against melanoma, colon cancer, non-small cell lung cancer and central nervous system (CNS) cancer with GI\(_{50}\) (50% growth inhibition) values ranging from low \(\mu\)M to nM (Table 2.1-2.4).\(^8\) Continuing investigation of the anticancer activity of \textbf{NQM111} is currently being carried out also by NCI.
Scheme 2.3. Mechanism for multiple product formation in scheme 2
Scheme 2.4. New Method of synthesis of compound NQM111

Scheme 2.5. Proposed mechanism of NQM111 formation from 4a'
Scheme 2.6. Proposed mechanism of NQM111 formation from 4c’

Table 2.1: GI\textsubscript{50} values of NQM111 for different types of melanoma

<table>
<thead>
<tr>
<th>Melanoma</th>
<th>GI\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOX IMVI (Malignant)</td>
<td>0.50</td>
</tr>
<tr>
<td>MALME-3M (Malignant)</td>
<td>0.15</td>
</tr>
<tr>
<td>M14</td>
<td>0.68</td>
</tr>
<tr>
<td>MDA-MB-435 (Adenocarcinoma)</td>
<td>0.20</td>
</tr>
<tr>
<td>SK-MEL-2 (Malignant)</td>
<td>1.57</td>
</tr>
<tr>
<td>SK-MEL-28 (Malignant)</td>
<td>1.61</td>
</tr>
<tr>
<td>SK-MEL-5 (Malignant)</td>
<td>0.38</td>
</tr>
<tr>
<td>UACC-257</td>
<td>1.79</td>
</tr>
</tbody>
</table>
**Table 2.2:** $GI_{50}$ values of **NQM111** for different types of colon cancer

<table>
<thead>
<tr>
<th>Colon Cancer</th>
<th>$GI_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLO 205 (Adenocarcinoma)</td>
<td>1.01</td>
</tr>
<tr>
<td>HCC-2998 (Carcinoma)</td>
<td>1.24</td>
</tr>
<tr>
<td>HCT-116 (Carcinoma)</td>
<td>0.68</td>
</tr>
<tr>
<td>HCT-15 (Adenocarcinoma)</td>
<td>1.61</td>
</tr>
<tr>
<td>HT29 (Adenocarcinoma)</td>
<td>1.14</td>
</tr>
<tr>
<td>KM12 (Adenocarcinoma)</td>
<td>0.79</td>
</tr>
<tr>
<td>SW-620 (Adenocarcinoma)</td>
<td>1.15</td>
</tr>
</tbody>
</table>

**Table 2.3:** $GI_{50}$ values of **NQM111** for different types of non-small cell lung cancers

<table>
<thead>
<tr>
<th>Non-small Cell Lung Cancers</th>
<th>$GI_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549/ATCC (Adenocarcinoma)</td>
<td>0.32</td>
</tr>
<tr>
<td>EKVX (Adenocarcinoma)</td>
<td>1.21</td>
</tr>
<tr>
<td>HOP-62 (Adenocarcinoma)</td>
<td>1.54</td>
</tr>
<tr>
<td>NCI-H226 (Squamous)</td>
<td>0.51</td>
</tr>
<tr>
<td>NCI-H23 (Adenocarcinoma)</td>
<td>0.62</td>
</tr>
<tr>
<td>NCI-H322M (Carcinoma)</td>
<td>0.28</td>
</tr>
<tr>
<td>NCI-H460 (Large cell carcinoma)</td>
<td>0.30</td>
</tr>
<tr>
<td>NCI-H522 (Adenocarcinoma)</td>
<td>1.41</td>
</tr>
</tbody>
</table>
Table 2.4: GI<sub>50</sub> values of NQM111 for different types of CNS cancer

<table>
<thead>
<tr>
<th>CNS Cancer</th>
<th>GI&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-268 (Astrocytoma)</td>
<td>0.95</td>
</tr>
<tr>
<td>SF-295 (Gliolastoma-Multiforme)</td>
<td>1.05</td>
</tr>
<tr>
<td>SF-539</td>
<td>1.35</td>
</tr>
<tr>
<td>SNB-19 (Glioblastoma)</td>
<td>1.59</td>
</tr>
<tr>
<td>SNB-75 (Astrocytoma)</td>
<td>1.68</td>
</tr>
<tr>
<td>U251 (Glioblastoma)</td>
<td>1.27</td>
</tr>
</tbody>
</table>

2.5. Conclusion

In summary, we have successfully developed a convenient and safe route for the large-scale synthesis of 4,9-dioxo-1,3-dimethylnaphthо[2,3-d][1,2,3]triazol-3-ium triflate with significant anticancer activities. This new method is essential for providing sufficient amount of material for ongoing anticancer investigations *in vivo*, which could lead to the development of new chemotherapeutic agents. This protocol can also be applicable for the synthesis of other 1,3-dimethyl-1,2,3-triazolium salts of various applications without the use of potentially explosive methyl azide. Finally, this discovery can also augment the application of ‘Click’ chemistry that involves the use of small molecules of organic azide.
2.6. Experimental Section

**General procedure for the preparation of alkyl azides.** All the alkyl azides were prepared following the general procedure mentioned in ref 9.

**General procedure for [3+2] cycloaddition/oxidation: synthesis of compounds 4a’-c’.** To a solution of alkyl azides (estimated 3.2 mmol) in DMF (10 mL), 1,4-naphthoquinone (1.0 g, 6.4 mmol) was added, and the resulting solution was heated at 80 ºC for 2 days. After removal of solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred until the products precipitated. The precipitated products were collected with Hirsh funnel, washed with more diethyl ether (25 mL), and dried under vacuum. The yields were calculated based on the estimated amount of aryl azides used.

**General procedure for N-3 alkylation: synthesis of NQM111 and 9.** To a solution of compounds 3a-c (0.35 mmol) in anhydrous toluene (10 mL), methyl trifluoromethanesulfonate (0.15 mL, 1.40 mol) was added, and the resulting solution was heated at 100 ºC overnight. After removal of solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred for 30 min. The precipitated products were collected with Hirsh funnel, washed with more diethyl ether (25 mL), and dried under the vacuum.

**1-[(4-methoxyphenyl)methyl]-1H,4H,9H-naphtho[2,3-d][1,2,3]triazole-4,9-dione(4a).** This is a known compound and was synthesized according to our general procedures with 81% yield.
1-benzyl-1H,4H,9H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (4b'). This is also a known compound previously synthesized in our laboratory according to our general procedures.

1-[(2-methoxyethoxy)methyl]-1H,4H,9H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (4c'). This compound was synthesized according to the general procedures with 59% yield. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.3 - 8.4 (m, 1H), 8.2 - 8.3 (m, 1H), 7.8 - 7.9 (m, 2H), 6.2 (s, 2H), 3.8 - 3.9 (m, 2H), 3.5 - 3.6 (m, 2H), 3.3 (s, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 176.8, 175.2, 145.7, 135.4, 134.5, 133.9, 133.4, 128.0, 127.6, 79.1, 71.4, 70.1, 59.2. ESI/APCI calcd for C$_{14}$H$_{14}$N$_3$O$_4$+$^+$ (MH$^+$) m/z 288.0984; measured m/z 288.0985.

1-benzyl-3-methyl-4,9-dioxo-1H,4H,9H-naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (9). This is a known compound previously synthesized in our laboratory according to our general procedures.

1,3-dimethyl-4,9-dioxo-1H,4H,9H-naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (NQM111). This is a known compound and synthesized following our new general protocol with 99% yield from 4a and 81% yield from 4c'.
2.7. References


CHAPTER 3

SYNTHESIS, ANTICANCER STRUCTURE ACTIVITY RELATIONSHIP

INVESTIGATION OF CATIONIC ANTHRAQUINONE ANALOGS

3.1. Abstract

We have synthesized a series of novel 4,9-dioxo-4,9-dihydro-1\textit{H}-naphthol[2,3-\textit{d}][1,2,3]triazol-3-i-um salts, which can be viewed as analogs of cationic anthraquinones. Unlike the similar analogs that we have reported previously, these compounds show relatively weak antibacterial activities but exert strong anticancer activities (low \textmu M to nM GI\textsubscript{50}), in particular, against melanoma, colon cancer, non-small cell lung cancer and central nervous system (CNS) cancer. These compounds are structurally different from their predecessors by having the aromatic group, instead of alkyl chains, directly attached to the cationic anthraquinone scaffold. Further investigation of the structure–activity relationship (SAR) reveals the significant role of electron donating substituents on the aromatic ring in enhancing the anticancer activities via resonance effect. Steric hindrance of these groups is disadvantageous but is less influential than the resonance effect. The difference in the attached groups at the N-1 position of the cationic anthraquinone analog is the main structural factor for the switching of biological activity from antibacterial to anticancer. The discovery of these compounds may lead to the development of novel cancer chemotherapeutics.

\textsuperscript{a}Adapted with permission from [Shrestha, J. P.; Fosso, M. Y.; Bearss, J.; Chang, C. W. \textit{Eur J Med Chem} \textbf{2014}, \textit{77}, 96-102.] Copyright \textbf{2014}. Elsevier B.V.
3. Introduction

The development of novel antitumor agents has long been the focused area in cancer chemotherapy.\(^1\) Molecules bearing an anthraquinone motif have attracted great interest due to their potential uses as cancer chemotherapeutics.\(^2\) We have previously reported the synthesis of a library of cationic anthraquinone analogs, their antibacterial activities, and the mode of action study.\(^3\) Most of these compounds exhibit strong antibacterial activities against Gram-positive (G+) bacteria with minimum inhibitory concentrations (MIC's) lower than 1 µg/mL against *Staphylococcus aureus* (ATCC 25923). By comparison, two analogs, compounds 9 and NQM11, exert only modest antibacterial activity with MIC's of 2 and 4-8 µg/mL, respectively (Figure 3.1). However, these two compounds manifest significant anticancer activities with GI\(_{50}\) values ranging from low µM to nM in the five-dose 60 cell lines assay performed by National Cancer Institute (NCI). Further derivatization of Compound NQM11 will be relatively difficult. In addition, derivatives of compound 9 having substituents, such as a \(\rho\)-methoxy group on the benzene ring have been found to be unstable. Therefore, we designed compound 1c to serve as an analog of NQM11. Our initial goal is to investigate the importance of methylene group between the cationic anthraquinone scaffold and the benzene ring.

![Figure 3.1. Cationic Anthraquinone Analogs](image-url)
3.3. Design, Synthesis, and Evaluation of Anticancer Activities

Using commercially available 1,4-naphthoquinone (1) and phenyl azide (1A), compound 1c can be synthesized following the protocol reported previously (Scheme 3.1). The first step is a cycloaddition using excess naphthoquinone followed by an oxidation to yield compound 1b. Methylation of compound 1b provided compound 1c as a triflate salt, which can be converted into the corresponding chloride salt after ion-exchange column chromatography. To our delight, compound 1c displays anticancer activities comparable to 1, which proves that the methylene bridge between the cationic anthraquinone scaffold and aromatic ring can be omitted. Without the presence of this methylene group, it is expected that substituents at the ortho or para positions on the benzene ring of compound 1c will have higher electronic influence due to the resonance effect. Since ortho-substituents may cause higher steric hindrance toward the naphthoquinone core, we used compound 1c as the new lead and synthesized a library of derivatives of compound 1c with para-monosubstituted benzene rings.

Scheme 3.1. Synthesis of cationic anthraquinone analogs
The synthesis started with the preparation of the corresponding diazonium salts from para-substituted anilines, 2a-6a using NaNO₂ and HCl (Scheme 3.2), followed by a nucleophilic aromatic substitution using NaN₃ to obtain the desired para-substituted phenyl azides 2d-6d. Due to their relatively low boiling points, these aryl azides are used as concentrated solutions in ether without further purification or complete removal of solvents, which contribute to the relatively lower yields for the cycloaddition/oxidation step for the formation of compounds 2b-6b. Meanwhile, we have also discovered that the counter ion of the cationic anthraquinone analogs has no effect on the anticancer activities. Thus, these new members were prepared and tested as trifluoromethanesulfonate (OTf⁻) salts following the N-methylation step.

Scheme 3.2. Synthesis of cationic anthraquinone analogs with substituted aryl groups.
Compounds 3a-e were tested in the single dose 60 cell lines assay conducted through the Developmental Therapeutic Program (DTP) of NCI (Table 3.1). The results of mean cell growth percentages strongly indicate that the strong electron-donating methoxy group, as in compound 2c, significantly increases the anticancer activities. Compound 3c is less active than compound 2c because it has a moderate electron-donating methyl group that can only provide an inductive effect to increase the electron density on the benzene ring. Electron-withdrawing groups, such as trifluoromethyl and chloro groups, further decrease the activities. Interestingly, the fluoro derivative 6c bearing the most strongly electron-withdrawing group that we have introduced does not follow this trend. It is possible that smaller steric hindrance of the fluoro group attributes to the partial restoration of the anticancer activities.

**Table 3.1. Single dose 60 cell lines assay of compounds 1c-6c**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean cell growth percentage(^a)</th>
<th>N-1 Substituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>-10.18</td>
<td>Phenyl</td>
</tr>
<tr>
<td>2c</td>
<td>-24.70</td>
<td>4-methoxyphenyl</td>
</tr>
<tr>
<td>3c</td>
<td>11.53</td>
<td>4-methylphenyl</td>
</tr>
<tr>
<td>4c</td>
<td>58.69</td>
<td>4-trifluoromethylphenyl</td>
</tr>
<tr>
<td>5c</td>
<td>101.70</td>
<td>4-Chlorophenyl</td>
</tr>
<tr>
<td>6c</td>
<td>43.38</td>
<td>4-Fluorophenyl</td>
</tr>
</tbody>
</table>

\(^a\) = Mean cell growth percentage on NCI 60 cell line assay
After confirming the positive role of the strong electron-donating group, we decided to further explore the effect of having multiple methoxy groups. The designs and syntheses of these new members are shown in Scheme 3.3.

Although all the desired aryl azides can be prepared accordingly, the cycloaddition/oxidation step using aryl azides 10d and 11d offered products mixed with inseparable by-products. Therefore, only compounds 7a, 8a and 9a were converted to the corresponding cationic anthraquinone analogs 7c, 8c, and 9c, respectively.

Scheme 3.3. Synthesis of cationic anthraquinone analogs with substituted aryl groups (aryl group substituted with multiple methoxy group).

The results confirm the importance of resonance effect from the electron-donating methoxy group. For example, the methoxy groups attached at the meta positions of benzene ring on compound 7c can only increase the electron density of benzene ring and the cationic anthraquinone scaffold
through the inductive effect. However, the 3,5-dimethoxy groups increase the steric hindrance. As a result, the activities of \( 7c \) are even lower than \( 2c \). The methoxy group attached to the para position can increase the electron density of benzene ring and the cationic anthraquinone scaffold through the resonance effect. As predicted, both \( 8c \) and \( 9c \) showed better activities than \( 7c \) and even \( 2c \). With less steric hindrance, compound \( 9c \) is slightly more active than compound \( 8c \).

**Table 3.2.** Single dose 60 cell lines assay of compounds \( 7c-9c \)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean cell growth percentage</th>
<th>N-1 Substituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 7c )</td>
<td>23.23</td>
<td>3,5-dimethoxyphenyl</td>
</tr>
<tr>
<td>( 8c )</td>
<td>-27.06</td>
<td>3,4,5-trimethoxyphenyl</td>
</tr>
<tr>
<td>( 9c )</td>
<td>-31.51</td>
<td>3,4-dimethylphenyl</td>
</tr>
</tbody>
</table>

Compounds \( 1c-4c \) and \( 6c-9c \) were selected to five-dose cell lines assay also conducted through the DTP of NCI. Overall, these compounds displayed higher anticancer activities (low \( \mu \)M to nM \( GI_{50} \)) against melanoma, colon cancers, non-small cell lung cancers and central nervous system (CNS) cancers.\(^5\) The \( GI_{50} \) (50% growth inhibition) from the test results are summarized in Tables 3-6. In general, the activities are consistent with the results from single dose assay with \( 1c, 2c \) and \( 9c \) as the most active analogs. However, slight differences have been noted. For example, based on the average \( GI_{50} \), \( 9c \) is most active against melanoma, non-small cell lung cancer
and CNS cancer followed by either 2c or 1c whereas 1c and 2c are more active against colon cancer than 9c.

We have also noticed that certain cell lines appear to be less susceptible to these cationic anthraquinone analogs. For example, among the cell lines of melanoma, these compounds are more active against MDA-MB-435 than LOX IMVI. A similar tendency has also been found in the anticancer studies of spongistatin 1. Nevertheless, even with the available information on the mutations of cell lines, it is difficult to provide a satisfactory explanation for the difference. For instance, SK-MEL-5 that expresses mutant B-Raf (V600E) and wild-type N-Ras is sensitive to the anticancer action of cationic anthraquinone analogs. A similar cell line, SK-MEL-28 that also expresses mutant B-Raf (V600E) and wild-type N-Ras is much less susceptible to these compounds.

3.4. Discussion of Anticancer and Antibacterial SAR

We are currently in the process of investigating the possible anticancer mode of action and the potential cellular targets of these cationic anthraquinone analogs. Based on the structure-activity relationship (SAR) that has been revealed, we believe that electron density via the resonance effect and steric hindrance of the N-1 aryl groups play important roles in the anticancer activities. From the mean cell growth percentage and average GI50 values, a general tendency that indicates the presence of electron-donating substituents at the N-1 aryl group, enhance the anticancer activity when the tested compounds are arranged in order of decreasing electron density (Figures 3.2 and 3.3). Further analysis shows that only the groups that can
increase the electron density via the resonance effect can also enhance the anticancer activity. As mentioned previously, both compounds 7c and 3c, which contain electron-donating groups but cannot increase the electron density via the resonance effect, are less active than 1c, which has no substituent attached. This observation suggests the adverse steric effect in anticancer activity.

**Figure 3.2.** Comparison of mean cell growth percentage

**Figure 3.3.** Comparison of average GI (nM)
Table 3.3. Anticancer activities (G50) against melanoma

<table>
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<tr>
<th>Cell Line</th>
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<th></th>
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<td>3c</td>
<td>4c</td>
<td>6c</td>
<td>7c</td>
<td>8c</td>
<td>9c</td>
</tr>
<tr>
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<td>1.35</td>
<td>1.71</td>
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<td>0.35</td>
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<td>2.27</td>
<td>2.08</td>
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<td>12.8</td>
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</tr>
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<td>-</td>
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<td>0.17</td>
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<td>1.55</td>
<td>1.41</td>
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<td>1.22</td>
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<td>Average (G50)</td>
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<td>0.85</td>
<td>1.36</td>
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<td>1.22</td>
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Table 3.4. Anticancer activities (Glo) against Colon Cancer

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</tr>
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<td>1.13</td>
<td>1.52</td>
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<td>1.65</td>
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<td>1.35</td>
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<td>2.51</td>
<td>1.10</td>
<td>0.34</td>
<td>0.55</td>
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<tr>
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<td>A549/ATCC (Adenocarcinoma)</td>
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<td>2.34</td>
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<td>1.88</td>
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</table>

Table 3.5: Anticancer activities (Gbe) against non-small cell lung cancer
Table 3.6. Anticancer activities (G1so) against CNS cancer

<table>
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<th>Cell line</th>
<th>Compound (µM)</th>
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<td>SF-268 (Astrocytoma)</td>
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<td>SF-295 (Gliolastoma-Multiforme)</td>
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</tr>
<tr>
<td>SF-539</td>
<td>1.09</td>
</tr>
<tr>
<td>SNB-19 (Glioblastoma)</td>
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</tr>
<tr>
<td>SNB-75 (Astrocytoma)</td>
<td>0.64</td>
</tr>
<tr>
<td>U251 (Glioblastoma)</td>
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</tr>
<tr>
<td>Average G1so</td>
<td>1.04</td>
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</table>
Table 3.7. Anticancer activities (LC50s) against melanoma

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<th>Cell line</th>
<th>Compound (µM)</th>
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<td>LOX IMV1 (Malignant)</td>
<td>5.55</td>
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<tr>
<td>MALME-3M (Malignant)</td>
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<tr>
<td>M14</td>
<td>0.55</td>
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<tr>
<td>MDA-MB-435 (Adenocarcinoma)</td>
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<tr>
<td>SK-MEL-2 (Malignant)</td>
<td>6.44</td>
</tr>
<tr>
<td>SK-MEL-28 (Malignant)</td>
<td>6.47</td>
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<tr>
<td>SK-MEL-5 (Malignant)</td>
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<td>UACC-257</td>
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<td>UACC-62</td>
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### Table 3.8. Anticancer activities (LC50) against colon cancer

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<th>7c</th>
<th>8c</th>
<th>9c</th>
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<tbody>
<tr>
<td>COLO 205 (Adenocarcinoma)</td>
<td>0.62</td>
<td>0.96</td>
<td>4.43</td>
<td>5.73</td>
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<td>5.35</td>
<td>5.05</td>
<td>5.71</td>
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</tr>
<tr>
<td>HCC-2998 (Carcinoma)</td>
<td>3.69</td>
<td>4.72</td>
<td>5.40</td>
<td>12.9</td>
<td>8.22</td>
<td>8.99</td>
<td>8.22</td>
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<tr>
<td>HCT-116 (Carcinoma)</td>
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<td>41.9</td>
<td>8.19</td>
<td>48.6</td>
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<td>84.0</td>
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<td>ND</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>42.9</td>
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<td>&gt;100</td>
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### Table 3.9. Anticancer activities (LC50) against non-small cell lung cancer

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<td>A549/ATCC/Adenocarcinoma</td>
<td>5.46</td>
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<td>EKVX (Adenocarcinoma)</td>
<td>12.6</td>
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<tr>
<td>HOP-G2 (Adenocarcinoma)</td>
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</tr>
<tr>
<td>HOP-G2 (Large cell)</td>
<td>9.15</td>
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<td>NCI-H226 (Squamous)</td>
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<tr>
<td>NCI-H23 (Adenocarcinoma)</td>
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<td>NCI-H232M (Large cell)</td>
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<td>NCI-H322 (Adenocarcinoma)</td>
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Table 3.10. Anticancer activities (LC50) against CNS Cancer

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<td></td>
<td>1c</td>
</tr>
<tr>
<td>SF-266 (Astrocytoma)</td>
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</tr>
<tr>
<td>SF-295 (Glioblastoma-Multiforme)</td>
<td>4.97</td>
</tr>
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<td>SF-539</td>
<td>4.72</td>
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<tr>
<td>SNB-19 (Glioblastoma)</td>
<td>4.34</td>
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<tr>
<td>SNB-75 (Astrocytoma)</td>
<td>5.87</td>
</tr>
<tr>
<td>U251 (Glioblastoma)</td>
<td>4.33</td>
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</table>
We have reported the synthesis and antibacterial activity of a library of cationic anthraquinone analogs bearing two alkyl groups at the N-1 and N-3 positions (Figure 3.4).\textsuperscript{3b, 4} One of the lead compounds, \textbf{NQM108}\textsuperscript{3a} which is similar to compound \textbf{NQM111} but has an octyl group instead of a methyl group at the N-1 position, exerts only poor anticancer activities (mean cell growth percentage = 44.8\%, Table 3.11). These results are consistent with the negative effect of steric hindrance on the anticancer activities.

![Figure 3.4. Dialkylated cationic anthraquinone analogs](image)

**Figure 3.4.** Dialkylated cationic anthraquinone analogs

When comparing the SAR of anticancer and antibacterial activities (Table 3.11), we conclude that a small alkyl chain (CH\(_3\), entry 11, \textbf{NQM111}) or aromatic ring at the N-1 position increases the anticancer activities. A longer alkyl chain is crucial for antibacterial activities but not the anticancer activities (octyl, entry 12, \textbf{NQM108}).\textsuperscript{3a, 3b} Overall, reduced steric hindrance and increased electron density via resonance are crucial for tuning the biological activity of cationic anthraquinone analogs from being antibacterial to anticancer and vice versa. Although we are investigating the possible modes of anticancer activity and targets, we have noted that \textbf{NQM111} also has a
similar profile of anticancer activities: particularly active against melanoma, colon cancer, non-small cell lung cancer, and CNS cancer. This interesting finding implies that the cationic anthraquinone is the structural motif responsible for the anticancer activity and that the N-1 substituent plays an auxiliary role. It also points to two possible modes of action: intercalation of nucleic acids and disruption of cellular redox processes. The former is favored by small steric hindrance as in the case of NQM111, and the latter can be affected by the electronic property (redox potential) of the compounds.

Finally, for compounds with substituted benzene rings at N-1 position, we have summarized the SAR as following: 1) a methylene group between the benzene and triazole moieties is not needed 2) electron-donating substituents at the para position of the benzene ring increase the anticancer activities, and 3) reduced steric hindrance is beneficial for anticancer activities.
Table 3.11. Comparison of antibacterial and anticancer activities

<table>
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<tr>
<th>Entry</th>
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<th>MIC against S. aureus $^a$ (µg/mL)</th>
<th>Mean cell growth percentage (%)</th>
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$^a$ Staphylococcus aureus (ATCC 25923)

3.5. Conclusion

In conclusion, we have developed a concise synthesis of cationic anthraquinone analogs bearing aryl substituents. The simplicity in the synthesis can enable a scale-up production when needed and greatly
increase the chance of developing practical therapeutics. Investigation of the SAR also reveals the structural factors for tuning the biological activities (anticancer vs. antibacterial) of these compounds. These discoveries may lead to the development of novel anticancer therapeutics. It will also help to the development of antibacterial agents with less cytotoxicity.

3.6. Experimental Section

**General procedure for the preparation of aryl azides.** To a solution of substituted anilines (46.7 mmol) in water (50 mL), conc. HCl (11.2 mL, 37% v/v) was added. The reaction mixture was cooled to 0°C and NaNO₂ (46.7 mol) dissolved in 10 mL water was added. After being stirred for 10 min., NaN₃ (60.0 mmol) was added portion wise. The mixture was stirred for 1.5 h at r.t. Formation of an oily compound was observed. The mixture was extracted with ether (3 x 25 mL), and then the ether extracts were washed with water (2 x 50 mL) and once with brine (50 mL). The organic layer was dried over anhydrous MgSO₄ or Na₂SO₄, filtered and concentrated to about 1/10 of the original volume. The resulting aryl azides were used without further purification.

**General procedure for [3+2] cycloaddition/oxidation: synthesis of compounds 1b-9b.** To a solution of aryl azides (estimated 3.2 mmol) in DMF (10 mL), 1,4-naphthoquinone (1.0 g, 6.3 mmol) was added, and the resulting solution was heated at 60°C for 2 days. After removal of the solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred until the products precipitated. The precipitated products were collected with a Hirsh
funnel, washed with more diethyl ether (25 mL), and dried under vacuum. The yields were calculated based on the estimated amount of aryl azides used.

1-Phenyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (1b). This is a known compound\(^9\) and was synthesized according to our general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 27% yield. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.4 (m, 1H), 8.2 (m, 1H), 7.8 - 7.9 (m, 2H), 7.7 - 7.8 (m, 2H), 7.6 - 7.7 (m, 3H). \(^1\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 177.2, 174.5, 146.2, 135.5, 135.4, 134.7, 133.6, 133.4, 133.3, 131.1, 129.5 (2C), 128.0, 127.9, 125.3 (2C).

1-(4-Methoxyphenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (2b). This is a known compound\(^10\) was synthesized according to our general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 33% yield.

1-p-Tolyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (3b). This compound was synthesized according to the general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 45% yield. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.38 (dd, \(J = 7.5, 1.4\) Hz, 1H), 8.21 (dd, \(J = 7.4, 1.4\) Hz, 1H), 7.84 (m, 2H), 7.61 (d, \(J = 8.4\) Hz, 2H), 7.41 (d, \(J = 8.2\) Hz, 2H), 2.50 (s, 3H); \(^1\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 177.1, 174.4, 145.6, 141.4, 135.2, 134.6, 133.4, 133.3, 133.2, 132.9, 129.9 (2C), 127.8, 127.7, 124.9 (2C), 21.5; ESI/APCI calcd for C\(_{17}\)H\(_{11}\)N\(_3\)O\(_2\)^+ ([MH]^+) m/z 290.0924; measured m/z 290.0931.

1-(4-Trifluoromethylphenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (4b). This compound was synthesized according to the general procedures for
the preparation of aryl azides and [3+2] cycloaddition/oxidation with 30% yield. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.4 (m, 1H), 8.2 (m, 1H), 7.8 - 8.0 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 176.5, 174.5, 146.0, 133.8, 133.0, 133.7, 135.6, 134.9, 133.2, 128.1, 127.9, 126.7 (2C), 125.7 (2C); ESI/APCI calcd for C$_{17}$H$_9$F$_3$N$_3$O$_2$ $^+$ ([MH]$^+$) m/z 344.0641; measured m/z 344.0643.

1-(4-Chlorophenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (5b). This compound was synthesized according to the general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 32% yield. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.37 (dd, $J = 7.6, 1.7$ Hz, 1H), 8.21 (dd, $J = 7.6, 1.7$ Hz, 1H), 7.83 (dq, $J = 7.6, 1.7$ Hz, 2H), 7.6 - 7.7 (m,2H), 7.5 - 7.6 (m, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 176.9, 174.9, 146.1, 137.1, 135.4, 134.8, 134.7, 133.8, 133.4, 133.1, 129.6 (2C), 127.9, 127.7, 126.4 (2C); ESI/APCI calcd for C$_{16}$H$_9$ClN$_3$O$_2$ $^+$ ([MH]$^+$) m/z 310.0378; measured m/z 310.0381.

1-(4-Fluorophenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (6b). This compound was synthesized according to the general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 30% yield. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.38 (dd, $J = 7.6, 1.7$ Hz, 1H), 8.21 (dd, $J = 7.5, 1.4$ Hz, 1H), 7.8 - 7.9 (m, 2H), 7.7 - 7.8 (m, 2H), 7.3 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 177.1, 174.5, 163.9 (d, $J_{C-F} = 251$ Hz), 146.1, 135.5, 134.8, 133.8, 133.5, 133.2 (2C), 131.5, 128.0, 127.9, 127.4 (d, $J_{C-F} = 9$ Hz, 2C), 116.6 (d, $J_{C-F} = 23.5$ Hz, 2C). ESI/APCI calcd for C$_{16}$H$_9$FN$_3$O$_2$ $^+$ ([MH]$^+$) m/z 394.0673; measured m/z 394.0678.
1-(3,5-Dimethoxyphenyl)-1\textit{H}-naphtho[2,3-d][1,2,3]triazole-4,9-dione (7b).
This compound was synthesized according to the general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 41% yield. \textit{\textsuperscript{1}}\textit{H} NMR (3200 MHz, CDCl\textsubscript{3}) \(\delta\) 8.37 (d, \(J = 7.3\) Hz, 1H), 8.22 (d, \(J = 8.2\) Hz, 1H), 7.8 – 7.9 (m, 2H), 6.88 (d, \(J = 1.9\) Hz, 2H), 6.6 – 6.7 (m, 1H), 3.87 (s, 6H); \textit{\textsuperscript{13}}\textit{C} NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) 177.2, 174.3, 161.2, 146.1, 136.8, 135.3, 134.7, 133.5 (3C), 127.9, 103.7, 103.1, 56.0 (2C); ESI/APCI calcd for C\textsubscript{18}H\textsubscript{14}N\textsubscript{3}O\textsubscript{4}\textsuperscript{+} ([\textit{MH}]\textsuperscript{+}) m/z 336.0979; measured m/z 336.0971.

1-(3,4,5-Trimethoxyphenyl)-1\textit{H}-naphtho[2,3-d][1,2,3]triazole-4,9-dione (8b). This compound was synthesized according to the general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 43% yield. \textit{\textsuperscript{1}}\textit{H} NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 8.7 (dd, \(J = 1.7, 7.2\) Hz, 1H), 8.04 (dd, \(J = 1.7, 6.8, 1\)H), 7.8 – 7.9 (m, 2H), 7.01 (s, 2H), 3.97 (s, 3H), 3.95 (s, 3H); \textit{\textsuperscript{13}}\textit{C} NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 177.1, 174.3, 153.4 (2C), 145.9, 139.9, 135.3, 134.6, 133.3, 133.0, 130.7, 127.8 (2C), 102.8 (2C), 61.2, 56.6; ESI/APCI calcd for C\textsubscript{19}H\textsubscript{16}N\textsubscript{3}O\textsubscript{5}\textsuperscript{+} ([\textit{MH}]\textsuperscript{+}) m/z 366.1084; measured m/z 366.1084.

1-(3,4-Dimethoxyphenyl)-1\textit{H}-naphtho[2,3-d][1,2,3]triazole-4,9-dione (9b). This compound was synthesized according to the general procedures for the preparation of aryl azides and [3+2] cycloaddition/oxidation with 45% yield. \textit{\textsuperscript{1}}\textit{H} NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 8.6 (d, \(J = 7.2\) Hz, 1H), 8.21 (d, \(J = 7.6\) Hz, 1H), 7.8 (m, 2H), 7.32 (d, \(J = 8.6\) Hz, 2H), 7.03 (d, \(J = 8.6\) Hz, 1H), 3.99 (s, 3H), 3.96 (s, 3H); \textit{\textsuperscript{13}}\textit{C} NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 177.1, 174.4, 150.9, 149.2, 145.9, 135.2, 134.5, 133.3, 133.1, 128.2, 127.8 (3C), 117.7, 110.7, 108.6, 56.4, 56.3;
ESI/APCI calcd for C_{18}H_{14}N_{3}O_{4}^{+} ([MH]^{+}) m/z 336.0979; measured m/z 336.098.

**General procedure for N-3 alkylation: synthesis of compounds 1c-9c.**

To a solution of compounds 1b-9b (0.35 mmol) in anhydrous toluene (10 mL), methyl trifluoromethanesulfonate (0.15 mL, 1.38 mol) was added, and the resulting solution was heated at 100˚C overnight. After removal of the solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred for 30 min. The precipitated products were collected with a Hirsh funnel, washed with more diethyl ether (25 mL), and dried under the vacuum.

**4,9-Dioxo-3-methyl-1-phenynaphtho[2,3-d][1,2,3]triazol-3-ium chloride (1c).** This compound was synthesized according to the general procedure for N-3 alkylation with 74% yield. $^1$H NMR (300 MHz, CD$_3$OD) δ 8.4 (m, 1H), 8.3 (m, 1H), 7.9 - 8.1 (m, 4H), 7.8 - 7.9 (m, 1H), 7.7 - 7.8 (m, 2H), 4.83 (s, 3H); $^{13}$C NMR (75 MHz, CD$_3$OD) δ 172.7, 171.0, 136.2, 136.1, 135.9, 138.8, 133.9, 133.1, 132.8, 132.3, 129.6 (2C), 128.0, 127.7, 125.8 (2C), 40.3; ESI/APCI calcd for C$_{17}$H$_{12}$N$_3$O$_2$ $^{+}$ ([M$^{+}$]) m/z 290.0924; measured m/z 290.0924.

**4,9-Dioxo-1-(4-methoxyphenyl)-3-methylnaphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (2c).** This compound was synthesized according to the general procedure for N-3 alkylation with 80% yield. $^1$H NMR (300 MHz, CD$_3$OD) δ 8.4 (m, 1H), 8.3 (m, 1H), 8.0 - 8.1 (m, 2H), 7.8 - 7.9 (m, 2H), 7.2 - 7.3 (m, 2H), 4.81 (s, 3H), 3.98 (s, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 172.9, 171.1, 163.4, 136.2 (2C), 135.9, 135.6, 133.2, 132.3, 128.1, 127.7,
127.2 (2C), 126.3, 114.7 (2C), 55.4, 40.2; ESI/APCI calcd for C₁₈H₁₄N₃O₃⁺ ([M⁺] m/z 320.1030);

4,9-Dioxo-3-methyl-1-<p>-tolyl-naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (3c). This compound was synthesized according to the general procedure for N-3 alkylation with 79% yield. ¹H NMR (300 MHz, CD₃OD) δ 8.4 (m, 1H), 8.3 (m, 1H), 8.0 (m, 21H), 7.76 (d, J = 8.5 Hz, 2H), 7.57 (d, J = 8.2 Hz, 2H), 4.81 (s, 3H), 2.54 (s, 3H); ¹³C NMR (75 MHz, CD₃OD) δ 172.8, 171.0, 144.1, 136.1, 135.8, 133.6, 131.9, 130.6 (2C), 130.1, 127.9, 127.6, 125.2, 121.2, 65.6, 40.0, 20.1, 14.1; ESI/APCI calcd for C₁₈H₁₄N₃O₂⁺ ([M⁺] m/z 304.1081; measured m/z 304.1081.

4,9-Dioxo-3-methyl-1-(4-trifluoromethylphenyl)naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (4c). This compound was synthesized according to the general procedure for N-3 alkylation with 90% yield. ¹H NMR (300 MHz, CD₃OD) δ 8.3 (m, 1H), 8.1 – 8.2 (m, 1H), 7.8 - 8.0 (m, 6H), 4.67 (s, 3H); ¹³C NMR (100 MHz, CD₃OD) δ 172.6, 171.1, 136.6, 136.3, 136.2, 132.9, 132.3, 128.2, 127.9, 127.1 (2C), 126.9, 40.5; ESI/APCI calcd for C₁₈H₁₁F₃N₃O₂⁺ ([M⁺] m/z 358.0798; measured m/z 358.0788.

1-(4-Chlorophenyl)-4,9-dioxo-3-methylnaphtho[2,3-d][1,2,3] triazol-3-ium trifluoromethanesulfonate (5c). This compound was synthesized according to the general procedure for N-3 alkylation with 99% yield. ¹H NMR (300 MHz, CD₃OD) δ 8.1 - 8.2 (m, 1H), 7.9 (m, 2H), 7.7 - 7.9 (m, 3H), 7.7 (m, 2H), 4.76 (s, 3H). ¹³C NMR (75 MHz, CH₃OD) δ 172.5, 170.9, 139.2, 136.2, 135.9, 135.8, 132.9, 132.2 (2C), 129.9 (2C), 128.0, 127.7, 127.2 (2C), 40.3;
ESI/APCI calcd for $\text{C}_{17}\text{H}_{11}\text{ClN}_{3}\text{O}_{2}^+$ ([M]$^+$) m/z 324.0534; measured m/z 324.0544.

1-(4-Fluorophenyl)-4,9-dioxo-3-methylnaphtho[2,3-$d$][1,2,3]triazol-3-ium trifluoromethanesulfonate (6c). This compound was synthesized according to the general procedure for N-3 alkylation with 69% yield. $^1$H NMR (300 MHz, CD$_3$OD) δ 8.4 (m, 1H), 8.3 (m, 1H), 8.0 - 8.1 (m, 2H), 7.9 - 8.0 (m, 2H), 7.9 - 8.0 (m, 2H), 7.5 (m, 2H), 4.80 (s, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 171.2, 169.7, 163.7 (d, $J_{C,F}$ = 252 Hz), 134.7(2C), 134.5 (2C), 131.5, 128.4, 127.0 (d, $J_{C,F}$ = 9 Hz, 2C), 126.6, 126.3, 115.3, (d, $J_{C,F}$ = 24.1 Hz, 2C), 38.8. ESI/APCI calcd for $\text{C}_{17}\text{H}_{11}\text{FN}_{3}\text{O}_{2}^+$ ([M]$^+$) m/z 308.0832; measured m/z 308.0838.

1-(3,5-Dimethoxyphenyl)-4,9-dioxo-3-methylnaphtho[2,3-$d$][1,2,3]triazol-3-ium trifluoromethanesulfonate (7c). This compound was synthesized according to the general procedures for N-3 alkylation with 95% yield. $^1$H NMR (300 MHz, CD$_3$OD) δ 8.3-8.4 (m, 1H), 8.04 (m, 1H), 8.0 (m, 2H), 7.04 (d, $J = 2.4$ Hz, 2H), 6.91 (t, $J = 2.0$ Hz, 1H), 4.81 (s, 3H), 3.88 (s, 6H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 173.3, 171.4, 161.4 (2C), 137.1, 136.7, 135.9, 135.0, 133.4, 132.1, 128.5, 128.2, 104.7 (2C), 56.7 (2C), 40.3; ESI/APCI calcd for $\text{C}_{19}\text{H}_{16}\text{N}_{3}\text{O}_{4}^+$ ([M]$^+$) m/z 350.1135; measured m/z 350.1137.

4,9-Dioxo-3-methyl-1-(3,4,5-trimethoxyphenyl)naphtho[2,3-$d$][1,2,3]triazol-3-ium trifluoromethanesulfonate (8c). This compound was synthesized according to the general procedure for N-3 alkylation with 99% yield. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.4 (m, 1H), 8.3 (m, 1H), 8.0 (m, 2H), 7.19 (s, 2H), 4.7-4.8 (m, 3H), 3.88 (s, 9H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ
173.4, 171.5, 153.7, 148.4 (2C), 139.9, 137.0, 136.6, 135.9, 133.4, 132.2, 128.6, 128.1, 124.2, 104.1 (2C), 60.9, 57.1 (2C), 40.3. ESI/APCI calcd for C_{20}H_{18}N_{3}O_{5}^{+} ([M]^{+}) m/z 380.1241; measured m/z 380.1231.

**1-(3,4-Dimethoxyphenyl)-4,9-dioxo-3-methylnaphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (9c).** This compound was synthesized according to the general procedure for N-3 alkylation with 94% yield. $^1$H NMR (300 MHz, DMSO-$_d$6) δ 8.3 (m, 1H), 8.2 (m, 1H), 8.0 – 8.1 (m, 2H), 7.56 (s, 1H), 7.50 (dd, J = 2.0, 8.6 Hz, 1H), 7.33 (dd, J = 8.6 Hz, 1H), 4.75 (s, 3H), 3.90 (s, 3H), 3.80 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-$_d$6) δ 173.4, 171.5, 152.5, 149.4, 137.0, 136.6, 136.1, 136.0, 133.4, 132.2, 128.5, 128.2, 123.2, 118.9, 112.1, 109.5, 56.7, 56.6, 41.7. ESI/APCI calcd for C$_{19}$H$_{16}$N$_{3}$O$_{4}$ ([M]^{+}) m/z 350.1135; measured m/z 350.1129.

**Procedure for MIC determination**

A solution of selected bacteria (*S. aureus*) was inoculated in the Trypticase Soy broth at 35°C for 1 - 2 h, after which the bacterial concentration was found and diluted with broth, if necessary, to an absorption value of 0.08 to 0.1 at 625 nm. The adjusted inoculated medium (100 µL) was diluted with 10 mL of broth, and then applied to a 96-well microtiter plate (50 µL). A series of solutions (50 µL each in 2-fold dilution) of the tested compounds was added to the testing wells. The 96-well plate was incubated at 35 °C for 12 - 18 h. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound needed to inhibit the growth of bacteria. The MIC results are repeated at least three times.
3.7. References


(8) Shrestha, J. P.; Chang, C. W., Safe and easy route for the synthesis of 1,3-dimethyl-1,2,3-triazolium salt and investigation of its anticancer activities. *Bioorg med chem lett* **2013**, *23* (21), 5909-11.


CHAPTER 4

A MODE OF ACTION STUDY OF CATIONIC ANTHRAQUINONE ANALOGS: A
NEW CLASS OF HIGHLY POTENT ANTICANCER AGENTS

4.1. Abstract

Previously we reported the synthesis and structure-activity relationship (SAR) study of a series of novel 4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium salts, which had very potent anti-proliferative activities (low µM to nM GI$_{50}$) against a broad range of cancer cells. These compounds, which can be viewed as cationic anthraquinone analogs (CAA's), are selective against cancer cells over bacteria or fungi as compared to the antibacterial CAA's that have also been reported by our group. Herein, we report the mode of action study of CAA's, which reveals at these compounds trigger apoptosis by generating extensive reactive oxygen species (ROS). The generation of extensive ROS species causes oxidative stress, a decrease in mitochondrial membrane potential, depletion of glutathione (GSH), and release of caspase-3; which ultimately kills cancer cells by programmed apoptosis. Furthermore, we have also showed that CAA's have an activity of 8-fold higher against A549 cell over the non-cancerous MRC-5 cell.

\textsuperscript{a}Adapted with permission from [Shrestha, J. P.; Subedi, Y. P.; Chen, L.; Chang, C.-W. T. MedChemComm 2015, 6 (11), 2012-2022. Copyright 2014. The Royal Society of Chemistry.]
4.2. Introduction

The development of novel antitumor agents has long been a focused area in cancer chemotherapy.\(^1\) Molecules bearing quinone motifs have attracted great interest due to their potential uses as cancer chemotherapeutics.\(^2\) Several anticancer drugs containing quinone motifs, e.g. daunomycin, doxorubicin, mitomycin C, and mitoxantrone (Figure 4.1, A), have been approved by the U.S. Food and Drug Administration (FDA) for clinical use. Natural compounds containing quinone motifs, such as cribrostatin, streptonigrin, and β-lapachone, are further being studied for their anti-cancer activities,\(^3\) of which streptonigrin and β-lapachone are already being used as experimental drugs. Our laboratory is dedicated to synthesizing small molecules containing 1,4-naphthoquinone or other heterocyclic motifs as antimicrobial or anticancer drugs. These compounds can be prepared in 2–3 steps from an inexpensive starting material (1,4-naphthoquinone), and the synthetic protocol can be scaled up for large quantities (5–10 g) without the requirement of cumbersome purification, such as column chromatography. Furthermore, various anionic counter ions of these compounds can be conveniently exchanged to improve the bioavailability. Previously, we have reported the syntheses of two different sets of CAAs that possess predominantly either antibacterial activities or anticancer activities.\(^4\) The first set of compounds (Fig. 4.1,B, left) exhibits strong antibacterial activity against Gram-positive (G\(^+\)) bacteria with minimum inhibitory concentrations (MICs) lower than 1 μg mL\(^{-1}\) against Staphylococcus aureus (ATCC 25923). The second set of compounds (Figure 4.1, B, right) exhibits strong anti-cancer activity with GI\(_{50}\)
values ranging from low \(\mu\)M to nM in the five-dose 60-cell line assay performed by the National Cancer Institute (NCI). By comparison, the first set of compounds only possesses strong antibacterial activity and weak anti-cancer activity, whereas the second set of compounds exhibits strong anticancer activity and weak antibacterial activity. The syntheses and SAR studies have already been reported for both sets of compounds.\(^4\) The SAR study has revealed that an 8-carbon alkyl chain at the 3-N position is the optimum chain length for antibacterial activity, whereas the electron donating group with low steric hindrance at the 3-N aryl group is the best group for anticancer activity. More SAR study details are presented in Figure 4.1, B; and a mode of action study for the first set of antibacterial compounds have already been reported.\(^5\) Herein, we wish to report the possible modes of action for the second set of compounds that possess anticancer activity.

**Figure 4.1.** (A) FDA-approved anticancer drugs containing quinone motifs; (B) Cationic 1,4-naphthoquinone analogs from our laboratory

\(^{4}\) SAR Study: When \(Z = EDG\), very active; When \(Z = EWG\), not active or moderately active; When \(Z = \) multiple group, steric hindrance lowers the bioactivity. Compound 2c (scheme 1) with strong electron donating group and low seric hindrance is the ideal compound.
Several studies have shown that redox cycling properties of quinone are responsible for their anti-cancer activities. One-electron reduction of quinone forms a semiquinone radical and two-electron reduction of quinone forms...
dihydroquinone. Both the semiquinone radical and the dihydroquinone can be re-oxidized back to their parent quinone by molecular oxygen forming ROS in a redox cycle.\(^7\) The superoxide anions formed during the redox cycle are known to cleave DNA, peptides, and proteins.\(^8\) Once cells are unable to repair these damages, apoptosis is triggered. The redox cycle can be facilitated by cellular reductases, electron leakage from mitochondria, or another hydroquinone.\(^9\) Our mode of action studies focuses on the quantification of cationic 1,4-naphthoquinone analogs that induced ROS, oxidative stress, mitochondrial membrane potential, glutathione (GSH) and caspase-3, all of which are related to programmed apoptosis.

4.3. Results and discussion

4.3.1. Cell viability analysis

All the compounds (1c–9c) were tested against NCI-60 cell lines, and their GI\(_{50}\) and LC\(_{50}\) values were reported in our earlier publication.\(^4c\) For the purpose of the mode of action study, here we chose to study the growth-inhibitory effect of compound 2c on the human lung alveolar epithelial cancer cell line (A549) and lung fibroblast cell line (MRC-5) as the control. Various doses of compound 2c were added to cells for 48 h of incubation, and the curve of cell growth was determined by means of a 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC\(_{50}\) (4.2 μM) value obtained under our experimental conditions is much higher than the GI\(_{50}\) (0.34 μM) value determined by the NCI. The growth medium used in our experiment (DMEM 1×) has a higher level of
glutathione than the medium used by the NCI (RPMI), and the higher level of glutathione is believed to be responsible for the observed higher IC\textsubscript{50} value. Similarly, the IC\textsubscript{50} value obtained for etoposide is 3.9 μM (Table 4.1) for the A549 cell line under similar conditions and was found to be at least 4 fold higher than the reported GI\textsubscript{50} (0.99 μM) value from the NCI. As shown in Figure 4.2, compound 2c exhibits a strong cytotoxic effect on the A549 cell line and moderate cytotoxic effects on the MRC-5 cell line. The selectivity is at least 8 fold higher for the A549 cell line vs. the MRC-5 cell line. This selective cytotoxic effect on the assayed cancer cell line greatly enhances the potential of these CAAs as chemotherapeutic agents. As a control experiment, we also tested the anticancer activity of antibacterial compound NQM108 on A549 under similar conditions as 2c. The compound showed very low cytotoxicity with an IC\textsubscript{50} value of 83.33 μM.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC\textsubscript{50} 2c</th>
<th>IC\textsubscript{50} Etoposide</th>
<th>IC\textsubscript{50} NQM108</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>4.2 ± 0.5</td>
<td>3.9</td>
<td>83 ± 15</td>
</tr>
<tr>
<td>MCR-5</td>
<td>35 ± 9.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 4.2.** 2c, NQM108, and etoposide dose-response curves
4.3.2. Compound 2c does not affect the cell cycle

Many anticancer compounds arrest cells in a certain phase of a cell cycle, for instance, cisplatin arrests cells in the G2 phase,\textsuperscript{10} doxorubicin arrests cells in the G2/M phase,\textsuperscript{11} camptothecin arrests cells in the G2 phase\textsuperscript{12} and some anticancer compounds do not arrest cells at all.\textsuperscript{13} The cell cycle arrest provides some mechanistic information on the mode of action, which makes this assay very important. The cell cycle analysis was carried out after incubation with different concentrations of compound 2c over 24 h. The cells were then analyzed by flow cytometry. The cell cycle analysis showed no significant effect on any stages of the cell cycle (Figure 4.4), and the percentage of cells in each phase of the cell cycle was not affected by drug concentration. This result also indicates that the mode of action of CAAs is not related to growth and/or growth regulating factors. These compounds are also unlikely to be topoisomerase inhibitors because all the reported topoisomerase inhibitors (e.g., doxorubicin and camptothecin) are known to arrest the cell cycle at a certain phase. For example, cribrostatin 6 does not affect the cell cycle and does not inhibit topoisomerase. Even though compounds arresting the cell cycle are desirable for a specific target, they are not very useful for targeting quiescent cells mostly present in the tumor.
4.3.3. Compound 2c induces apoptosis in cancer cells

FITC annexin-V (A)/propidium iodide (PI) double staining assay was utilized to study the mode of cell death. The assay takes advantage of the earliest apoptotic process, when phosphatidylserines (PS) from the inner side of the plasma membrane translocate to the outer surface. FITC annexin V is a
fluorochrome dye conjugated with Ca\(^{2+}\) dependent phospholipid-binding protein with a higher affinity for PS. This high affinity for FITC annexin-V allows detection of early apoptotic cells.\(^{14}\) Once the integrity of the cell membrane is compromised, the PI can get inside the cell and bind with DNA. The level of PI intake directly correlates with the severity of membrane damage. The cells were treated with various concentrations of \(2c\) (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 μM) over 24 h and then analyzed by flow cytometry for the cells stained with annexin-V and PI (Figure 4.5). Three distinctive populations of apoptotic cells (gate P12, \(A^+\)/PI\(^{-}\); gate 13, \(A^+\)/PI\(^{+}\); and gate P14, \(A^+\)/PI\(^{++}\); Figure 4.5) were observed. The total number of apoptotic cells increased from 7.70% to 8.74%, 11.06%, 11.07%, 35.44%, and 61.28% at 0.00 μM, 0.01 μM, 0.1 μM, 1.0 μM, 10 μM, and 100 μM concentrations of \(2c\), respectively. The progression of the apoptosis started from healthy cells (\(A^-\)/PI\(^{-}\)) to early apoptosis (\(A^+/\)PI\(^{-}\)) followed by late apoptosis (\(A^+/\)PI\(^{+}\)) and finally necrosis (\(A^+/\)PI\(^{++}\)). This process is a very clear indication of cell death via apoptosis. The progression trend of the apoptosis cycle can be observed in the bar diagram (Figure 4.5, B). The general morphological characteristics of apoptosis such as cell shrinkage, nuclear condensation, and mitochondrial membrane damage can be observed in Figure 4.6.
Figure 4.4. Annexin-V/PI assay for apoptosis (A and B), * = P ≤ 0.05, and **** = P ≤ 0.0001
Figure 4.5. (A) Morphological changes in A549 cells treated with 2c; (B) morphological changes in the A549 cell nucleus and mitochondria treated with 2c
4.3.4. Compound 2c induces oxidative stress by ROS generation

It is well documented that due to rapid metabolic processes, a level of endogenous ROS is elevated in cancer cells compared to normal healthy cells.\textsuperscript{15} Further elevation of the exogenous ROS pushes oxidatively stressed cancer cells over a critical redox threshold triggering apoptosis.\textsuperscript{16} Quinone motif-containing compounds (e.g., adaphostin, menadione, daunorubicin, and doxorubicin) are known to kill cancer cells by further elevating the ROS level. Selective killing of cancer cells over healthy cells is possible through this pathway by increasing the amount of ROS that pushes cancer cells over their critical threshold, while normal cell lines can survive this ROS level.\textsuperscript{16-17} The level of ROS production in A549 cells was determined by using a dichloro-dihydro-fluorescein diacetate (DCFH-DA) dye. The non-fluorescent DCFH-DA dye is cleaved by cellular esterase in the cell which upon oxidation forms a highly fluorescent adducts. The cells were then analyzed by flow cytometry for any increase in the fluorescence intensity. The incubation of 2c for 4 h showed a correlation between the concentration of compound 2c and the ROS level (Figure 4.6). The incubation of 2c at 10 nM and 100 nM showed no effect on ROS production, whereas the incubation of 2c at 10 \(\mu\)M and 100 \(\mu\)M showed a significant increase in mean fluorescence. Antibacterial compound NQM108 (Figure 1B, left) was also tested for ROS under similar conditions. It also generated ROS, but the level of ROS was much lower than that of ROS produced by 2c (Figure 4.6, C). The level of ROS at control was considered 100\%. At 10 \(\mu\)M and 100 \(\mu\)M, NQM108 only increased mean fluorescence by \~108\% and \~177\%, whereas 2c produced over
133% and 226%, respectively. It is likely that cells can cope with the lower level of ROS generated by \textbf{NQM108}, whereas ROS generated by \textbf{2c} exceeds the critical threshold resulting in apoptosis.

\textbf{Figure 4.6}. Reactive oxygen production in A549 after incubation of \textbf{2c} (A and B), and \textbf{NQM108} (C) for 4 h. \( \ast = P \leq 0.05; \ \ast \ast = P \leq 0.01, \text{ and } \ast \ast \ast = P \leq 0.0001 \)
4.3.5. Compound 2c depletes glutathione in cancer cells

Glutathione (GHS) is a natural antioxidant in many cells. GHS neutralizes harmful ROS produced in the cells. Although the majority of GHS is present in the cytosol, a small percentage of GHS exists in the mitochondria and is critical for cellular functions. Cellular redox processes that take place in the mitochondria are vulnerable to the detrimental effect of ROS, and depletion of mitochondrial GHS can have catastrophic consequences. For instance, it has been reported that GHS depletion can serve as a potent activator of apoptosis signaling. The depletion of GHS was measured using a glutathione fluorometric assay kit (Biovision, USA). A549 cells were incubated with 2c for 48 h, and the samples were analyzed in a 96-well plate with excitation at 360 nm and emission detection at 460 nm. The level of GHS was expressed as 100% for the control, and the decrease in GHS levels after drug treatment was compared to the control (Figure 4.7). Concentration-dependent GHS depletion was observed. Although incubation of 2c at 0.1 μM only decreased the GHS level to 99.35%, significant GHS depletion was noted at 1 μM and higher concentrations of 2c with the GHS level at 88.76%, 23.87%, and 12.13% after incubation at 1 μM, 10 μM, and 100 μM 2c, respectively. This result from the GHS depletion assay also supports our earlier results from the ROS assay as GHS depletion elevates ROS level in the cells and vice versa.
4.3.6. Compound 2c decreases mitochondrial membrane potential

Mitochondria play a key role in activating apoptosis. A decrease in mitochondrial membrane potential ($\Delta \Psi_m$) during apoptosis has been well reported. Therefore, we decided to examine the effect of 2c on $\Delta \Psi_m$. A decrease in $\Delta \Psi_m$ leads to matrix condensation and exposure of cytochrome c to the intermembrane space, facilitating the release of cytochrome c into the cytoplasm. This process is facilitated by the oligomerization of effectors BAK/BAX which forms pores in the mitochondrial membrane. The release of cytochrome c from the mitochondria promotes the activation of the executioner protein caspase cascade. We incubated different concentrations of 2c with A549 cells for 24 h. We used Rhodamine 123 for the $\Delta \Psi_m$ analysis, which distributes
electrophoretically into the mitochondrial matrix according to the electric potential
distribution across the inner mitochondrial membrane.\textsuperscript{20} The intensity of
Rhodamine 123 was analyzed by flow cytometry. The experiment showed a
concentration-dependent change in $\Delta \Psi_m$ even at a very low concentration (10
nM) of 2c. The gate P4 (Figure 4.8, A and B) represents the percentage of cells
with decreased $\Delta \Psi_m$. The percentage of cells with decreased $\Delta \Psi_m$ increased
from 1.7\% (control) to ~2.55\% in 10 nM and 100 nM of 2c. Significant changes
were only observed from 1 \mu M to 100 \mu M 2c. Overall, the percentage of cells
which showed a decrease in $\Delta \Psi_m$ increased to 4.10\%, 51.40\%, and 88.05\% at 1
\mu M, 10 \mu M, and 100 \mu M 2c, respectively. Under similar conditions, NQM108 only
increased the percentage of cells to 25\% at 10 \mu M and 50\% at 100 \mu M (Figure
4.8, C and D) with a decrease in $\Delta \Psi_m$. These data correlate with our earlier ROS
results where NQM108 produced a lower amount of ROS than 2c. A lower
amount of ROS damages a lower number of mitochondria resulting in less cell
death. The correlation of these two results clearly explains why 2c is active and
NQM108 is not against cancer.
Figure 4.8. Decrease in mitochondrial membrane potential in A549 cells at different concentrations of 2c (A and B). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001
Figure 4.9. Decrease in mitochondrial membrane potential in A549 cells at different concentrations of NQM108 (C and D). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001
4.3.7. Caspase-3 release assay

The release of the caspase family of protein is a well-known biomarker for apoptosis via the mitochondrial pathway. The release of mitochondrial cytochrome c into the cytoplasm activates initiator caspase-8/9. Once activated, they cleave and activate downstream effector caspase-3/-7.\textsuperscript{21} Caspase-9 activates executioner caspase-3 and caspase-7. They are the executioner proteins which upon activation cleave major cellular components. Caspase-3 is the key player in the digestion of the cellular component, while caspase-7 is responsible for the detachment of apoptotic cells.\textsuperscript{22} Therefore, identification of caspase-3 release is the key final step in the identification of the mode of action through this pathway. Different concentrations of \textbf{2c} (0.01 μM, 0.1 μM, 1.0 μM, 10.0 μM and 100 μM) were incubated with A549 cells for 4 h, 12 h, 24 h, and 48 h. The release of caspase-3 was detected by using a caspase-3 fluorometric assay kit (BioVision, USA) and analyzed by a 96-well plate reader. The result showed an increase in caspase-3 activity in a concentration and time-dependent manner (Figure 4.10, A and B). The highest level of caspase-3 activity was observed when compound \textbf{2c} (0.01 μM and 0.1 μM) was incubated with A549 for 24 h. The caspase-3 level had already decreased to the base level at 10.0 μM. Time-dependent release of caspase-3 was also observed at 0.1 μM drug concentration at 4 h, 12 h, and 48 h. The release of the caspase family of proteins is a biomarker for mitochondrial-dependent apoptosis; hence, the increased activities of caspase-3 in our experiment support the hypothesis that CAA generates apoptosis via the mitochondrial pathway.
Figure 4.10. (A) Caspase-3 release at different time intervals (1 h, 4 h, 12 h and 48 h); (B) caspase-3 release at different concentrations (0 μM, 0.01 μM, 0.1 μM, 1.0 μM, and 10.0 μM). * = P ≤ 0.05, and ** = P ≤ 0.01
4.3.8. Due to their structural similarities and redox properties, CAAs are expected to form toxic ROS by hijacking the function of ubiquinone (Q) in the mitochondrial electron transport chain

Having identified ROS generation as the key mechanism, it is crucial to identify the source of ROS. It is well known that ROS are generated by the reduction of molecular oxygen by semiquinone radicals. Semiquinone radicals are formed by one-electron reduction of a quinone. The process of formation of a semiquinone radical most likely takes place in the mitochondria, where they can hijack the function of electron transporter Q in the electron transport chain. It is well documented that diverse cationic dyes can readily penetrate the outer membrane of the mitochondria and accumulate in the inner mitochondrial membrane electrophoretically.\textsuperscript{20a, 23} The cationic nature of CAAs may facilitate this accumulation.\textsuperscript{20a, 23} Once accumulated in the membrane, CAAs can interfere with the function of ubiquinone due to the similarities in their structure and redox properties. The proposed mechanism is also supported by our previous work where we reported that antibacterial CAAs exert antibacterial activity by inhibiting the redox processes of bacteria.\textsuperscript{5} A direct correlation has been reported on the reduction potential and cytotoxicity of multiple heterocyclic quinones.\textsuperscript{24}

The redox potentials of CAAs were determined by cyclic voltammetry (Table 4.2). All these compounds can undergo redox reactions similar to the inter-conversion of quinones, semiquinone radicals, and dihydroquinones via one-electron or two-electron process (Scheme 4.2). All of the compounds tested
exhibited two reversible electrochemical reductions in tetrabutylammonium hexafluorophosphate (TBAF) buffer in anhydrous acetonitrile.

Scheme 4.2. Process of formation of semi-quinone and hydroquinone

We observed that all the active compounds have first $E_{1/2}$ higher than $−160$ mV, and the less active or inactive compounds have first $E_{1/2}$ lower than $−160$ mV. The most active compounds with the lowest percentage mean cell growth have greater than $−180$ mV first $E_{1/2}$. The active compounds contain electron donating groups while the inactive compounds contain electron withdrawing groups. The reduction potential of ubiquinone is approximately $−556$ mV vs. Ag/AgCl, $−602$ vs. SCE in an aprotic solvent. Since the redox potentials of CAAs are much lower than Q, they are much better electron acceptors compared to Q. Thus, it is quite possible that CAAs can replace Q and receive electrons directly from electron-transporting complex (ETC) I or II forming semiquinone radicals (SQRs). It has been reported that SQRs can readily reduce
molecular oxygen leading to the formation of ROS. Based on this information, a proposed mechanism is outlined in Scheme 4.3.

**Scheme 4.3.** Proposed mechanism of ROS formation in ETS by cationic anthraquinone analogs

The electron withdrawing groups on CAAs can better stabilize the SQRs and slow down the formation of ROS from the reduction of molecular oxygen, whereas electron donating groups on CAAs can destabilize the SQRs and facilitate the electron transfer to molecular oxygen forming ROS. Although both classes of CAAs can generate ROS, those compounds containing electron donating groups generate ROS more rapidly and contribute to their higher cytotoxicity. Previously published research also supports this claim where the substitution with electron withdrawing groups on quinone decreases the rate of formation of superoxide, while substitution of electron donating groups increases the rate of formation of superoxide. Since we only observed a direct correlation
between the first half wave reduction potentials ($E_{1/2}$) and anticancer activity, it is likely that these compounds work by one-electron reduction at the cellular level rather than a two-electron reduction process, and the redox process between the anthraquinone and semiquinone radicals is responsible for the anticancer activity of CAAs.

**Table 4.2.** Comparison of redox potentials with antibacterial and anticancer activities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>First wave $E_{1/2}$ (mV) vs (Ag/AgCl, 3M NaCl)</th>
<th>Second wave $E_{1/2}$ (mV) vs (Ag/AgCl, 3M NaCl)</th>
<th>Mean cell growth percentage (%) in NCI-60 cell line assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>-163.5</td>
<td>-866.5</td>
<td>-10.18</td>
</tr>
<tr>
<td>2c</td>
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<td>-885.0</td>
<td><strong>-24.70</strong></td>
</tr>
<tr>
<td>4c</td>
<td>-176.0</td>
<td>-881.5</td>
<td>11.53</td>
</tr>
<tr>
<td>5c</td>
<td><strong>-130.5</strong></td>
<td>-823.5</td>
<td><strong>58.69</strong></td>
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<tr>
<td>3c</td>
<td>-148.5</td>
<td>-842.5</td>
<td>101.70</td>
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<tr>
<td>6c</td>
<td>-156.5</td>
<td>-886.0</td>
<td>43.38</td>
</tr>
<tr>
<td>8c</td>
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<td>-867.0</td>
<td>23.23</td>
</tr>
<tr>
<td>7c</td>
<td><strong>-182.5</strong></td>
<td>-762.0</td>
<td><strong>-27.06</strong></td>
</tr>
<tr>
<td>9c</td>
<td><strong>-182.0</strong></td>
<td>-885.5</td>
<td><strong>-31.51</strong></td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>-567$_{25}^{25}$</td>
<td>-</td>
<td>-</td>
</tr>
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</table>
4.4.8. DNA alkylation experiment

Mitomycin C, a quinone motif bearing anticancer drug is known to alkylate nucleic acid after bio-reductive activation. These cationic 1,4-naphthoquinone analogs may also function as alkylating agents (methylation) via the N-3 methyl group. The alkylation experiment was carried out with guanosine monophosphate (GMP). GMP was ion exchange with cyclohexylamine to increase its solubility in methanol. The mixture of 2c and GMP were incubated at 37 °C and analyzed by 1 H NMR at different time intervals (Figure 4.6). It is expected that the methylation of GMP will result in the appearance of new methyl peak; and a

Table 4.3. Summary of cyclic voltammetry

<table>
<thead>
<tr>
<th>Compound</th>
<th>first wave</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
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<td>Pa</td>
<td>AEp</td>
<td>Electron</td>
<td>E1/2</td>
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<td>Pa</td>
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<td>Electron</td>
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<td>-832</td>
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</tr>
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<td>-0.79</td>
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</table>
change in the chemical shift of anomeric proton. The loss of methyl from 2c will result in the formation of new compound 2b, which has a very different pattern in the ¹H NMR chemical shift. No shift in anomeric proton and no appearance of new signals in ¹H NMR demonstrate that they are not an alkylating agent.

4.3.9. DNA cleavage experiment

Numerous studies have reported that compounds bearing quinone motif are capable of causing photo-induced, transition metal complex-assisted, or oxidative DNA cleavage. We selected compound 1c for the initial DNA cleavage study using supercoiled pUC 19 DNA, which has been commonly used in DNA cleavage studies. The experiments were conducted using Tris buffer (pH 8). To our surprise, compound 1c manifests a concentration-dependent DNA cleavage without the presence of transition metal complex, light irradiation or oxidative reagent (Figure 4.11, top). One dominant form of nicked supercoiled DNA (form II) was noted with concentrations of 1c at 240 and 480 μM while no or very little DNA cleavage at the lower tested concentrations (15 and 60 μM). We then tested the remaining compounds at 240 μM, and all the cationic 1,4-naphthoquinone analogs were capable of cleaving DNA (Figure 4.11). However, there is no obvious correlation between the anticancer activities and the extent of DNA cleavage. For example, compound 2c has similar anticancer activity as 1c but cause much less DNA cleavage as compared to compound 1c. The lack of
correlation and DNA cleavage only at much higher concentration than IC_{50} can be attributed to the possibility that the direct DNA damage may not be the primary mode of its anticancer activity. However, this property is unique to these cationic 1,4-naphthoquinone analogs and is different from other reported classes of DNA-cleaving agents.

Figure 4.11. DNA cleavage experiment with different concentration of 1c (Top); DNA cleavage experiment for other CAAs at 240 μM concentration (Bottom)

4.4. Conclusion

We have identified the anticancer mode of action of cationic anthraquinone analogs. This mode of action study has revealed that these compounds generate ROS and deplete the natural antioxidant glutathione in the cell. These active compounds possibly hijack the function of ubiquinone due to their similarities in structure and redox properties, thus generating semiquinone radicals. The semiquinone radicals, in turn, reduce molecular oxygen to form
highly reactive ROS. The generation of extensive ROS and the depletion of the level of glutathione lead to oxidative stress and damage to the mitochondrial membrane lowering its membrane potential. The programmed apoptosis is caspase dependent and triggered by the release of caspase-3. To unambiguously identify the mode of action, we have performed. We have observed direct DNA cleaving capability of these compounds at a higher concentration. Although the DNA cleaving properties of CAAs without light and transition metals are unique to these specific compounds, the DNA cleavage was only observed at a much higher concentration than the IC$_{50}$ value and, therefore, may not be attributed as the primary mode of action. Other experiments conducted in our laboratory have also ruled out the possibility that CAAs can function as alkylating agents. In summary, our results have confirmed the anticancer mode of action for this novel CAAs and may pave the way for the development of new chemotherapeutic agents.

4.5. Experimental Section

Proton nuclear magnetic resonance spectra were recorded using JOEL 300 OR Bruker ascend 500 spectrometers. Chemical shifts were reported as parts per million (ppm) downfield from tetramethylsilane in δ unit and coupling constants were given in cycles per seconds (Hz). Signal multiplicities were indicated by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). $^{13}$C NMR spectra were obtained using JOEL 300 at 75 MHz or Bruker ascend 500 at
125 MHz. Routine $^{13}$C NMR spectra were fully decoupled by broad-band WALTZ decoupling. All NMR spectra were at ambient temperature. Atmospheric pressure chemical ionization (APCI) or electron spray ionization (ESI) was provided by the mass spectrometry facilities, University of California, Riverside. Flow cytometry analysis was carried out using BD Biosciences Special Order FACS Aria™ II and the service was provided by Center for Integrated Biosystems (CIB), Utah State University.

Chemical reagents and chromatography solvents were purchased from Aldrich Chemical Co. or Acros Chemical Co. and were used without further purification unless otherwise noted. Dichloromethane was freshly distilled from calcium hydride under a nitrogen atmosphere. Pyridine and triethylamine were stored over 4 Å molecular sieves. Column chromatographic purifications were carried out on silica gel 230x450 mesh, Sorbent Tech. Analytical TLC was performed on Sorbent Technologies silica gel glass TLC plates. Visualization was accomplished with UV light (254 nm).

**General Procedure for Cell Culture.** A549 and MRC-5 cells were grown in commercial DMEM 1X (Gibco) with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO$_2$. The cells were allowed to adhere for 48 h before drug treatment.

**General Procedure for Cell Viability Assay.** The cells were seeded in 96-well microtiter plates (3000/200 μL). After 48 h of incubation in the
corresponding media, cells were treated with various concentrations of test compounds (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) for 48 h. 20 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Upon completion of incubation, the media was carefully removed and washed twice with 100 mL of pbs buffer. Then, 100 µL of DMSO was added to each well; agitated on an orbital shaker for 15 min., and the absorbance at 590 nm with 620 nm filter was determined with a microplate reader. The results were expressed as viability compared with that of control. The experiment was carried out in triplicate in three independent experiments.

**General Procedure for Cell Cycle Analysis.** A549 cells (5x10^4 cells/2 mL) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of test compounds (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 24 h. The adherent cells were removed by 0.25% Trypsin/EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The cells were washed with 1 mL of PBS buffer and collected by centrifugation. The pelleted cells were then re-suspended in 0.3 mL of PBS buffer, fixed with 0.7 mL of ice-cold ethanol and left at 4 °C for overnight. The cells were then centrifuged and collected, washed with PBS buffer and re-centrifuged. The pelleted cells were then re-suspended in 0.25 mL of PBS buffer and 5µL of 10 mg/mL RNase A was added. The solution was incubated at 37 °C for 1 h. 10 µL of 1 mg/mL PI solution was added and left in the dark for FACS analysis (488nm).
**General Procedure for Annexin-V/PI Apoptosis Assay.** A549 cells (5x10^4 cells/2mL) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of test compound (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 24 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The cells were washed with 1 mL of PBS buffer and collected by centrifugation. The cell pellet was resuspended in 500 µL of 1X binding buffer (Annexin V/PI assay kit, Biovision, USA.); 5 µL of annexin V-PI was added and incubated at room temperature for 5 min in the dark. The cells were then analyzed by flow cytometry at ex. 488 nm and em. 578 nm.

**General Procedure for ROS Measurement Assay.** A549 cells (5x10^4 cells/2 mL) were cultured in 6-well plates and allowed to adhere for 48 h. 2 mL solution of DCFH-DA (10 µM) in serum free media was added and incubated for 1 h. The cells were washed with 1 mL of PBS buffer and different concentrations of test compound (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) in normal media were added and incubated for 4 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The pelleted cells were washed once with 1 mL of PBS buffer and centrifuged. The cell pellets were then diluted with 500 µL of PBS buffer and analyzed in FACS scan at Ex/Em: ~ 492 – 495/517 – 527 nm.
**General Procedure for Mitochondrial Membrane Potential Measurement.** A549 cells (5x10^4 cells/2 mL) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of test compound (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 24 h. 100 mg/mL rhodamine 123 stock solution was directly added to the culture media and incubated at 37 °C for 30 min. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The pelleted cells were washed twice with 1 mL of PBS buffer and centrifuged. The cell pellets were then diluted with 500 µL of PBS buffer analyzed in FACS scan.

**General Procedure for Glutathione Depletion Assay.** A549 cells (5x10^4 cells/2 mL) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of test compound (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 48 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The level of glutathione was measured with modified Glutathione Fluorometric assay kit (Biovision, catalog number: K51-100). Fluorescence intensities were measured using 96 well plate readers (BioTek Synergy H4 reader with Gen5 software) with 360 nm excitation and 461 nm emission.

**General Procedure for Caspase-3 Release Assay.** A549 cells (1x10^6 cells/2 mL) were cultured in 6-well plates and allowed to adhere for 48 h.
Different concentrations of test compound (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 4, 12 h and 48 h. The adherent cells were removed by 0.25% Trypsin-EDTA and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The level of Caspase-3/CPP32 was measured with Caspase-3/CPP32 fluorometric assay kit (Biovision, catalog number: K105-25). Fluorescence intensities were measured using 96 well plate reader (BioTek Synergy H4 reader with Gen5) with 400 nm excitation and 505 nm emission.

**General Procedure for Fluorescence Imaging.** Following the same procedure as cell viability, cells are seeded in µ-Slide 8 well plate (CVRGLS LABKITKII 8 Well#1.5). The morphological studies in cells were studied in phase contrast mode while nuclear condensation (DAPI nuclear stain) and mitochondrial morphology (Rhodamine 123) were studied by fluorescence microscopy (Olympus IX70). The images were recorded by spot RT color camera (model: 2.2.1) and processed with spot 5 software.

**General Procedure for Cyclic Voltammetry Measurement.** Cyclic Voltammetry was performed in a BASi-Epsilon EC system using 0.1 M Tetrabutylammonium hexafluorophosphate (TBAF) for electrochemical analysis (purity ≥99.0 %) in an anhydrous Acetonitrile as a supporting electrolyte. The 3 mm glassy carbon electrode was used as the working electrode, together with a platinum counter-electrode and an Ag/AgCl reference electrode. The working electrode was polished with 0.05 µM alumina, washed with dH2O, and sonicated
for 5 min. before using for each sample. 2 mM samples in supporting electrolyte were prepared just before the electrochemical experiment, degassed with \( \text{N}_2 \) for 15 min., and readings were taking under an \( \text{N}_2 \) blanket.

**General Procedure for Statistical data analysis.** All results are presented as the mean ± standard deviation of three independent experiments using GraphPad Prism software using an unpaired t-test. The results were considered significant at \( P < 0.05 \) (*) = \( P \leq 0.05 \); **(*) = \( P \leq 0.01 \); ***(*) = \( P \leq 0.001 \); and ****(*) = \( P \leq 0.0001 \).

**General Procedure for DNA Cleaving Experiment.** The DNA cleavage experiments of supercoiled pUC19 plasmid DNA 0.75 µL (~125 ng) by 1c (15, 30, 60, 120, 240, and 480 µM) containing ~3% DMSO in (10mM Tris-HCl, 1mM EDTA Buffer and 50mM NaCl) buffer at pH 8.0 were performed by agarose gel electrophoresis (Fisher Biotech Electrophoresis FB-SB-170 System). The samples were incubated for 24 h at 37 °C in the dark. A 3.3 µL loading dye containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol was added and electrophoresis was carried out at 65 V for 55 min in Tris–Acetate–EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA) using 1% agarose gel containing 0.5 µg/mL ethidium bromide. The gels were viewed in an Alpha Innotech Corporation Gel doc system at 302 nm and photographed using an 8 MP Olympus digital camera. Following the same condition, electrophoresis was carried for rest of the compounds at 240 µM concentrations.
4.6. References


(22) Porter, A. G.; Janicke, R. U., Emerging roles of caspase-3 in apoptosis. 


5.1. Abstract

In search of broad spectrum synthetic antimicrobials, we have developed a series of synthetic 1,1'-(hexane-1,6-diyl)bis(3-alkyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) salts, which have very potent antimicrobial activity against a broad range of susceptible and resistant species of fungi and bacteria. These compounds possess azole, quinone, and quaternary ammonium complex (QAC). Quinone moieties can act as a redox inhibitor in bacteria while multi-cationic head with alkyl chain can disrupt membrane integrity. The in vitro biological evaluation has already shown that they are very active against a series of fungal and bacterial strains. The cytotoxicity assay in SKOV-3, a cancer cell line, has already demonstrated that they are selectively toxic to fungi and bacteria over human cells.
5.2. INTRODUCTION

Amphiphilic molecules have attracted greater interest as antifungal or antibacterial agents for many years. Recently, many studies on aminoglycosides based amphiphilic molecules have been published.\(^1\) The protonated amines on an aminoglycoside form a cationic head and together with a long alkyl chain form an amphiphilic molecule. Amphiphilic molecules based on quaternary ammonium salts (QAC) have been known for many years, and it is one of the most common antimicrobial agents used as disinfectants.\(^2\) They are widely used in domestic, industrial, agricultural and clinical applications.\(^3\) Several different types of QACs either possessing mono- or multi-cationic functional groups can be obtained in the market. Several studies have shown that bacteria can not develop resistance against multi-cationic QACs but can develop resistance against mono-cationic QAC easily. Due to the strong positively charged cationic head and long lipophilic tail, this compound acts as an amphiphilic molecule similar to the phospholipids found in the cell membrane. Disruption of the phospholipid bilayer to compromise cell membrane is the primary mode of action.\(^4\)

Our laboratory is dedicated to synthesizing cationic amphiphiles based on aminoglycoside or 1,4-naphthoquionone moiety. We have already published several QAC types of compounds and their mode of action.\(^5\) These compounds are antimicrobial when substituted with an alkyl chain,\(^5\) but anticancer when substituted with the aromatic ring.\(^6\) These antimicrobial cationic anthraquinone
analogs (CAAs) act as a redox inhibitor at minimum inhibitory concentration (MIC) and act as a membrane disrupter at higher concentrations. The anticancer CAAs act as ROS-generating molecules.

In this article, we are presenting a small multi-cationic QAC type of molecule (MCAAs) which consists of redox active cationic anthraquinone head and an alkyl lipophilic tail. The redox-active quinone head is connected with alkyl chain via triazole moiety. Alkylation at triazole gives it cationic property, and the positive charge generated by alkylation is delocalized around triazole ring. The molecule is a hybrid in nature as it is the combination of several popular antimicrobial scaffolds, such as quinone, triazole, and QAC. All MCAAs are active against a broad range of resistant fungi and bacteria with low cytotoxicity against human cell lines. Synthesis, biological evaluation, and mode of action study are discussed here.
Figure 5.2. Structural comparison of CAAs from our laboratory

5.3. Result and Discussion

5.3.1 Design and Chemical Synthesis

Conferring multiple cationic functional groups and multiple alkyl chains in a single molecule was our primary focus while designing these compounds. For the ease of synthesis, the bridging alkyl chain connecting two cationic quinone heads was kept constant (6 carbon chain) to form **NQM123**, and the rest of the analogs were derivatized from it. The free alkyl chain lengths were varied from C$_1$ to C$_{10}$. Beyond C$_{10}$, we observed solubility issues in water for biological testing. So, no derivatives with carbon chain longer than C$_{10}$ were synthesized. The compounds can be synthesized from a very cheap starting material, 1,4-naphthoquinone, and
do not require cumbersome purification via column chromatography. Therefore, a large quantity can be synthesized easily.

The $S_N2$ reaction of 1,6-dibromohexane dissolved in DMF with sodium azide at room temperature resulted into a 1,6-diazidohexane (2). The compound was directly used for the next step without further purification. NQ123 could be synthesized by 1,3 dipolar cycloaddition using excess 1,4-naphthoquinone followed by oxidation (82% yield). NQ123 was methylated with methyl triflate to give NQM123C1. For the synthesis of NQM123C2-NQM123C10, first, the hydroxyl group on corresponding alkyl alcohol was converted to leaving group via triflic anhydride to form corresponding alkyl triflate. The alkyl triflates were directly used for alkylation to form corresponding compound NQM123C2-NQM123C10. All the compounds were characterized by $^1$H NMR, $^{13}$C NMR, and high-resolution mass spectrometry. HPLC analysis determined the purity level of all the final compounds and found >95% pure.
5.3.2. Antibacterial Activity

All the newly synthesized compounds were tested for antibacterial and antifungal activity. No biological activity was observed for intermediate compound NQ123 at 5 mg/mL, which emphasizes the importance of cationic property and alkylation. All the final compounds (NQM123C1-NQM123C10) exhibited strong activity against *Staphylococcus aureus* (ATCC25923). NQM123C1-NQM123C4 are the most active compounds against *S. aureus* with MIC 0.125 µg/mL. MIC
decreased by 32-64 fold for \textit{S. aureus} and the chain length of free alkyl chain at N3 increased to 6 or more. The trend was observed in reverse order for MRSA (ATCC 33591) and \textit{E. coli} (ATCC25922). In both cases, MICs for \textbf{NQM123C6-NQM123C10} are >250 µg/mL, but they become active with a longer chain. It is evident from this data that shorter MCAAs are more active against \textit{S. aureus} while MCAAs with longer alkyl chain are more active against MRSA and \textit{E. coli}. It is very likely that these compounds have a different mode of action when substituted with shorter or longer chain. Therefore, we observed a different trend in antibacterial activity. From our earlier publication on the similar compounds with a shorter alkyl chain, we showed that these compounds interfere with bacterial redox process at MIC level, and also act as a membrane disrupter at higher concentration level.\textsuperscript{7}

5.3.3. Antifungal Activity

Azoles and QAC’s are also known to have potent antifungal activity.azole-based drugs, such as fluconazole, posaconazole, and itraconazole are considered first of the line of treatment for candidiasis caused by the yeast that belongs to the genus \textit{Candida}. As a matter of fact, our new analogs possess both azole moiety and QAC property. This hybrid compound may have superior antifungal activity, which prompted us to explore their \textit{in vitro} antifungal activity. The compounds were tested against major human pathogenic fungi. This fungi test includes \textit{Aspergillus}, \textit{Candida}, \textit{Cryptococcus}, and
### Table 5.1. Antibacterial activity of MCAAs (µg/mL)

<table>
<thead>
<tr>
<th>Compound</th>
<th>G⁺ Bacteria</th>
<th>G⁻ Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus (ATCC25923) MIC (µg/mL)</td>
<td>MRSA (ATCC33591) MIC (µg/mL)</td>
</tr>
<tr>
<td>NQM123C1</td>
<td>0.125</td>
<td>&gt;250</td>
</tr>
<tr>
<td>NQM123C2</td>
<td>0.125</td>
<td>&gt;250</td>
</tr>
<tr>
<td>NQM123C4</td>
<td>0.125</td>
<td>&gt;250</td>
</tr>
<tr>
<td>NQM123C6</td>
<td>4</td>
<td>8-16</td>
</tr>
<tr>
<td>NQM123C8</td>
<td>4</td>
<td>4-8</td>
</tr>
<tr>
<td>NQM123 C10</td>
<td>8</td>
<td>4-8</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>2-4</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>2-4</td>
<td>-</td>
</tr>
</tbody>
</table>

Rhodotorula species. The MIC values of NQM123C1 -NQM123C10, K20, and fluconazole against various fungi is summarized in Table 5.2. NQM123C1-NQM123C6 did not exhibit any antifungal activity against Aspergillus flavus, Candida albicans 64124, and Candida albicans MYA2876. However, they exhibited mild to moderate antifungal activity against Cryptococcus neoformans and Rhodotorula pilimanae with MIC values ranging from 125 µg/mL and 62.5-
15.6 µg/mL, respectively. Both NQM123C8 and NQM123C10 exhibited excellent antifungal activity for all the tested fungal species. The MIC values ranging from 15.63 to 7.81 µg/mL, 3.91 µg/mL, 3.91 µg/mL, 3.91-2.0 µg/mL, and 2.0 µg/mL for Aspergillus flavus, Candida albicans 64124, Candida albicans MYA2876, Cryptococcus neoformans, and Rhodotorula pilimanae, respectively.

NQM123C10 is the most active compound with a slight edge over NQM123C8, the MIC values for Aspergillus flavus and Cryptococcus neoformans decreased to half for NQM123C10. The result showed that antifungal activity depended on free alkyl chain length where said activity exhibited free alkyl chain lengths that were longer than 6. Aspergillus species are one of the most emerging causes of invasive aspergillosis in immunocompromised individuals, and Aspergillus flavus is one of the leading cause of the reduction in crop yield. Similarly, Candida albicans are one of the most prevalent fungal pathogens. The results are very significant as both NQM123C8 and NQM123C10 are active against Aspergillus species and azole-resistant Candida albicans.

5.3.4. Fungal Mechanism of Action

Disruption of the cell membrane is the primary mode of action for antimicrobial amphiphiles including aminoglycoside amphiphiles and QAC amphiphiles. The effect of our most active antifungal candidate, NQM123C10, was used to observe
its effect on the fungal membrane. Sytox Green, a dye that gives off fluorescence upon penetrating membrane-compromised cells and binding to nucleic acid, was used to investigate the cell membrane disruption. The study was performed on *Candida albicans* ATCC 64124 and filamentous fungi, *Fusarium graminearum* B4-A5. The fungi were exposed to **NQM123C10** at MIC level for 1 h. The result was as expected and the compound functioned as a membrane disrupter in both fungi (Figure 5.3 and 5.4).

**Table 5.2.** Antifungal activity of MCAAs (µg/mL)

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>Aspergillus flavus</em></th>
<th><em>Candida albicans</em> ATCC 64124</th>
<th><em>Candida albicans</em> MYA2876</th>
<th><em>Cryptococcus neoformans</em></th>
<th><em>Rhodotorula pilimanae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>NQM123C1</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>125</td>
<td>62.5</td>
</tr>
<tr>
<td>NQM123C2</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NQM123C4</td>
<td>&gt;250</td>
<td>250</td>
<td>&gt;250</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NQM123C6</td>
<td>&gt;250</td>
<td>250</td>
<td>&gt;250</td>
<td>125</td>
<td>15.6</td>
</tr>
<tr>
<td>NQM123C8</td>
<td>15.63</td>
<td>3.91</td>
<td>3.91</td>
<td>3.9</td>
<td>2.0</td>
</tr>
<tr>
<td>NQM123C10</td>
<td>7.81</td>
<td>3.91</td>
<td>3.91</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>K20</td>
<td>7.8</td>
<td>31.3</td>
<td>15.6</td>
<td>7.8</td>
<td>-</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>&gt;250</td>
<td>1.56</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.3. *Candida albicans*, control (top). *Candida albicans* incubated with NQM123C10 for 1h (bottom).

Figure 5.4. *Fusarium graminearum* single hypha experiment Control (top). Incubated with NQM123C10 for 1h (bottom).
5.3.5. Cytotoxicity

A cytotoxicity assay was carried out against a human ovary cancer cell, SKOV3 to evaluate the cytotoxicity of our new compounds. This assay is critical as it determines how selectively these compounds kill desired pathogens over human cells. The results are shown in Figure 5.5. At a concentration of 10 µg/mL, NQM123C1 displayed mild toxicity while other derivatives displayed no toxicity. All the MIC values for antifungal activity are lower than 10 µg/mL except NQM123C8 MIC for *Aspergillus flavus*. The most active compounds NQM123C8 and NQM123C10 showed the least toxicity to SKOV3.

![Cytotoxicity assay for all MCAA analogs]

*Figure 5.5. Cytotoxicity assay for all MCAA analogs*
5.4. Conclusions and Significance

In conclusion, we successfully synthesized a new series of QAC type of antimicrobial agent. Compounds substituted with shorter alkyl chain were very active against \textit{S. aureus} while compounds substituted with longer alkyl chain were active against \textit{E.Coli} and MRSA. For compounds with alkyl chain longer than 6, they showed excellent antifungal activity against a broad range of fungal species, including resistant species. The cytotoxicity assay showed little to no cytotoxicity against a human cell line.

5.5. Experimental Section

**General procedure for the preparation of alkyl azides.** All the alkyl azides were prepared as previously reported.

**General procedure for [3+2] cycloaddition/oxidation: synthesis of compounds NQ123C1-NQM123C10.** To a solution of alkyl azides (estimated 3.2 mmol) in DMF (10 mL), 1,4-naphthoquinone (2.0 g, 12.8 mmol) was added, and the resulting solution was heated at 80 °C for two days. After removal of solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred until the products precipitated. The precipitated products were collected with
The yields were calculated based on the estimated amount of alkyl azides used.

**General procedure for N-3 alkylation: synthesis of NQM123C1.** To a solution of compounds NQ123C1 (0.35 mmol) in anhydrous toluene (10 mL), methyl trifluoromethanesulfonate (0.30 mL, 2.80 mol) was added, and the resulting solution was heated at 100 °C overnight. After removal of solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred for 30 min. The precipitated products were collected with Hirsh funnel, washed with more diethyl ether (25 mL), and dried under the vacuum.

**General procedure for N-3 alkylation: synthesis of NQM123C2-NQM123C10.** The alcohol (4 equiv) and pyridine (8 equiv) were dissolved in anhydrous toluene (10 mL) and cooled in an ice–water bath before Tf₂O (8 equiv) was slowly added. The mixture was stirred at 0 °C for 2 h. The reaction mixture was passed through a sodium sulfate filter. The sodium sulfate filter cake was further washed with another 10 mL of anhydrous toluene. The toluene fractions were combined, and NQM123C2-NQMC10 (0.11 g, 1 equiv) was then added. This mixture was then refluxed at 110 °C for overnight. After removal of solvent, diethyl ether (25 mL) was added and the reaction mixture was stirred for 30 min. The precipitated products were collected with Hirsh funnel, washed with more diethyl ether (25 mL), and dried under the vacuum.
1,1’-(hexane-1,6-diyl)bis(1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione) (NQ123). This compound was synthesized according to the general procedures with 84% yield. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.3 - 8.4 (m, 1H), 8.2 - 8.3 (m, 1H), 7.8 - 7.9 (m, 2H), 6.2 (s, 2H), 3.8 - 3.9 (m, 2H), 3.5 - 3.6 (m, 2H), 3.3 (s, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 176.8, 175.2, 145.7, 135.4, 134.5, 133.9, 133.4, 128.0, 127.6, 79.1, 71.4, 70.1, 59.2.; ESI/APCI calcd for C\(_{14}\)H\(_{14}\)N\(_3\)O\(_4\)\(^+\) ([M]\(^+\)) m/z 325.1784; measured m/z 325.1773.

1,1’-(hexane-1,6-diyl)bis(3-methyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate (NQ123C1). This compound was synthesized according to the general procedures with 84% yield. \(^1\)H NMR (300 MHz, CD\(_3\)OD) \(\delta\) 8.4 - 8.3 (m, 2H), 8.1 - 8.0 (m, 2H), 5.1 (t, \(J = 6.9\) Hz, 2H), 2.1 - 1.9 (m, 2H), 4.7 (s, 3H), 2.2 - 2.1 (m, 2H), 1.5 - 1.5 (m, 2H); \(^{13}\)C NMR (125 MHz, DMSO - D6) \(\delta\) 173.9 (2H), 137.7, 137.6, 136.9, 136.8, 133.5 (2C), 129.1, 129.0, 54.9, 50.1, 29.4, 25.9, 15.1; ESI/APCI calcd for C\(_{30}\)H\(_{30}\)N\(_6\)O\(_4\)\(^{++}\) ([M]\(^{++}\)) m/z 269.1159; measured m/z 269.1146.

1,1’-(hexane-1,6-diyl)bis(3-ethyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate (NQ123C2). This compound was synthesized according to the general procedures with 84% yield. \(^1\)H NMR (300 MHz, DMSO - D6) \(\delta\) 8.4 - 8.2 (m, 2H), 8.1 - 8.0 (m, 2H), 5.0 - 5.1 (m, 4H), 2.1 - 1.9 (m, 2H), 1.60 (t, \(J = 7.2\) Hz, 2H), 1.5 1.4 (m, 2H); \(^{13}\)C NMR (125 MHz, DMSO - D6) \(\delta\) 173.9 (2H), 137.7, 137.6, 136.9, 136.8, 133.5 (2C), 129.1, 129.0, 54.9,
50.1, 29.4, 25.9, 15.1; ESI/APCI calcd for C$_{30}$H$_{30}$N$_6$O$_4^{++}$ ([M]$^{++}$) m/z 269.1159; measured m/z 269.1146.

1,1'-(hexane-1,6-diyl)bis(3-butyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate (NQM123C4). This compound was synthesized according to the general procedures with 84 % yield. $^1$H NMR (300 MHz, DMSO) δ 8.4 - 8.2 (m, 2H), 8.1 - 8.0 (m, 2H), 5.0 - 5.1 (m, 4H), 2.1 - 1.9 (m, 4H), 1.5 - 1.3 (m, 4H), 0.90 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (125 MHz, DMSO) δ 172.6, 172.5, 135.9, 135.8, 135.7 (2C), 132.6, 132.5, 127.7, 127.6, 54.1, 53.9, 30.4, 28.2, 25.1, 19.1, 12.2; ESI/APCI calcd for C$_{34}$H$_{38}$N$_6$O$_4^{++}$ ([M]$^{++}$) m/z 297.1472; measured m/z 297.1462.

1,1'-(hexane-1,6-diyl)bis(3-hexyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate (NQM123C6). This compound was synthesized according to the general procedures with 84 % yield. $^1$H NMR (300 MHz, DMSO) δ 8.4 - 8.2 (m, 2H), 8.1 - 8.0 (m, 2H), 5.0 - 5.1 (m, 4H), 2.1 - 1.9 (m, 4H), 1.5 - 1.2 (m, 8H), 0.84 (t, $J$ = 6.8 Hz, 3H); $^{13}$C NMR (125 MHz, DMSO) δ 173.1 (2C), 136.8 (2C), 136.1 (2C), 132.7 (2C) 128.3, 128.1, 54.2, 54.1, 30.9, 28.7, 28.5, 25.4, 25.2, 22.3, 14.3; ESI/APCI calcd for C$_{14}$H$_{14}$N$_3$O$_4^{+}$ ([M]$^{++}$) m/z 325.1784; measured m/z 325.1773.

1,1'-(hexane-1,6-diyl)bis(3-octyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate (NQ123C8). This compound was
synthesized according to the general procedures with 84 % yield. $^1$H NMR (300 MHz, DMSO) δ 8.3 - 8.2 (m, 2H), 8.1 - 8.0 (m, 2H), 5.0 - 5.1 (m, 4H), 2.1 - 1.9 (m, 4H), 1.5 - 1.1 (m, 12H), 0.81 (t, $J = 6.9$ Hz, 3H); $^{13}$C NMR (125 MHz, DMSO) δ 173.1 (2C), 136.9, 136.8, 136.1 (2C), 132.7 (2C), 128.3, 128.2, 54.2, 54.1, 31.6, 28.9, 28.8, 28.7, 28.5, 25.8, 25.1, 22.5, 14.4; ESI/APCI calcd for C$_{42}$H$_{54}$N$_6$O$_4$$^{++}$ ([M]$^{++}$) m/z 353.2098; measured m/z 353.2083.

1,1'-(hexane-1,6-diyl)bis(3-decyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium triflate (NQM123C10). This compound was synthesized according to the general procedures with 84 % yield. $^1$H NMR (500 MHz, DMSO) δ $^{13}$C NMR (125 MHz, DMSO) δ 173.1 (2C), 136.8 (2C), 136.1 (2C), 132.7 (2C), 128.3 (2C), 128.1, 54.2, 54.1, 31.7, 39.4, 29.4, 29.1, 28.7(2C), 28.5, 25.8, 25.1, 22.6, 14.4.; ESI/APCI calcd for C$_{46}$H$_{62}$N$_6$O$_4$$^{++}$ ([M]$^{++}$) m/z 381.2411; measured m/z 381.2395.
5.6. References


APPENDICES
APPENDIX A $^1$H and $^{13}$C NMR Spectra for Synthesized New Compounds
$^1$H NMR of 1-Phenyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (6)

X parts per Million : 110
$^{13}$C NMR of 4,9-Dioxo-3-methyl-1-phenylnaphtho[2,3-d][1,2,3]triazol-3-ium chloride (3)
$^{13}$C NMR of 1-p-Tolyl-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (6b)
$^1$H NMR of 4,9-Dioxo-3-methyl-1-p-tolyl-naphtho[2,3-d][1,2,3]triazol-3-imin trifluoromethanesulfonate (3b)
$^{13}$C NMR of 4,9-Dioxo-3-methyl-1-$p$-tolyl-naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (3b)
$^{13}$C NMR of 1-(4-Trifluoromethylphenyl)-1$H$-naphth[2,3-d][1,2,3]triazole-4,9-dione (6c)

**Standard JC Experiment**

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**Chemical Shifts (ppm)**

- 1H: 7.31 ppm
- 13C: 128.7, 135.2, 145.6, 152.0
$^{1}H$ NMR of 4,9-Dioxo-3-methyl-1-(4-trifluoromethylphenyl)naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (Gc)

X: parts per Million (ppm)
$^{13}$C NMR of 4,9-Dioxo-3-methyl-1-(4-trifluoromethylphenyl)naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (3c)
$^{1}H$ NMR of 1-(4-Chlorophenyl)-$1H$-naphtho[2,3-$d$][1,2,3]triazole-4,9-dione (6d)
$^1$H NMR of 14-Chlorophenyl-1,9-dioxo-3-methylindan[2,3-d][1,2,4]triazolo[3,4-a]pyrimidinium (3b)
H NMR of 1-(4-Fluorophenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (6c)
$^{13}$C NMR of 1-(4-Fluorophenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (6c)
$^1$H NMR of 1-(3,4,5-Trimethoxyphenyl)-1H-naphtho[2,3-d][1,2,3]triazole-4,9-dione (6g)
$^{13}$C NMR of 4,9-Dioxo-3-methyl-1-(3,4,5-trimethoxyphenyl)naphtho[2,3-d][1,2,3]triazol-3-ium trifluoromethanesulfonate (3g)
1.1\textsuperscript{1}-(hexane-1,6-diyl)bis(3-methyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate
1,1'-[[hexane-1,6-diyl]bis[3-ethyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium]] triflate
1,1''-(hexane-1,6-diyldi)(bis(3-ethyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate
1,1'-(hexane-1,6-diyl)bis(3-butyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate
1,1'-(hexane-1,6-diyl)bis(3-butyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate
1,1'-[hexane-1,6-diyl]bis(3-hexyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium) triflate
C13

Bruker

Current Data Parameters
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PROCNO 1

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1,1'-hexane-1,6-diyl)bis(3-decyl-4,9-dioxo-4,9-dihydro-1H-naphtho[2,3-r][1,2,3]triazol-3-ium triflate
APPENDIX B Cyclic Voltammogram for compound 1c-9c
CV Run for BASi-Epsilon

Data File Name
C:\Users\BASI Custom\Desktop\CVfinalmnm120.CV3

Title
CV Run for BASi-Epsilon

Experiment Type
Cyclic Voltammetry (CV)

Printed
4/28/2013 10:21:44 PM

Date & Time of the run
4/28/2013 10:20:42 PM

Number of data points
2700

Display Convention
POLAROGRAPHIC

Initial Potential
100 (mV)

Switching Potential 1
-1250 (mV)

Final Potential
0 (mV)

Number of segments
2

Scan rate
100 (mV/s)

Current Full Scale
100 (uA)

Filter
10 Hz

Quiet Time
2 (sec)

Sample Interval
1 mV

Peak Data:
peak [1]: -198 (mV), 41.7675 (uA), 25.5020 (uC)
peak [2]: -833 (mV), 40.5371 (uA), 25.4973 (uC)
peak [3]: -767 (mV), 42.7680 (uA), 26.6588 (uC)
peak [4]: -128 (mV), 41.3672 (uA), 24.9104 (uC)

1c

O
Me
N
N
Cl
CV Run for BASi-Epsilon

Data File Name: C:\Users\BASi Customer\Desktop\jayCVfr\new21\light.CV0
Title: CV Run for BASi-Epsilon
Experiment Type: Cyclic Voltammetry (CV)
Printed: 4/29/2013 3:25:22 PM
Date & Time of the run: 4/29/2013 3:24:15 PM
Number of data points: 2700
Display Convention: POLAROGRAPHIC
Initial Potential: 100 (mV)
Switching Potential 1: -1250 (mV)
Final Potential: 100 (mV)
Number of segments: 2
Scan rate: 100 (mV/s)
Current Full Scale: 100 uA
Filter: 10 Hz
Quiet Time: 2 (sec)
Sample Interval: 0.1 mV

Peak Data:
peak [1] @ -217 (mV), 39.5439 (uA), 24.2799 (uC)
peak [2] @ -921 (mV), 27.7234 (uA), 23.8666 (uC)
peak [3] @ -846 (mV), 30.6342 (uA), 24.7551 (uC)
peak [4] @ -146 (mV), 30.8474 (uA), 24.7087 (uC)

![Chemical Structure 2c]
CV Run for BASi-Epsilon

Data File Name: C:sers\BASi Customer\Desktop\JayCV\final\nom124.png

Title: CV Run for BASi-Epsilon
Experiment Type: Cyclic Voltammetry (CV)
Date & Time of the run: 4/29/2013 4:17:56 PM
Number of data points: 2700
Display Convention: POLAROGRAPHIC
Initial Potential: 100 (mV)
Switching Potential 1: 1260 (mV)
Final Potential: 100 (mV)
Number of segments: 2
Scan rate: 100 (mV/s)
Current Full Scale: 100 uA
Filter: 10 Hz
Quiet Time: 2 (sec)
Sample Interval: 1 mV

Peak Data:
peak [1]: [211 (mV), 50.3700 (uA), 31.1904 (uA)]
peak [2]: [848 (mV), 48.6000 (uA), 32.6000 (uA)]
peak [3]: [775 (mV), 49.2368 (uA), 29.8285 (uA)]
peak [4]: [141 (mV), 49.1392 (uA), 20.2944 (uA)]

4c
Data File Name: C:sers\BASI\Customer\Desktop\jap\CV1finalvqm\12\CV1

Title: CV Run for BASI-Epsilon

Experiment Type: Cyclic Voltammetry (CV)

Printed: 4/26/2013 4:49:38 PM

Date & Time of the run: 4/26/2013 4:48:48 PM

Number of data points: 2700

Display Convention: POLAROGRAPHIC

Initial Potential: 100 (mV)

Switching Potential 1: -1250 (mV)

Final Potential: 100 (mV)

Number of segments: 2

Scan rate: 100 (mV/s)

Current Full Scale: 100 uA

Filter: 10 Hz

Quiet Time: 2 (960)

Sample Interval: 1 mV

Peak Data:

peak [1] @ -165 [mV], 41.0773 (uA), 25.0185 (uC)

peak [2] @ -858 [mV], 42.3143 (uA), 27.6188 (uC)

peak [3] @ -780 [mV], 38.5575 (uA), 24.0871 (uC)

peak [4] @ -96 [mV], 39.6392 (uA), 24.1764 (uC)

5c
Data File Name: C:\Users\BAS1 Customer\Desktop\CVfinalname122.CV0
Title: CV Run for BASi-Epsilon
Experiment Type: Cyclic Voltammetry (CV)
Printed: 4/29/2013 3:50:49 PM
Date & Time of the run: 4/29/2013 3:53:49 PM
Number of data points: 2700
Display Convention: POLAROGRAPHIC
Initial Potential: 100 (mV)
Switching Potential 1: 1283 (mV)
Final Potential: 100 (mV)
Number of segments: 2
Scan rate: 100 (mV/s)
Current Full Scale: 100 (uA)
Filter: 10 Hz
Quiet Time: 2 (sec)
Sample Interval: 1 (mV)

Peak Data:
peak [1] @ -192 (mV), 40.527 (uA), 25.2514 (uC)
peak [2] @ -878 (mV), 30.4387 (uA), 20.8922 (uC)
peak [3] @ -500 (mV), 34.4702 (uA), 22.7076 (uC)
peak [4] @ -115 (mV), 42.8847 (uA), 27.1775 (uC)

3c
Peak Data:
peak [1] @ -191 [mV], 31.7549 (μA), 19.5369 (μC)
peak [2] @ -900 [mV], 32.2240 (μA), 24.3378 (μC)
peak [3] @ -332 [mV], 30.2129 (μA), 18.7691 (μC)
peak [4] @ -122 [mV], 30.6188 (μA), 19.0504 (μC)
CV Run for BASi-Epsilon

Data File Name: C:sers\BASi\Customer\Desktop\pyCVfinal\figm130 CV0
Title: CV Run for BASi-Epsilon
Experiment Type: Cyclic Voltammetry (CV)
Printed: 4/29/2013 5:36:16 PM
Date & Time of the run: 4/20/2013 5:37:26 PM
Number of data points: 2700
Display Convention: POLAROGRAPHIC
Initial Potential: 100 (mV)
Switching Potential 1: -1250 (mV)
Final Potential: 100 (mV)
Number of segments: 2
Scan rate: 100 (mV/秒)
Current Full Scale: 100 uA
Filter: 10 Hz
Quiet Time: 2 (Sec)
Sample interval: 1 mV

Peak Data:
peak [1] @ -202 (mV), 34.8516 (uA), 21.2991 (uC)
peak [2] @ -902 (mV), 33.6653 (uA), 20.2068 (uC)
peak [3] @ -832 (mV), 33.6653 (uA), 21.0294 (uC)
peak [4] @ -134 (mV), 30.6542 (uA), 22.0638 (uC)
Data File Name: C:sers\RAS\Customer\Desktop\Jay\CVfine.txt\qm131.CV0
Title: CV Run for BASi-Epsilon
Experiment Type: Cyclic Voltammetry (CV)
Printed: 4/26/2013 6:57:32 PM
Date & Time of the run: 4/26/2013 6:57:32 PM
Number of data points: 2700
Display Convention: POLAROGRAPHIC
Initial Potential: 100 (mV)
Switching Potential 1: -1250 (mV)
Final Potential: 100 (mV)
Number of segments: 2
Scan rate: 100 (mV/s)
Current Full Scale: 100 µA
Filter: 10 Hz
Quiet Time: 2 (Sec)
Sample Interval: 1 mV

Peak Data:
peak [1] @ -517 (mV), 35.4437 (µA), 22.2590 (µC)
peak [2] @ -643 (mV), 10.7698 (µA), 13.7671 (µC)
peak [3] @ -148 (mV), 35.0591 (µA), 21.0684 (µC)

7c
CV Run for BASI-Epsilon

Data File Name: C:\Users\BASI Customer\Desktop\Jay\CVfinal\nm132\CV0\n
Title: CV Run for BASI-Epsilon

Experiment Type: Cyclic Voltammetry (CV)

Printed: 4/29/2013 6:13:50 PM

Date & Time of the run: 4/29/2013 6:13:31 PM

Number of data points: 2700

Display Convention: POLAROGRAPHIC

Initial Potential: 100 (mV)

Switching Potential 1: -1250 (mV)

Final Potential: 100 (mV)

Number of segments: 2

Scan rate: 100 (mV/s)

Current Full Scale: 100 uA

Filter: 16 Hz

Quiet Time: 2 (Sec)

Sample Interval: 1 mV

Peak Data:

peak [1] @ -217 [mV], 39.0509 (uA), 25.8808 (uC)

peak [2] @ -923 [mV], 28.7863 (uA), 22.4477 (uC)

peak [3] @ -848 [mV], 28.4581 (uA), 20.1368 (uC)

peak [4] @ -147 [mV], 41.1536 (uA), 25.4124 (uC)

9c

OCH3
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DOI: 10.1039/C5MD00314H

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Permission Letter

Dec 21, 2015
Jaya P Shrestha
Department of Chemistry and Biochemistry
0300 Old Main Hill
Logan, UT 8432-0300

Dear Prof. Liao hai Chen,

I am in the process of preparing my dissertation in the Department of Chemistry and Biochemistry at Utah State University. I plan to complete in the spring of 2016.

I am requesting your permission to include the following paper we co-authored in my doctoral dissertation.


Please indicate your approval by signing in the space provided, attaching any other form or instruction necessary to confirm permission.

Thank you for your cooperation.

Sincerely,

Jaya P. Shrestha

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I hereby give permission to Jaya P. Shrestha to reprint the following publication in part or in full in his doctoral dissertation.


Signed [Signature] Date 12/21/2015
Curriculum vitae

Oct 20, 2015

Jaya P Shrestha

ADDRESSES

Work: Department of Chemistry and Biochemistry
0300 Old Main Hill Utah State University Logan, Utah 84322-0300 Ph: (435) 797-1847
jpshrestha@aggiemail.usu.edu

Home: 839 N 700 E APT # 3
Logan, Utah 84321 Ph: (435)-213-6034

EDUCATION

Ph.D Organic Chemistry Utah State University, Logan, UT
(expected completion: Spring 2016) 84322
2011 Fall-Present

M.S. Chemistry Southern Illinois University
Chemical & Pharmaceutical Science Dublin City University, Dublin, Ireland
Science

(2009 Fall – 2011 Spring)

RESEARCH EXPERIENCE


2008 Undergraduate research assistant in the lab of Dr. Julian McMahon.
Research area: Surface Modification of gold AFM cantilever for the detection of Hydroxyapatite crystals in synovial fluid from osteoarthritis patient using AFM.
Curriculum vitae

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WORK EXPERIENCE

2007-2008  QC Analyst
Bristol Myers Squibb, Mulhuddart, Dublin-15, Ireland

- As a part of the 12 member QC department where quality tests were
  performed to ensure that intermediate and final products meet quality
  standards.
- Tested raw materials and finished products at various stages of the
  production process to ensure GMP compliance, and all the local laws and
  regulations.
- Under minimal supervision, performed analytical testing including
  stability and experimental testing on raw materials and finished products
  using HPLC or other instrumentation for Quality Control.

Training and Expertise

Organic Synthesis

- Synthesis, Purification, and characterization of Carbohydrates
- Synthesis, Purification, and characterization of Heterocycles
- Click Chemistry
- Synthesis, purification, and characterization of new analogs of bioactive
  natural products based on SAR study and molecular docking study.

Biological Study

- Human Cell culture and related assays (Cytotoxicity, ROS
  measurement, Mitochondrial membrane potential measurement,
  Caspase activity, Cell cycle analysis, apoptosis assay, Fluorescence
  Imaging etc.)
- Bacterial cell culture and related assays (Disc diffusion assay, MIC
determination, Synergistic study for FIC determination etc.)
- Fungal cell culture and related assays
- Determination of carbohydrates and other UV/fluorescence active drug
  in rat plasma sample using HPLC

Instrumentations

- 1D and 2D NMR, Mass spectrometry, IR, UV, AFM, LC-MS, HPLC,
  MPLC.

Computation

- Operating system: Linux and Windows
- Graphics: Adobe Photoshop, Adobe Illustrator, Pymol
- Additional software: Graph Pad Prism, Gaussian 9.0, Auto
dock Vina, Microsoft Word, Excel, and PowerPoint

Leadership and Management

- Trained undergraduate and new graduate students.
Curriculum vitae

J.P. Shrestha

- Mentored undergraduate research projects and undergraduate research proposal development.

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Research Activities

Publications

2015


2015


2015


2014


2014


2014


2013


Research Poster Presentations

2015


2015

Shrestha, J. P.; Subedi, Y.P.; Chen, L.L.; Chang, C.-W.T. Mode of Action Study of Cationic Anthraquinone Analog: A New Class of Highly Potent Anticancer Agent. SBI Meeting 2015, USU.
Curriculum vitae

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2013
Shrestha, J.P.; Chang, C.-W.T. “Anti-cancer properties of newly synthesized 1,4- Naphthoquinone analogs” presented at winter meeting of Synthetic Biomanufacturing Institute, 2013.

2012
Shrestha, J.P.; Chang, C.-W.T. “1,4- Naphthoquinone derivatives and their biological activities” presented at Hansen’s Life science retreat, USU, 2012.

2011

2011

2011

Conferences/Professional Meetings Attended

2015
American chemical Society National Meeting, Denver, Colorado.

2011

Funding/support

2012
Dept. of Chemistry/Biochemistry, Utah State University - Travel Award

2011
Dept. of Chemistry/Biochemistry, Southern Illinois University, Edwardsville, Illinois - Travel Award

Professional Memberships

2013-present
Member: American Chemical Society.

2007-2008
Member: Royal Society of Chemistry, UK.

Teaching Activities

Courses Taught

Utah State University, Logan Utah.

2015 (Fall)
Organic Chemistry 2300 - guest lecture

2015 (Spring)
Advanced Organic Chemistry Laboratory

2014 (Fall), 2013 (Fall) & 2012 (Fall)
Organic Chemistry Laboratory I

2014 (Spring), 2013 (Spring) & 2012 (Spring)
Organic Chemistry Laboratory II

2011 (Fall)
Principal of Chemistry Laboratory I

Southern Illinois University, Edwardsville, Illinois

2009 (Fall) & 2010 (Fall)
General Chemistry Laboratory I
Curriculum vitae

J.P. Shrestha

2010 (Spring) & 2011 (Spring), General Chemistry Laboratory II

Education Enrichment Activities

2013 Grant Writing Workshop, Getting Started as a Successful Proposal Writer and Academician seminar. Logan, UT.

References

Chang-Wei Tom Chang, Ph.D.
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