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Synthesis and Biological Activity of Novel Tu100 Derivatives

Oladotun J. Alao

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL TU100 DERIVATIVES

by

OLADOTUN JOHN-PAUL ALAO

(Under the direction of John DiCesare)

ABSTRACT

In an attempt to create more effective chemotherapeutic compounds, the naphthoquinone adduct, 12,13-dihydro-N-methyl-6,11,13-trioxo-5H-benzo[4,5]cyclohepta [1,2 b]naphthalen-5,12-imine (hereafter called TU100) was synthesized. Inspired by its unique and novel mechanism of action, a series of structural derivatives were synthesized to explore structure-activity relationships. The analogues exhibited different cytotoxicity profiles, revealing the indicated regions are involved in cell death induction. Furthermore, the analogues had dramatically different effects on cellular ATP production, suggesting different molecular targets. Synthesis, biological activity, and SAR study of these analogues will be revealed.

INDEX WORDS: TU100, Benzoquinone adduct, GSU100, Anthraquinone adduct, N-ethyl naphthoquinone adduct, p-Phenylbenzoquinone adduct, Cancer, Antitumor activity

SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL TU100 DERIVATIVES

by

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B. PHARM., University of Ibadan, Nigeria, 2011

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial

Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

STATESBORO, GEORGIA

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL TU100 DERIVATIVES

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Electronic version approved:

July 2017

DEDICATION

To the blessed virgin mother of all graces for her unfailing intercessions and to the memory of an exceptional aunt- Mrs. Caroline Ayorinde- and a mother per excellence.

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CHAPTER 1

CANCER AND CANCER THERAPY OVERVIEW

1.1 Facts About Cancer

Cancer is major a health problem worldwide and the second leading cause of death in the United States, with ~1,600,000 new cases each year and 595,690 fatalities.[1] Although there was a gradual rise in the cancer death rate until 1991, currently rates are declining due to early detection and dramatic therapeutic advances.[1]

The term “cancer” encompasses a diverse group of diseases that all arise from accumulated genetic mutations that lead to cell immortalization, inappropriate proliferation, and avoidance of senescence and apoptosis.[2] Diverse mechanisms exist to ensure cells in the body maintain proper functioning for the greater good of the organism, and activate various host defense mechanisms to eliminate damaged or rogue cells. Some cells, however, may undergo mutations that render them insensitive to the body’s controlling signals. Consequently, these cells with genetic abnormalities can escape intra and intercellular defense mechanisms (e.g. checkpoints and the immune system), potentially forming a tumor that can metastasize and threaten organism survival. Men are more prone to prostate, lung, bronchus and colorectal cancers; in fact, these account for 44% of cancer cases in men. Females, on the other hand, are prone to breast, lung, bronchus, and colorectal cancers. The lifetime probability of developing cancer is 42% for men and 38% for women.[1] Thus, approximately one in every two males and one in every three females will develop cancer in their lifetime. Early detection and medical intervention can increase odds of survival, and thanks to advances in chemotherapeutics, many

people have ‘cheated’ death. Approximately 2 million deaths have been averted in men and more 500,000 thousand deaths averted in women in the last two decades.[1] As of January 1, 2016, more than 15.5 million Americans with a history of cancer were alive, and this number is projected to reach more than 20 million by January 2026.[1]

Normal cells divide when needed in response to appropriate signals.[3] This prevents too many cells from being made. Unfortunately, cancer cells do not obey this rule and will divide in the absence of appropriate signals or continue to divide in the presence of a stop signal. Proto-oncogenes are genes responsible for driving normal cell division, but mutations in these genes (creating an oncogene) can lead to inappropriate cell division. Tumor suppressor genes or anti-oncogenes are genes that slow down cell division, repair DNA mistakes or facilitate apoptosis. Mutation to these genes can also cause cells to grow inappropriately, which can lead to cancer. Tumor suppressor genes are mostly dominant genes. For those that are dominant to lose their function, the mutation must affect both copies of the gene. Tumors get nutrients and eliminate wastes by releasing signals that cause angiogenesis (blood vessel formation), thus, diverting abundant nutrients from the body to the cancerous cells.

1.2 Cell Cycle

Cell division is a critical process in multicellular organisms. It is essential to the process whereby a fertilized cell (zygote) develops into a fetus and subsequently matures. It also ensures regeneration of cells in the mature organism like skin, red blood cells, hair, intestinal lining etc. Many cells in a developed organism are differentiated and perform a specific function for a finite time period, so they have to be replaced periodically by proliferation of stem cells. However, in cancer these differentiated cells can be induced to proliferate inappropriately. The process of

cell proliferation is described by the cell cycle, which consists of four distinct phases: G₁, S, G₂, and M (Figure I-1)

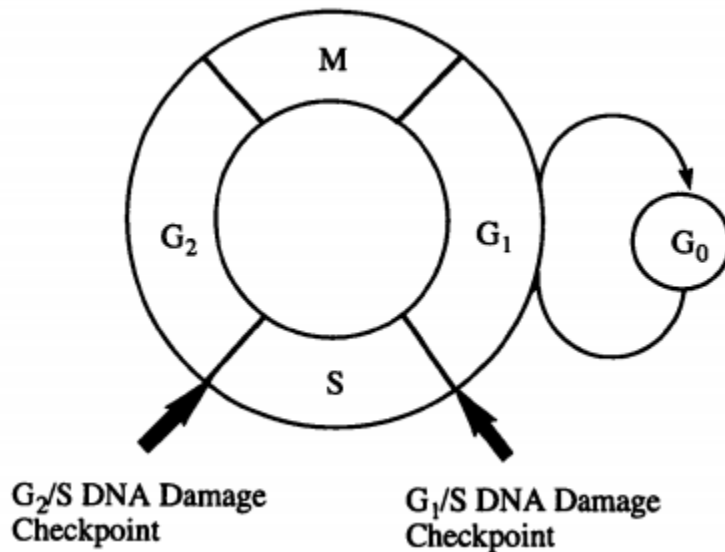


Figure I-1. The cell cycle

During G₁ (gap 1) phase the cell increases in size by synthesizing RNA and proteins in preparation for DNA synthesis. The G₁ checkpoint (Restriction point) ensures that the cell is adequately prepared to commit and complete cell division. S (synthesis) phase involves replicating the DNA in chromosomes to make an exact copy. The G₂ (gap 2) phase is a period of further protein synthesis and cell growth and a period to check accuracy of DNA replication and prepare for mitosis. The nucleus and the cytoplasm divide during the M (mitosis) phase to give two daughter cells; this phase is relatively short, complex and highly regulated. Unfortunately, in some instances genetic mutations can result in the loss of cell cycle controls mechanisms and inappropriate cell division resulting in tumor formation.[4]

1.3 Differences Between Cancer Cells and Normal Cells

Many differences have been identified between normal cells and cancer cells.

- **Growth-** Unlike normal cells that stop growing when there are sufficient cells present, cancerous cells may not. Continuous “firing” of growth factors is sometimes responsible for this. For example, a mutation could cause excess growth factor production driving cell division.[5]
- **Communication-** Cells interact by sending signals to each other. This prevents normal cells from encroaching on each other. Normal cells stop growing when a nearby signal from another cell is received. Cancer cells often fail to respond to such signals.
- **Cell repair and cell death-** The body immune system assesses the integrity of body cells, damaged cells are repaired or induced to undergo apoptosis. The P53 gene, a tumor suppressor that protects genome integrity and induces apoptosis if it cannot be repaired, is disrupted in almost all cancers.[5, 6]
- **Metastasis-** Normal cells secrete substances that makes them adhere together and stay at their location. Cancer cells lack this adhesion substance; hence, can metastasize to distant organs.
- **Differentiation-** Normal cells are well differentiated and mature, while cancerous cells are typically less well differentiated to facilitate rapid cell division.
- **Immune System Evasion-** Cancer cells are able to evade or trick the immune system long enough to grow into a tumor, either by escaping detection or by secreting chemicals that inactivate immune cells that come to the scene. Some of the newer immunotherapy medications enhance this host defense mechanism.[5]

- **Angiogenesis**- This is a normal body process of generating blood vessels for growth and healing processes. Cancer cells, however, cause excessive stimulation of angiogenesis to constantly supply nutrients to and eliminate waste from the tumor.
- **Immortality** –Normal cells except nerve cells typically have a fixed life span. Telomeres are responsible for regulating the number of times a cell can divide. They are present at the end of chromosomes, and their length shortens as the cell divides eventually halting cell division.[5] Cancer cells adopt ways to regenerate their telomeres so that the cell remains in proliferation. For instance, an enzyme, telomerase, works to lengthen the telomeres so that the cell can divide indefinitely—essentially becoming immortal. The enzyme p53 disruption also plays a major role here via loss of genome stability and failure to induce apoptosis.

1.4 Causes of Cancer

Cancers generally are caused by mutation in the DNA, but not all mutagenesis result in carcinogenesis. Mutagenesis can be triggered by many factors like replication error, reactive oxygen from species from metabolism, chemicals etc. focusing on the external factors, we have three classes of agents:[4]

1.4.1 Chemicals

Chemicals are one of the major culprits in causing cancers. Although there are diverse mechanisms through which carcinogens act, a popular feature is their ability to generate active electrophilic forms that attack nucleophilic sites on DNA[4]. The International Agency for

Research on Cancer (IARC) classified different agents into five categories based on the strength of evidence of their ability to cause cancer.[7]

- **Group 1:** Agents in this group are known to be carcinogenic to humans e.g ciclosporin 2-naphthylamine, aflatoxins, arsenic and inorganic arsenic compounds ,benzene, benzo[*a*]pyrene, chloromethyl methyl ether,1,3-butadiene, cadmium and cadmium compounds, chlorambucil, methyl-ccnu (1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; semustine)chromium(vi) compounds, ciclosporin, cyclophosphamide.
- **Class 2A:** For agents in this class, exposure to them poses the risk of cancer development. Examples are, chloral hydrate, ethylene bromide etc.
- **Class2B:** There is limited evidence to support that exposure to these agents might cause cancer in humans and not sufficient evidence in experimental animals. Examples include acetaldehyde, anthraquinone, isoprene, chloroform, P-diclorobenezene etc.
- **Class 3:** Agents in this class are not classifiable as to their carcinogenicity in humans. For example, anthracene, anthanthrene, aniline, p-benzoquinone dioxim, bromoethane, etc.
- **Group 4:** Compounds in this class are devoid of carcinogenic properties e.g caprolactam

Carcinogenic chemicals may cause activation of procarcinogen to the active form via activity of series of enzymes. For instance, benzo[*a*]pyrene a polycyclic aromatic hydrocarbon which is one of the procarcinogens in tobacco, might undergo enzymatic oxidation to an epoxide which can react with an amino group of guanine residues in DNA as shown in Figure I-2.[4]

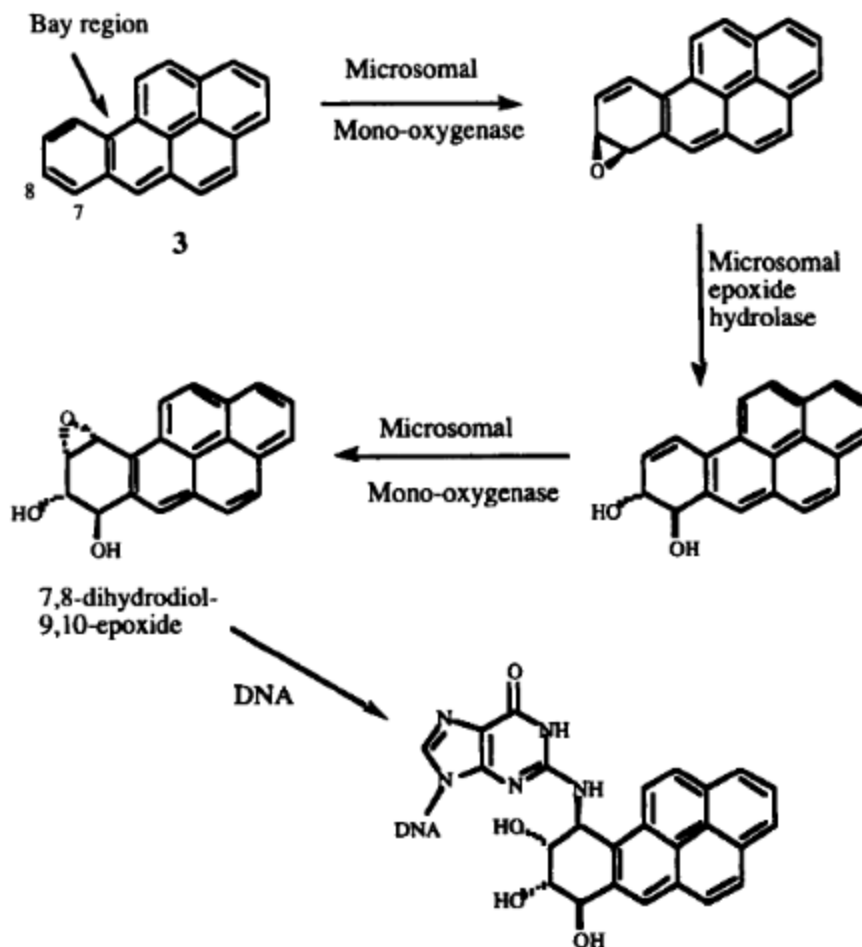


Figure I-2. Activation of benzo[a]pyrene

1.4.2 Radiation

Radiation of certain wavelengths, referred to as ionizing radiation, has sufficient energy to cause damage to the DNA.[4] Example of ionizing radiation includes radon, x-rays, gamma rays, and other forms of high-energy radiation. Ionizing radiation can cause base deletions and alteration or attack the sugar backbone that can cause DNA strand breakage.

1.4.3 Viruses

Via different mechanisms, viruses may cause cancer in human beings. Hepatitis B virus is known to cause liver cancer while papilloma virus can induce cervical cancer.

1.5 Treatment Options for Cancer

Advances in medicine and technology have increased the options available to manage cancer patients. In fact, there has never been more exciting ways to treat cancer than now. Most successful treatments require more than one intervention.

- **Surgery**- Cancers can be cured by entirely removing the tumor or in some cases the affected organ. This is sometimes not possible in some types of cancers or when the cancer has metastasized.
- **Chemotherapy**- This involves treatment with drugs that can kill cancer cells. Although drugs are the preferred treatment option in many cases, this approach is hampered by serious side effects.
- **Radiation therapy**- This uses ionizing radiation to kill cancer cells and shrink tumors. Modes of radiation can either be externally or internally.
- **Immunotherapy**. This treatment strategy involves stimulating the patients' immune system to fight the tumor cells.
- **Photodynamic therapy**- This is a ternary cancer treatment often involving photosensitizers, light, tissue oxygen and use of a laser or other light source.
- **Hormonal therapy**- The growth of some cancers can be inhibited by blocking the activity or providing hormones. Common examples of hormone sensitive tumors include some types of breast cancers and prostate cancers.

1.6 The challenge of developing effective anti-cancer drugs

Development of effective cancer treatments is difficult due to the origin of the disease within cells of the body. Due to many similarities between normal and transformed cells, designing highly specific therapeutics with few side effects has proven difficult.[8] Chemotherapeutics are not as safe as other conventional drugs like antibiotics that target foreign bacteria in the body. Inappropriate cellular division is a universal phenotype of cancer, so most chemotherapy drugs, even with distinct mechanisms of action, are directed against rapidly proliferating cells.[9] Unfortunately, all these drugs possess a relatively low therapeutic index resulting in a range of side effects (many quite severe) due to adverse consequences on normal cell populations also undergoing rapid proliferation (e.g. hair, blood, and intestinal cells).[8] Hence, clinicians operate within the context of a limited therapeutic index, which depends on the extent to which a drug preferentially targets cancerous cells to normal cells. An additional problem is that while these drugs may initially suppress cancer cell proliferation, eventually a drug-resistant clone will expand.[8] To address this, chemotherapeutics are often used in combination. There are some fourth-generation drugs that capitalize on the unique genetic signature of individual cancers (e.g. Gleevac).[8] Thus, there is a pressing need to further optimize chemotherapeutic agents that have proven effective and minimize side effects to develop more effective cancer chemotherapeutics

1.7 Mechanism of action of currently available chemotherapeutics

Current chemotherapeutic drugs can be divided into three general classes based on their molecular targets.[10] Some drugs limit synthesis of precursors required for DNA synthesis which requires building blocks such as folic acid, heterocyclic bases, and nucleotides.[10]

Clinically important drugs such as methotrexate, fluorouracil, hydroxyurea, and mercaptopurine all function by blocking formation of these DNA building blocks and thus inhibit DNA replication/cancer cell proliferation. The second class of chemotherapeutic drugs cause direct damage to nucleic acids by chemically altering DNA and RNA, thereby disrupting DNA replication and halting cancer cell proliferation.[10] Examples include cisplatin and antibiotics such as daunorubicin, doxorubicin, and etoposide. The final main category of chemotherapeutics target mitosis (i.e. cell division) in order to block cancer cell division. Many of these compounds disrupt formation or operation of the mitotic spindles that normally ensure proper chromosome segregation. Drugs in this class include Vinblastine, Vincristine, and Paclitaxel. For all three classes, the overall goal is to stop proliferation of the cancer cell and hopefully induce its death.

A number of antibiotics such as anthracyclines, dactinomycin, bleomycin, adriamycin, mithramycin, bind and disrupt normal DNA functioning.[11] Consequently, they modulate DNA metabolism, cause DNA damage, prevent replication, and/or block RNA synthesis. In some cases, these drugs intercalate (i.e. wedge) between DNA base pairs, altering DNA structure and inhibiting function of proteins/enzymes that bind DNA. Inhibition of DNA synthesis and transcription result in induction of mutations and eventually cell death. Carboplatin and Cisplatin are two well-known intercalating agents that preferentially bind guanine and adenine bases to generate debilitating cross-links. Other examples of the group cause strand breakage or interfere with the action of topoisomerases. Many of these drugs were originally isolated from natural sources, and while directed modification has in some cases significantly increased their ability to kill cancer cells, in most instances they still cause undesirable side effects.

With growing knowledge of cancer biology, cancer-critical genes and other features unique to cancer cells have been identified, and these may now be aimed at to provide a targeted

therapy.[3] This marked a shift to developing anticancer agents with lesser side effects. The first of such drugs approved is *Gleevec*. It blocks the activity of the Bcr-Abl enzyme; thus, halting proliferation of chronic myeloid leukemia.[3] Also, the estrogen antagonist Tamoxifen is used to prevent breast cancer re-occurrence. Other drugs in this class, approved or in clinical trials, targets unique cancer features like presence of Her2 proteins, absence of p53, blockage of angiogenesis etc. The major drawback to this class of compounds is the possibility of ‘‘multidrug resistance’’, a phenomenon in which a patient develops resistance not only to the drug being taken but also to other drugs he has never been exposed to.[3] This is due to overproduction of the gene Mdr1, a gene that codes for plasma-membrane-bound transport ATPase.[3] Over production of this or some other members of the family prevents intracellular accumulation of certain drugs by pumping them out of the cell.

1.8 TU100 Preliminary Studies

In cancer treatment, there is still much left to be done to find a lasting solution for those suffering from these insidious illnesses. Attempts to address this problem led to the synthesis of a novel antitumor class of compounds. Novel compounds have been synthesized by the 3+2 dipolar cycloaddition of N-methyl-4-hydroxyisoquinoline and 1,4-naphthoquinone and 1,4-benzoquinone to give ‘‘TU100’’ and ‘‘Benzoquinone adduct’’.[12] These agents, TU100 and benzoquinone adduct, bear structural similarities to two known classes of antitumor agents-anthracyclines and tetrahydroisoquinolines.[13]

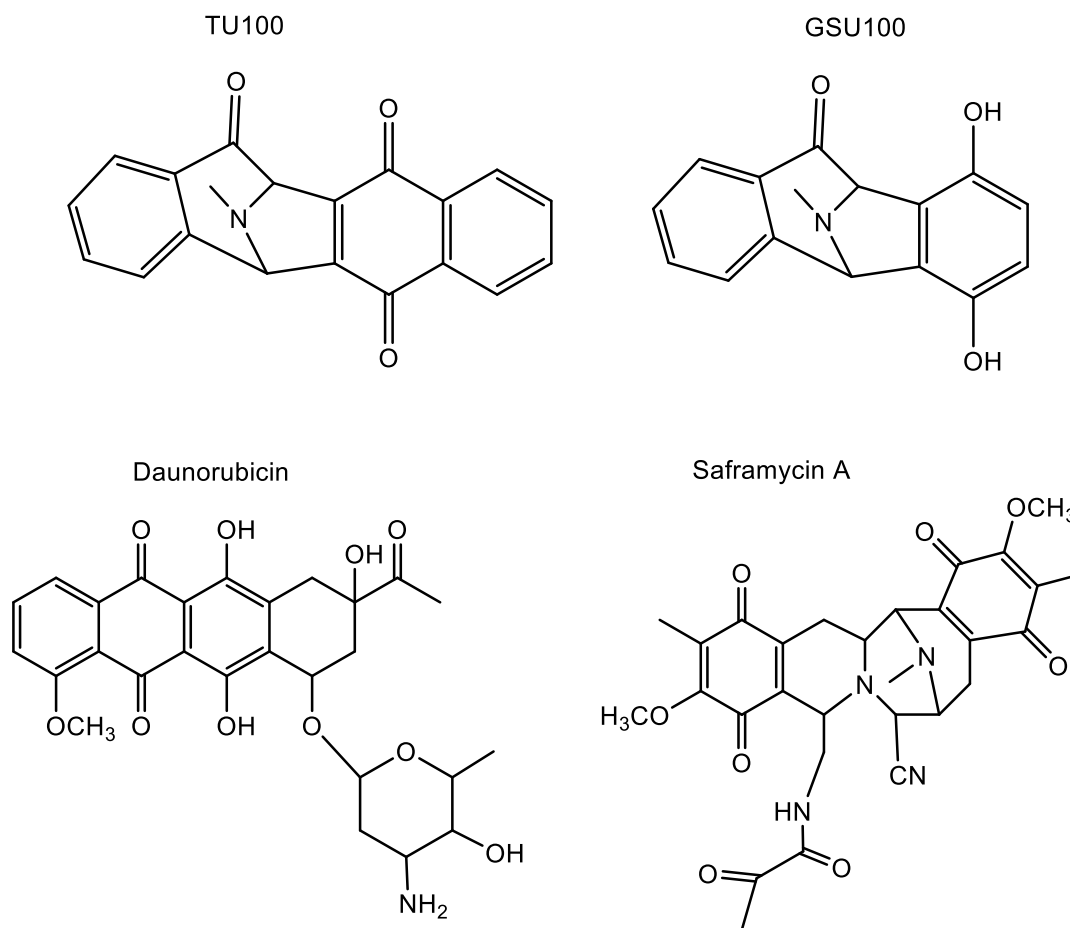


Figure I-3. Similarities between anthracyclines, tetrahydroisoquinoline and TU100

Anthracyclines are a large group of structurally related compounds that encompass an important therapeutic arsenal against many different types of cancer.[13] They are based upon daunosamine and tetrahydronaphthacenedione, and are used to treat a variety of cancers such as leukemias/lymphomas, breast, uterus, ovaries as well as lung cancers. Their mechanism of action involves disrupting DNA metabolism by intercalating between DNA base pairs and/or causing free radical damage to DNA ribose moieties[13] and also they inhibit topoisomerases and generate reactive oxygen species(ROS). These agents generally work in all phases of the cell cycle. Consequently, enzymes like DNA polymerases, helicases, and topoisomerases cannot function properly. Anthracyclines such as daunorubicin, doxorubicin, epirubicin, and idarubicin

inhibit topoisomerase, an enzyme crucial in DNA replication. It binds and cleaves DNA in order to alter twisting/supercoiling of the double strands. There is also some evidence of supplementary effects that may be important for therapeutic efficacy. For example, some members of this group create iron-mediated free oxygen radicals that damage the DNA and cell membranes, while other may also inhibit other cellular processes like protein turnover.[13] A major drawback associated with the use of these chemotherapeutics is the toxicity adverse effect of anthracyclines which include but not limited to cardiotoxicity, congestive heart failure, and vomiting. Although the precise mechanism of cardiotoxicity has not been identified, it may be caused in part by interference with signaling in heart muscle cells, free radical damage, or accumulation of anthracycline metabolic by-products.[14] This cardiotoxicity sets an upper limit on drug dosage that must be carefully monitored, and often limits potential benefits against the cancer. Modified versions of various anthracycline (e.g. Mitoxantrone and Iloxantrone) have been synthesized in an attempt to avoid this potentially serious complication. Liposomal variations of daunorubicin and doxorubicin have also been created, and may be somewhat less toxic to cardiac tissue.[14]

The tetrahydroisoquinilones on the other hand, are an equally diverse group of compounds defined by a quinone and aromatic core that function as an antibiotic.[14] These natural products are classified into the saframycin, naphthyridinomycin/bioxalomycin, and quinocarcin/tetrazomine families that includes therapeutically important compounds that exhibit antitumor, antimicrobial, and cytotoxic properties.[15] As with the anthracyclines, small structural changes in the tetrahydroisoquinilone antibiotics can drastically influence their biological activity, thus supporting further efforts to generate new derivatives.[15]

Due to the structural similarity of TU100 and Benzoquinone adduct to these agents, they were presumed to exhibit anti-tumor activity. Consequently, they were submitted to the National Cancer Institute for evaluation.

1.9 Testing by the National Cancer Institute Developmental Therapeutics Program

The compounds were submitted to the National Cancer Institute (NCI) Developmental Therapeutics Program (DTP) for screening of potential anti-tumor activity. The DTP uses a series of *in vitro* screens to reduce the number of compounds submitted for resource intensive and costly *in vivo* animal analysis.[16] The compound chemical structure is first evaluated to assess the uniqueness compared with other molecules in the database. If selected, a pure sample undergoes preliminary evaluation of cytotoxicity against three representative cancer cell lines. This pre-screening allows for elimination of about 50% of drug candidates without significantly increasing the probability of missing active compounds.[16] Compounds that scale through this screening are then evaluated against a 60 cell line of cancers from different origins such as blood, skin, lung, colon, brain, ovary, breast, prostate and kidney tissues. A minimum of five concentrations at 10-fold dilutions for 48 continuous hours are used. A sulforhodamine B protein concentration assay is used to quantitate total cellular protein and estimate effects on cell growth/viability.[16] TU100 and BA were evaluated against the three-cell line screen (data not shown), and they both passed the initial test. Both were subsequently submitted to the full 60 cell line screen. TU100 met these more rigorous criteria, the result was confirmed by repeating the evaluation using a new sample of the drug. TU100 dose–response is shown in Figure I-1. Bars extending to the right reflect the degree of increased sensitivity to the drug relative to all other cell lines tested [17]. The leukemia cell lines, for instance, were ~30 times more sensitive to TU100. Bars extending to the left indicate relative insensitivity (e.g., CNS-derived cancer

cells). In summary, overall results from the DPT screen indicate TU100 has above-average activity for leukemias, renal, and breast cancer cells; and below-average activity for lung, column, and CNS cancer cells.[17]

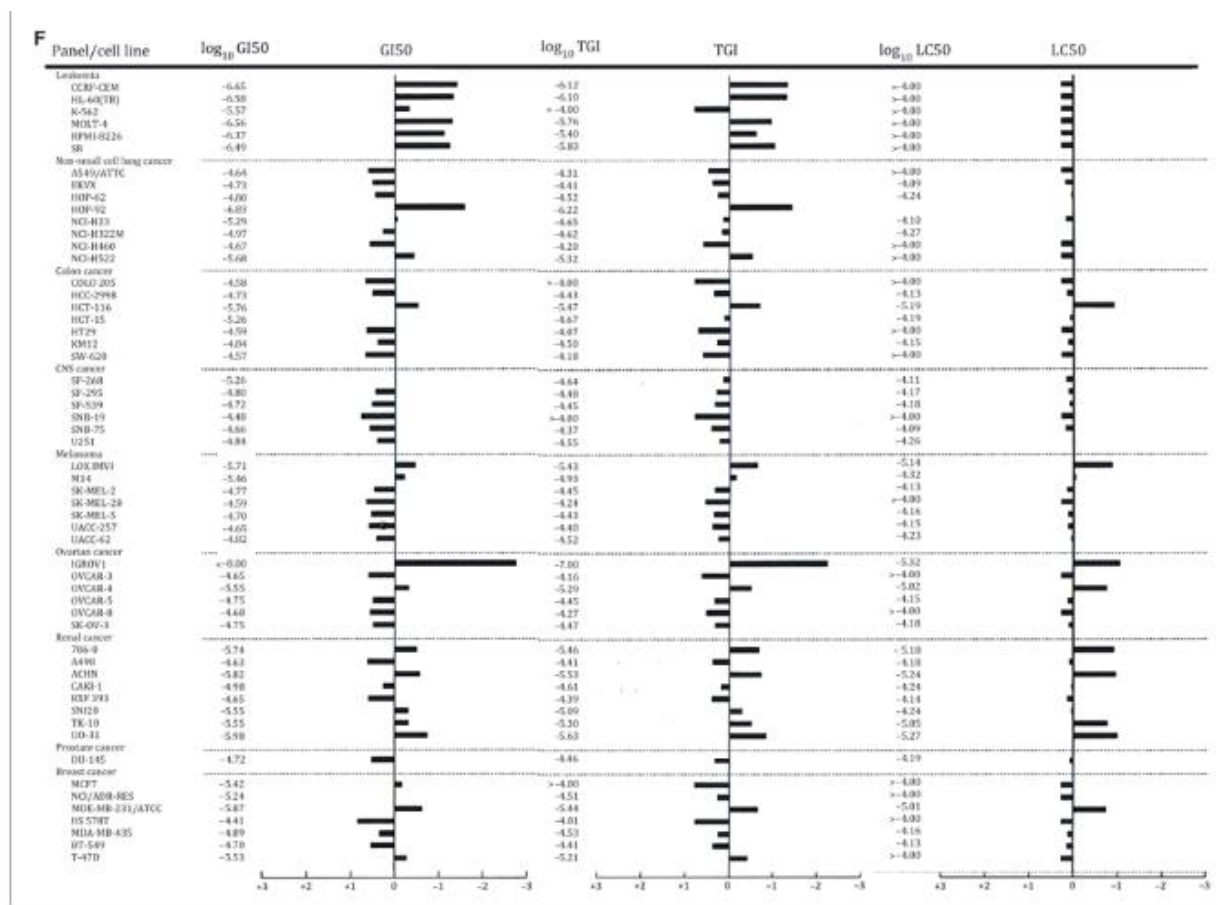


Figure I -4. TU100 evaluation against panels of tissue-specific cancer cell lines. Showing the 50% growth inhibition (GI₅₀), total growth inhibition (TGI), and lethal concentration (LC₅₀) for different cell lines. Data generated by the Developmental Therapeutics Program at the National Cancer Institute

To identify the mechanism of action, the compound was evaluated against approximately 600,000 compounds that DTP has previously analyzed using the COMPARE algorithm. This computer program compares patterns of cellular responses to identify potential similarities in activity profiles.[14] TU100 had at best a 60% correlation with Maytansine and Rhizoxin, both

of which are macrocyclic molecules that inhibit mitosis. [14] This low match correlation and the novel structure of TU100 suggests it may have a unique mechanism of action or act via combinations of mechanisms.

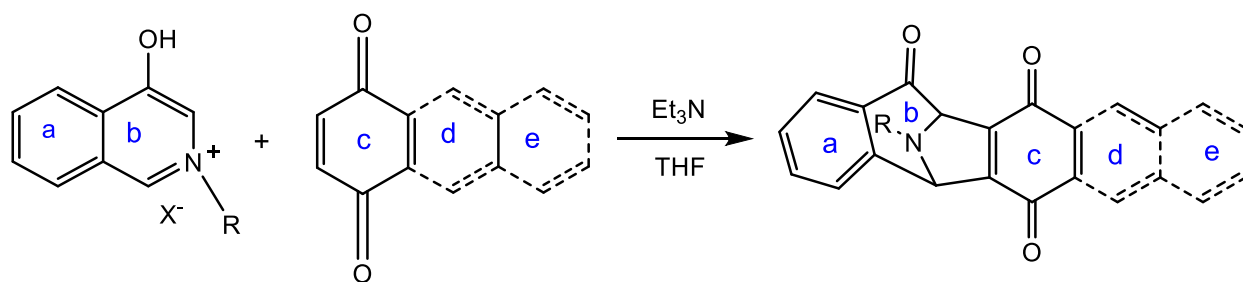
After the 60 cell line screen, the NCI subsequently tested TU100 activity in a more physiologically relevant hollow fiber *in vivo* assay using the leukemia and lymphoma cell lines K562, MOLT-4, HL-60(TB), SR, RPMI-8226 and CCRF-CEM [14]. The hollow fibers containing indicated tumor cell lines are implanted intraperitoneally in mice. After 4 days, the mice are treated with the experimental agent at two dose levels for an additional 4 days, followed by fiber collection and determination of percent net growth of the tumor lines relative to vehicle-treated controls. Compounds were given scores for intraperitoneal (i.p.) placement, subcutaneous (s.c.) placement, and net cell kill. A compound is considered to have met the criteria for further evaluation if the combined i.p. plus s.c. score is 10 or higher, the s.c. score is 4 or higher, or if there is any cell kill[17]. TU100 received an i.p. score of 2, a s.c. score of 6, and no cell kill. It was therefore considered to have met criteria for further evaluation by its s.c. score, which was >4 and indicated above-average activity against leukemias, renal cancers, and breast cancers. The next test is the xenograft testing. NCI requested further characterization of the drug before undertaking more extensive xenograft testing[17]. Following this request, attempts were made to determine the mechanism of action of TU100. It was discovered that it induces cytotoxicity via apoptosis[14] and equally it inhibits both topoisomerase I/II a rare effect among chemotherapeutics agents[18]. Most drugs either inhibit topoisomerase I or topoisomerase II. The potency of TU100 is comparable to commercially available antitumor agents like Daunorubicin and luteolin. In fact, it has a faster onset of action than these commercially available products.[14]

Due to the various unique properties of TU100, it becomes imperative for us to develop a structural activity relationship profile for this unique class of compound to determine the possibility of developing drugs with better selectivity for cancer cells and reduced side effects.

CHAPTER 2

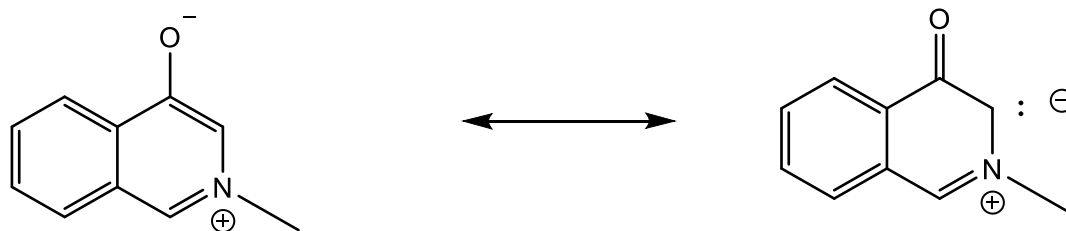
Results and Discussion.

The objective of our research was to develop structural analogues of TU100, determine their antitumor biological activities relative to TU100 and thus, develop a structural activity relationship for this class of compound. To achieve this, we labeled the ring system as shown in Scheme II-1a. In general, a completely oxidized product undergoes a 4-stage process. The first is the deprotonation of the 4-hydroxyisoquinoline by trimethylamine, and the deprotonated form can also exist in his resonance form shown in scheme II-1b. This is followed by 1, 3 dipolar cycloaddition reaction between the dienophile and the diene to give a cycloadduct product- a compound highly susceptible to keto-enol tautomerisation to form a more stable hydroquinone. If the hydroquinone is sufficiently activated towards air oxidation, the later proceeds to give the corresponding quinone which is our desirable product. Different modification was done on these rings as will be discussed thereafter.



R= Methyl, Ethyl or Benzyl

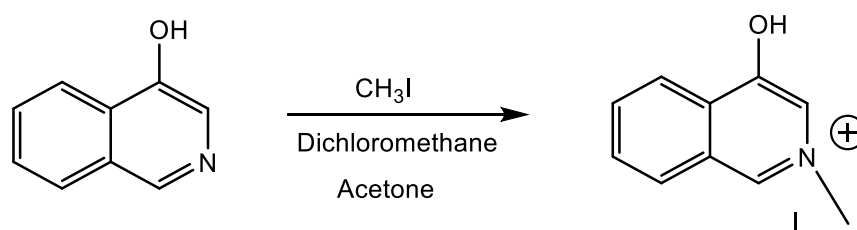
Scheme II-1a. General reaction schematic



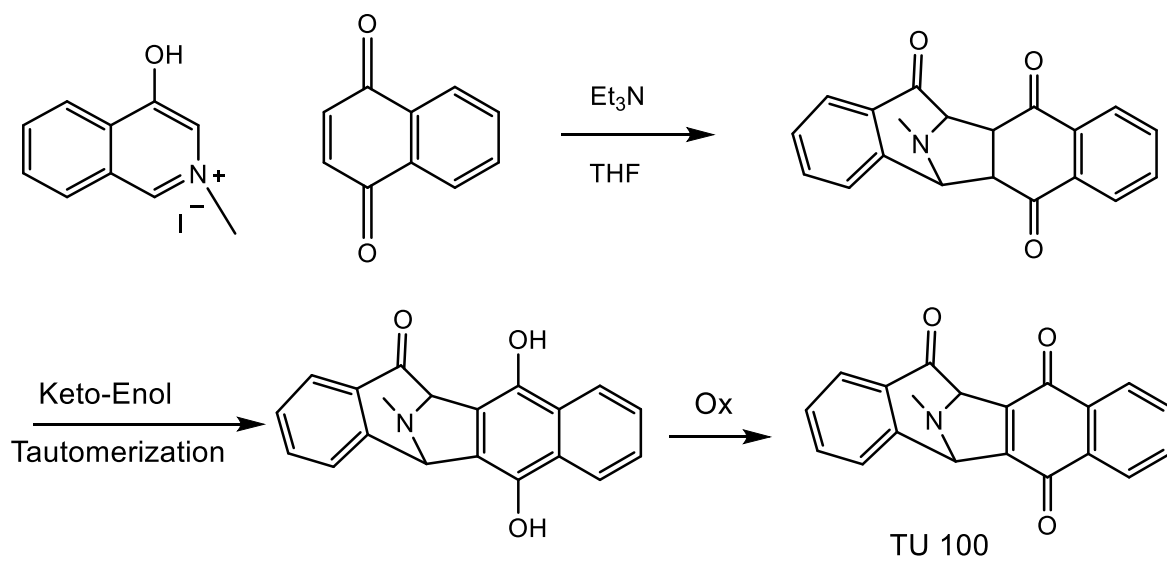
Scheme II-1b Deprotonation step

2.1 Synthesis of TU100

We synthesized TU100 by following the method previously used by DiCesare et al.[12] which involves [3+2] dipolar cycloaddition of N-methyl-4-hydroxyisoquinolinium iodide and 1,4-naphthoquinone. As expected, the initial product from the cycloaddition reaction, a hydroquinone, air oxidized to the quinone adduct upon standing. The initial crude product was found to contain some levels of impurity. The impurity bears structural characteristics to the desired product and hence complicated the ease of purification. Several methods such as recrystallization (cyclohexane) flash column chromatography, combi-flash and chromatotron chromatography methods didn't completely separate the impurity from the product. After successive purification with three rounds of flash column chromatography runs (chloroform) an appreciable pure compound was achieved with a yield of 70%. Product formation was monitored with TLC and product purity was confirmed by ^1H NMR and ^{13}C NMR (figure II -1a&b). This sample was sent for biological analysis.



Scheme II-2a Synthesis of N-methyl-4-hydroxyisoquinolinium iodide



Scheme II-2a Synthesis of TU100

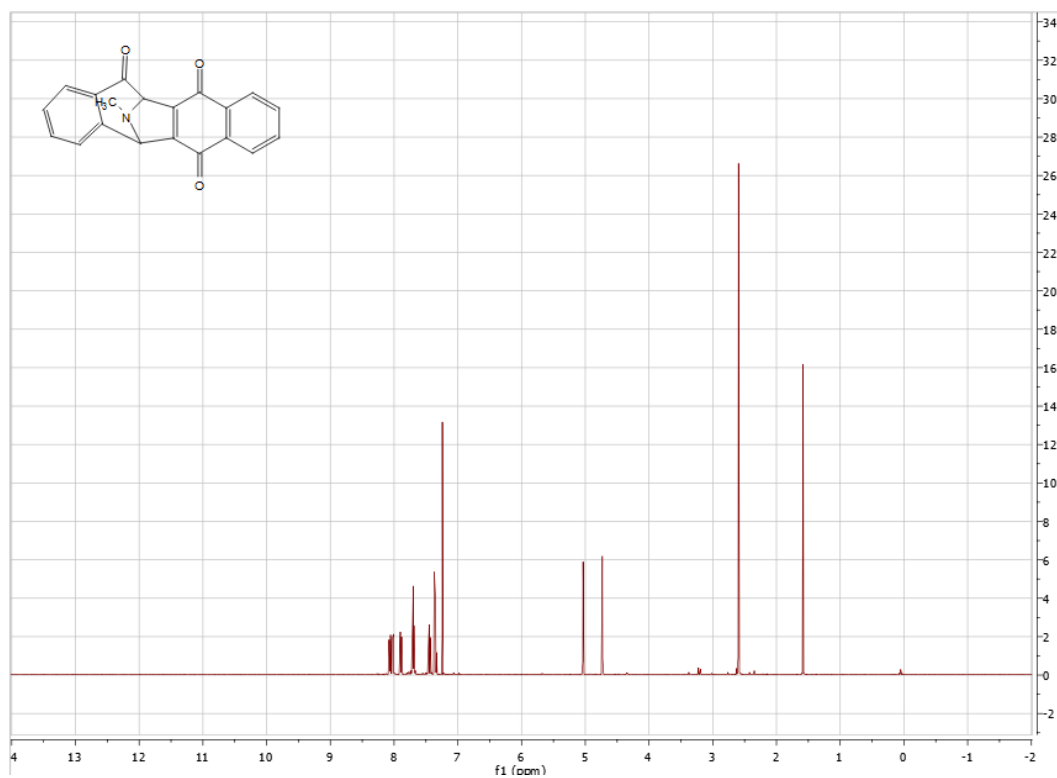


Figure II-1a ^1H NMR of TU100

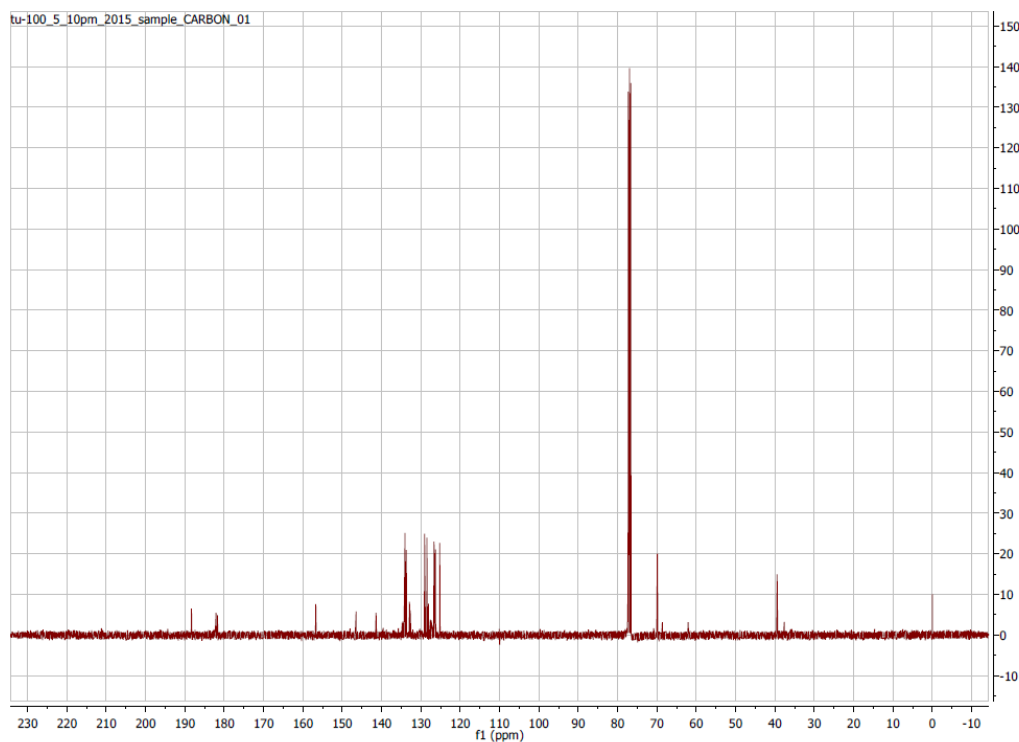
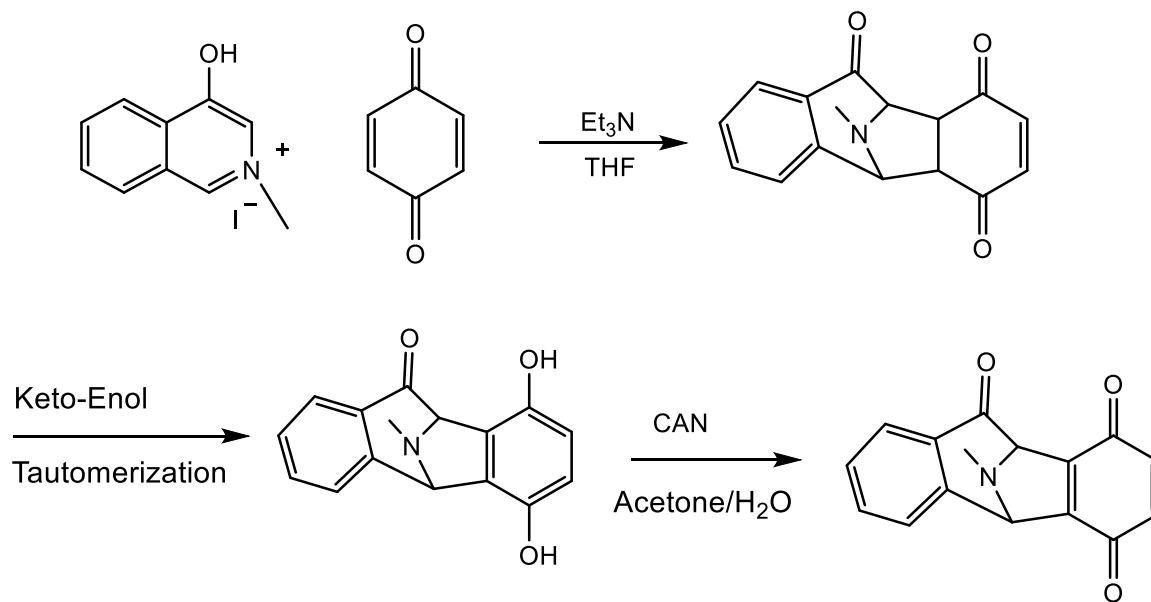


Figure II-1b ¹³C NMR of TU100

2.2 Synthesis of GSU100

The idea behind the synthesis of GSU100 was that the benzoquinone adduct, a hydroquinone, previously synthesized[12] and submitted to NCI for evaluation did not pass the 60cell line evaluation. We suspected this was due to the fact that it did not oxidize to the quinone. Hence, we expected oxidizing the hydroquinone to the quinone would increase the activity of the compound. To achieve this, the process described for the synthesis of the benzoquinone adduct was followed; [3 + 2] dipolar cycloaddition of N-methyl-4-hydroxyisoquinolinium iodide and benzoquinone gives the hydroquinone adduct (scheme II-3). Unlike the naphthoquinone adduct, this initial product didn't undergo air oxidation on standing.



Scheme II-3 Synthesis of benzoquinone adduct and GSU100

Several attempts were made to force the product to oxidation. We bubbled air through it and also dissolved it in sodium bicarbonate. Simple air-oxidation proved futile because the hydroquinone is not sufficiently activated towards oxidation[19]. Being mindful of the delicate nitrogen bridge and not wanting to sacrifice the labile but crucial nitrogen linkage during the process of oxidation, we restrained from using the conventional oxidizing agents and opted for the possibility of using mild oxidizing agents. Literature review shows that cerium ammonium nitrate (CAN) or 2,3-Dichloro-5,6-Dicyanobenzoquinone (DDQ) might be helpful.[20, 21] CAN is a one-electron oxidation reagent and cerium (IV) reagents are relatively mild oxidation reagents in comparison with other metal-based oxidation reagents such as potassium permanganate and chromium (VI) salts. [21] After several attempts, the right ratio of hydroquinone to CAN, 1:2, was determined which afforded a purified yield of 71.2%. Oxidation of the hydroquinone was confirmed by structural characterization by ¹H NMR and ¹³C NMR. A shift in the nitrogen-bridge ¹H NMR signals were observed from 4.48 ppm and 4.95 ppm in the hydroquinone to 4.57 ppm and 4.89 ppm in the quinone product (figure II-2a and c). The signals

in the carbonyl region of the ^{13}C NMR changed from one signal in the hydroquinone sample to three signals after reacting with CAN (figure II-2b and d). Hence, in addition to determining the biological effect of oxidizing the hydroquinone, GSU100 also provides the opportunity to examine the effect of removal of the ring labeled “d” in scheme II-1.

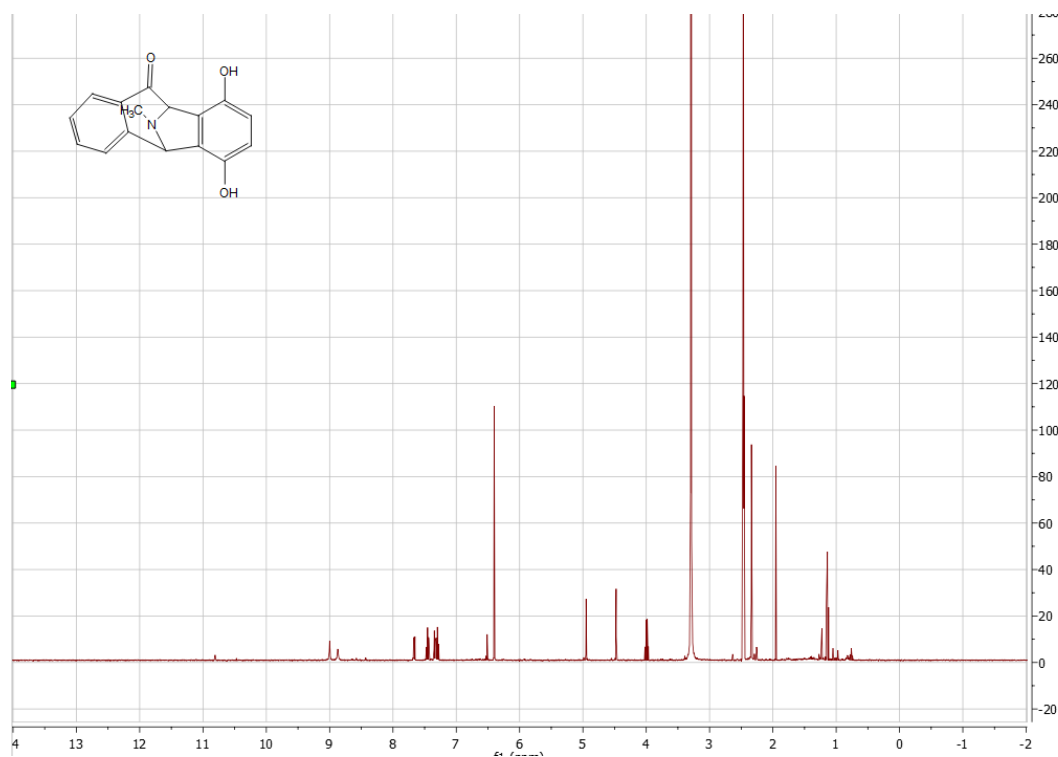


Figure II-2a ^1H NMR of benzoquinone adduct

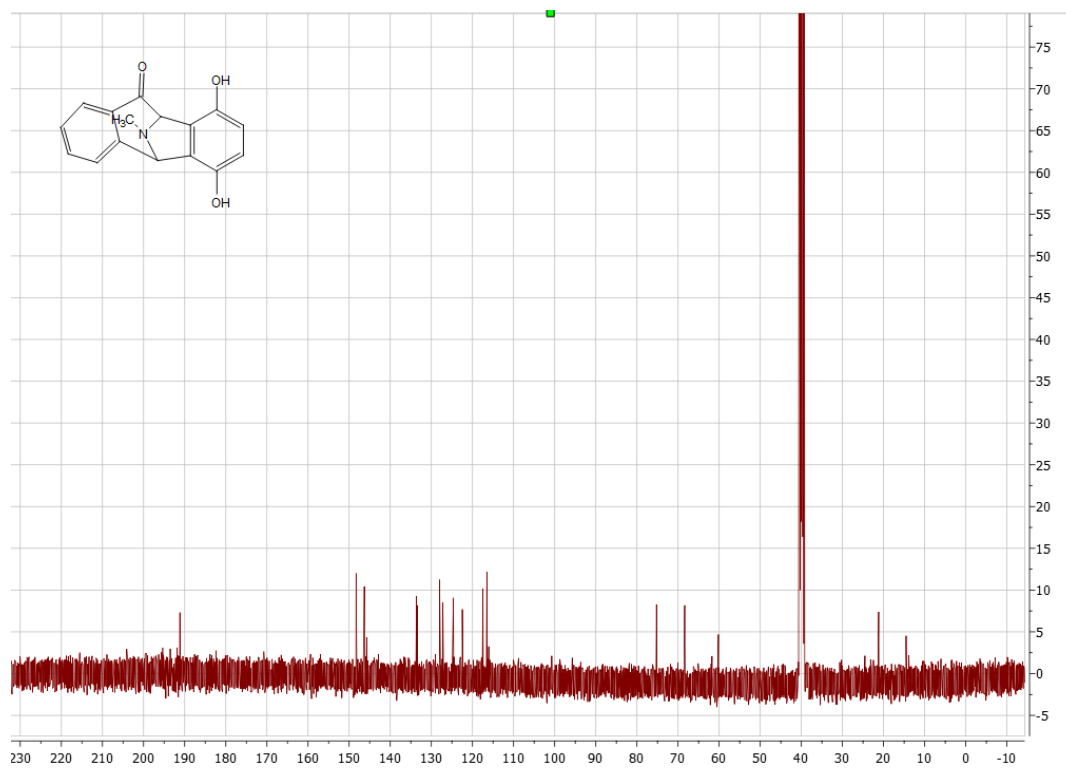


Figure II-2b ^{13}C NMR of benzoquinone adduct

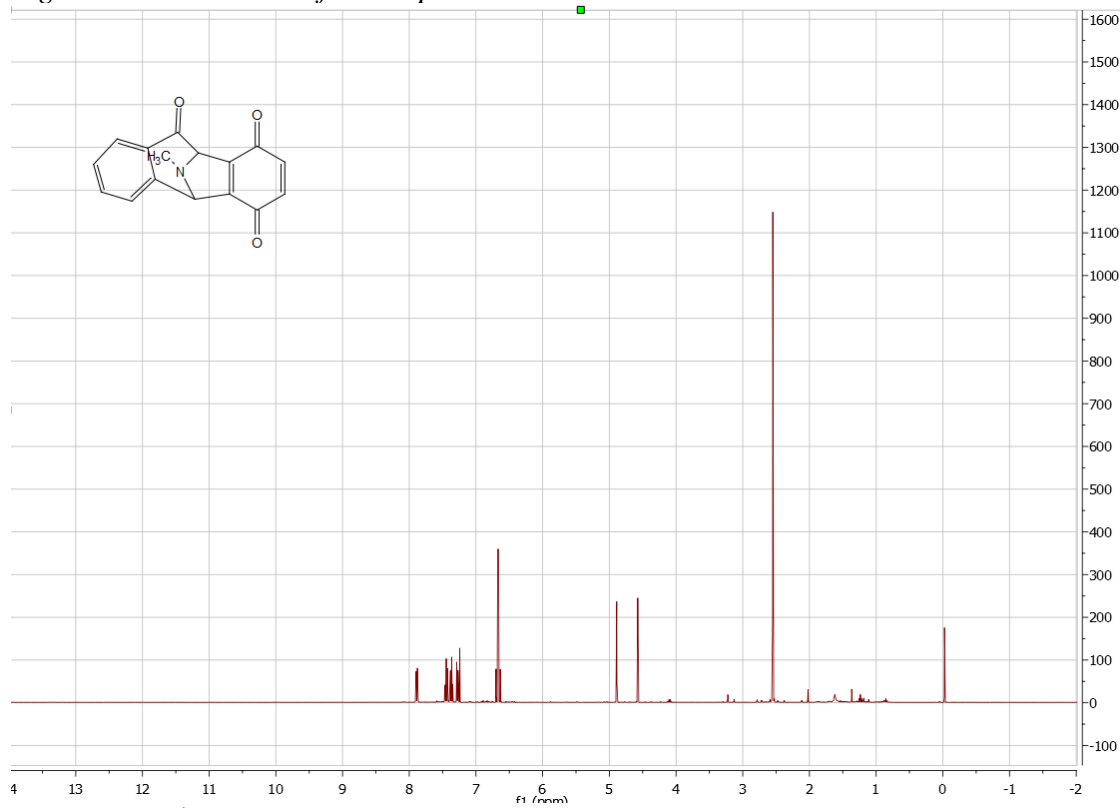


Figure II-2c ^1H NMR of oxidized benzoquinone adduct/GSU100

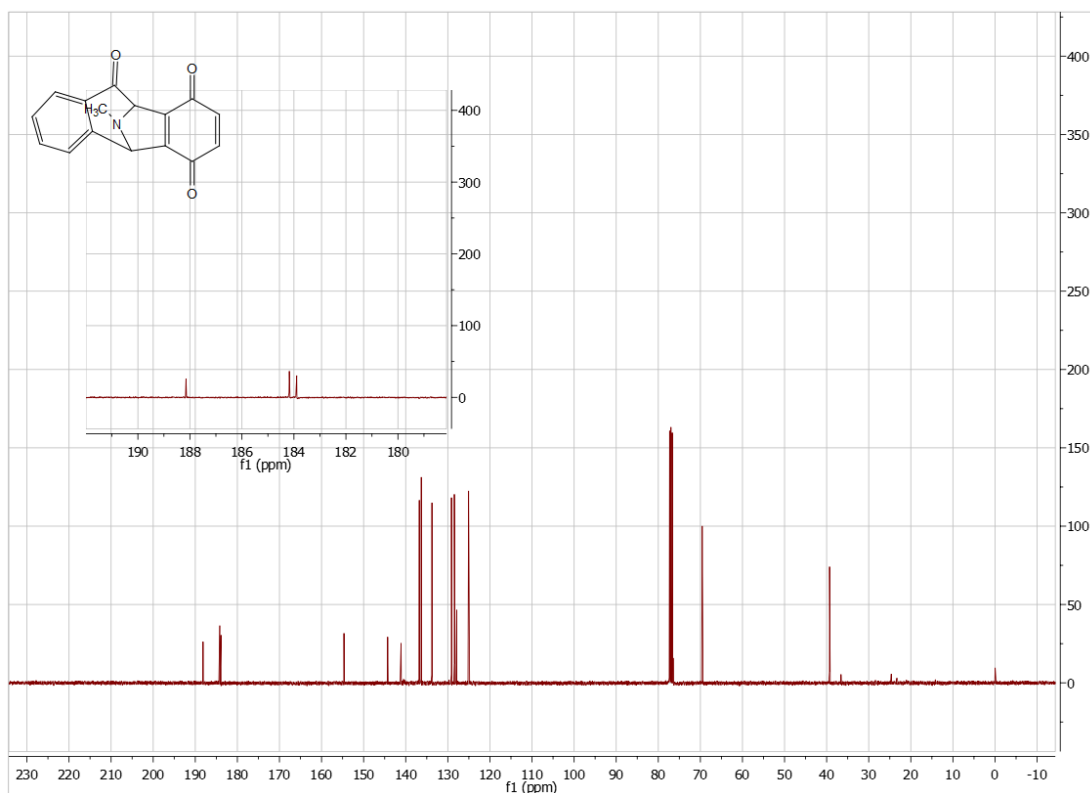
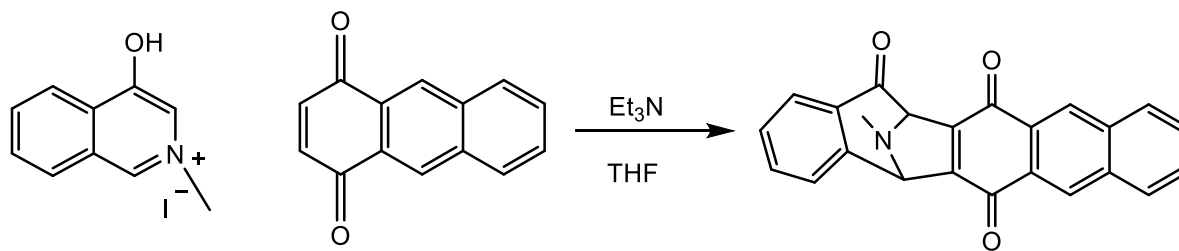


Figure II-2d ^{13}C NMR oxidized benzoquinone adduct/GSU100

2.3 Synthesis of anthraquinone adduct

Having successfully synthesized a compound that has one ring less than TU100, we proceeded to add an extra ring labeled “e” in scheme II-1. This was achieved by substituting one of the starting materials in the synthesis of TU100 - 1,4-naphthoquinone, with 1,4-antraquinone in the reaction (Scheme II-4). The reaction followed the same process as the synthesis of TU100. The initial hydroquinone undergoes air-oxidation to give a quinone adduct. The product was purified by flash column chromatography. The ^1H NMR and ^{13}C NMR is shown in figure II-3 and a yield of 72.7 % was achieved.



Scheme II-4 Synthesis of anthraquinone adduct

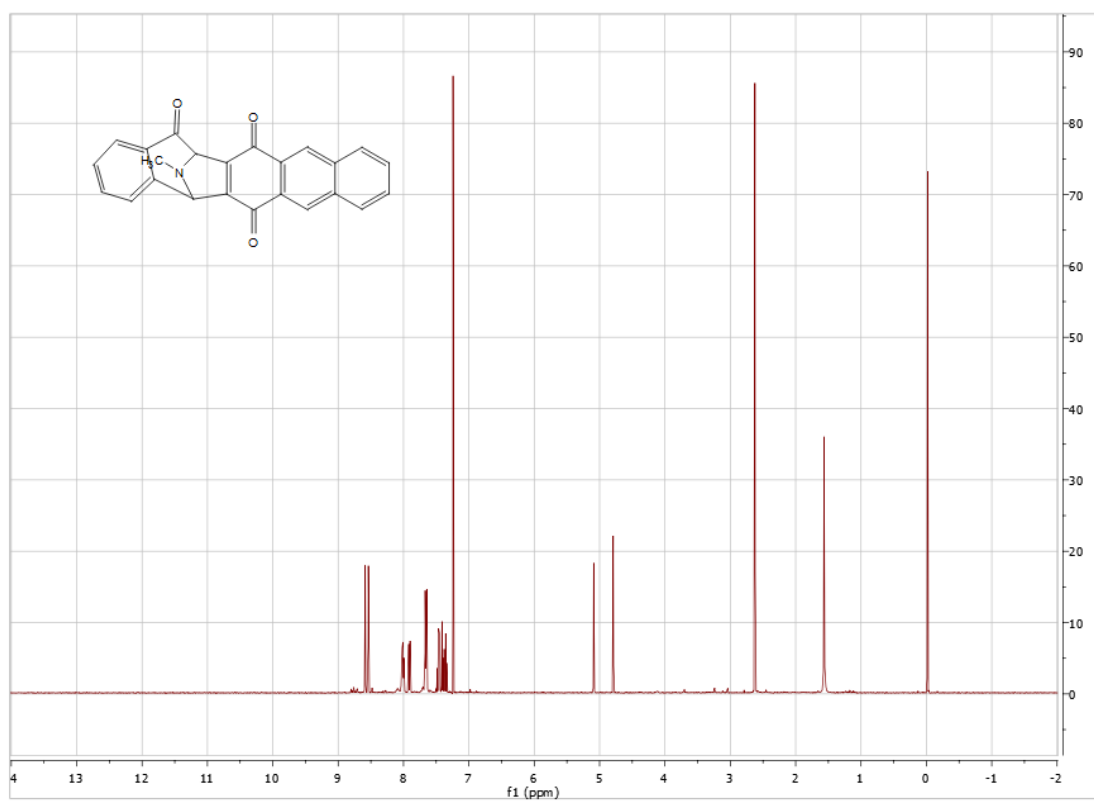


Figure II-3a ^1H NMR of anthraquinone adduct

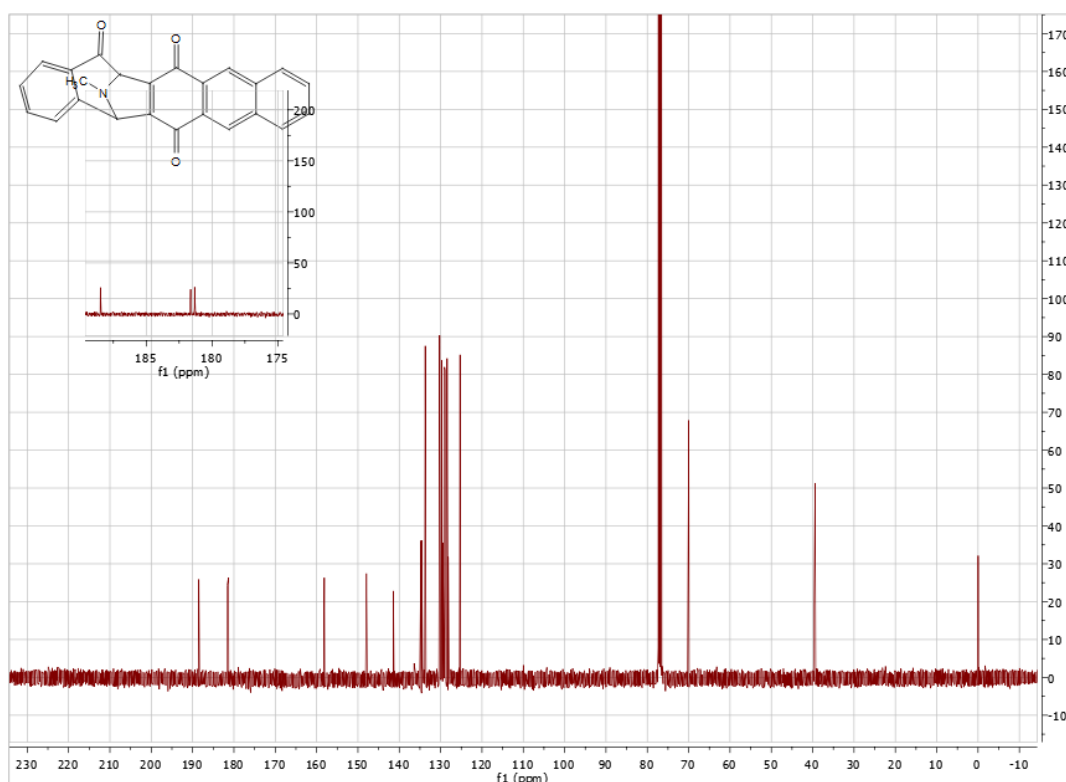
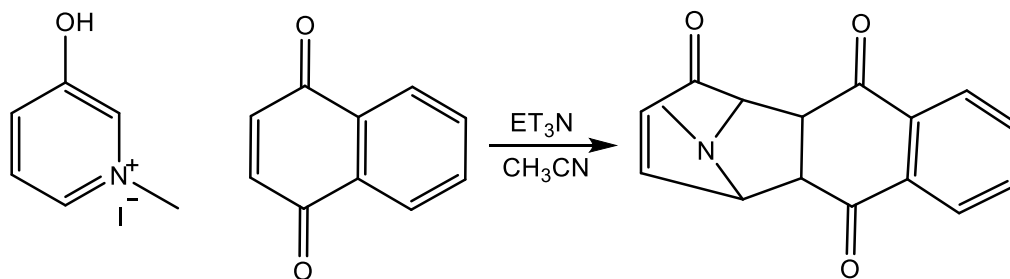


Figure II- 3b ^{13}C NMR of anthraquinone adduct

2.4 Methy-3-oxypyridyl-betaine hydriodide and its adduct

We set out to remove the ring labeled “a” in scheme II-1. To achieve this, we synthesized methyl-3-oxypyridyl-betaine hydroxide and reacted it with 1,4-naphthoquinone to form the cycloadduct product shown in scheme II-5. This reaction did not yield the expected product as shown by the absence of the nitrogen bridge protons on the ^1H NMR. We tried changing the base and were still not successful. We suspected polymerization of the product was taking place. Some literatures cited the use of hydroquinone to reduce such polymerization problems.[22] We added a small quantity, 10%, hydroquinone to the reaction mixture but still didn’t yield the expected result.



Scheme II-5 Synthesis of methy-3-oxypyridyl-betaine hydriodide adduct

We further thought of having a bulky group such as a benzyl group at the nitrogen terminal along with addition of a small quantity of hydroquinone to the reaction to eliminate or reduce polymerization. This approach was not successful as there was no formation of the desired cycloadduct product. We opted to suspend this part of the project temporarily and proceed with other reactions.

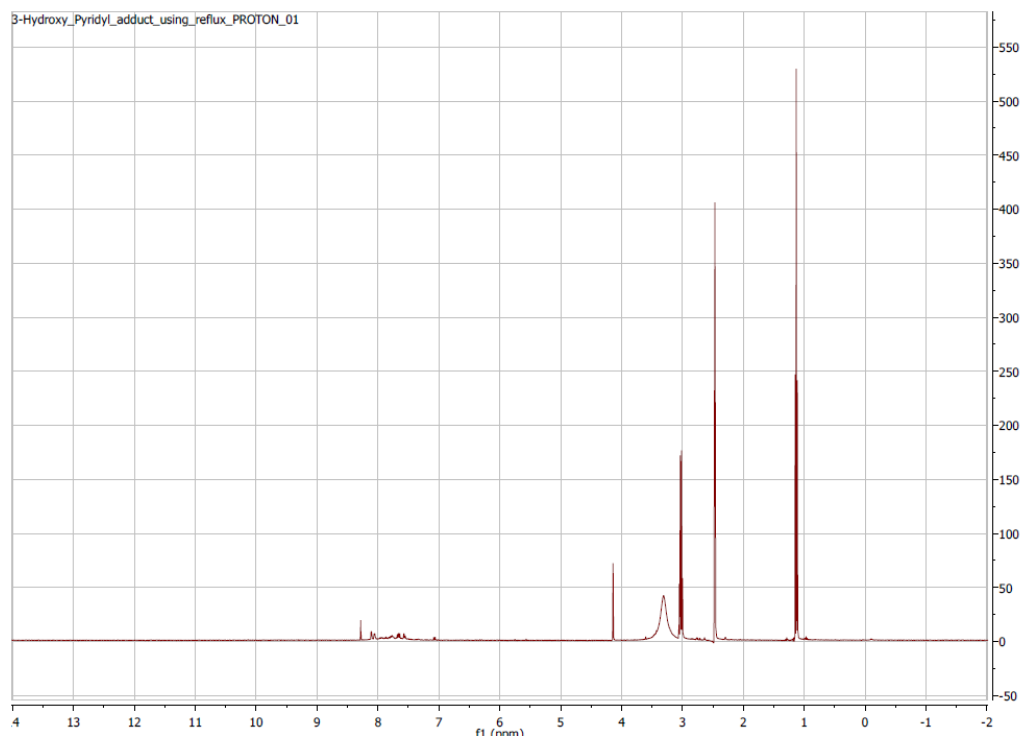


Figure II-4a ^1H NMR of product of 3-hydroxypyridyl hydriodide adduct reaction using refluxing acetonitrile as solvent

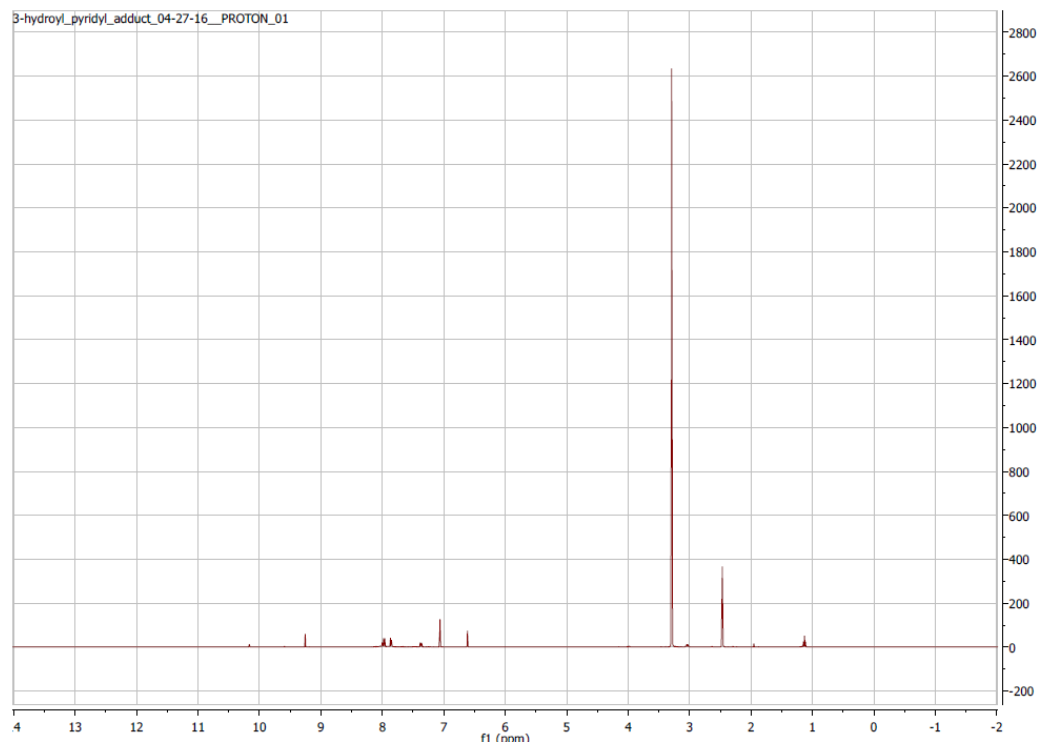
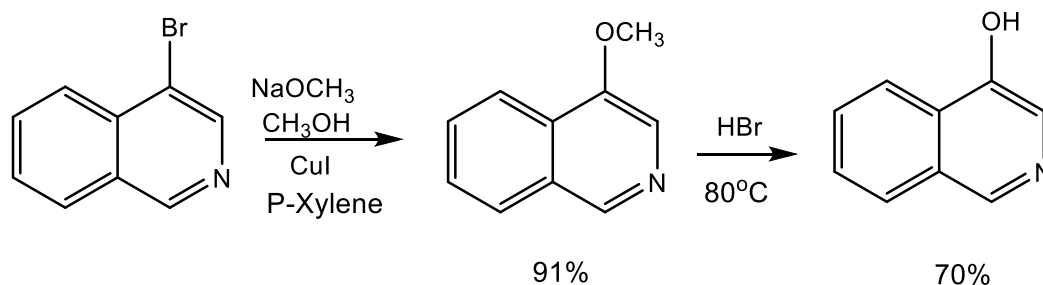


Figure II-4b ^1H NMR of product of 3-hydroxypyridyl hydriodide adduct reaction using Acetonitrile solvent at room temperature

2.5 Modification at the nitrogen terminal

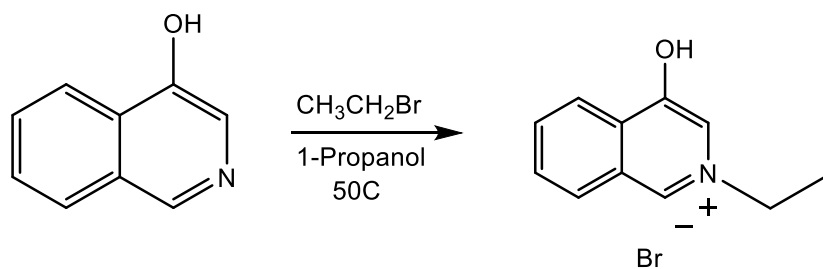
Subsequently, we shifted our attention to replacing the methyl group on the nitrogen bridge with a bulkier substituent. This was achieved by changing the nitrogen substituent on 4-hydroxyisoquinoline before the cycloaddition reaction. We were particularly interested in substituting the methyl group with either ethyl or a benzyl group.

Because previous researchers in our laboratory were not able to synthesize new products with the available 4-hydroxyisoquinoline, which was only 50% pure in the first place, we decided to start the entire synthesis from scratch. 4-hydroxyisoquinoline is not commercially available; we resolved to synthesize it from bromoisoquinoline, Scheme II-6. A pure product (Figure II-5a) was eventually synthesized after a time-consuming process. The reaction was undertaken in a large scale and it took over a month to complete.

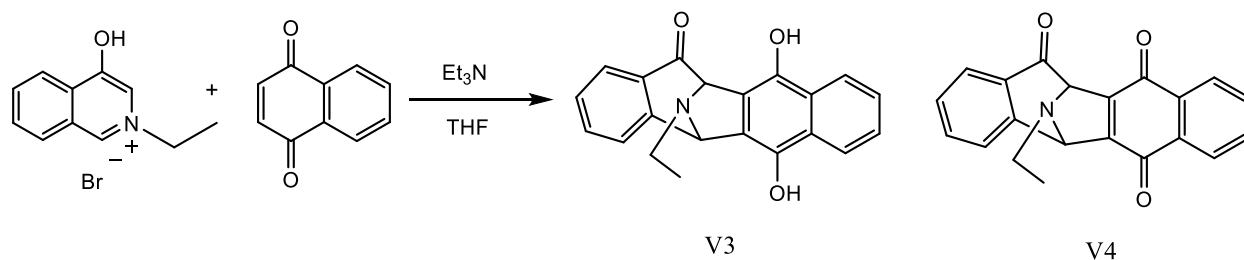


Scheme II-6 Synthesis of N-ethyl naphthoquinone adduct

The first thing we tried was substituting with an ethyl group. A pure N-ethyl-4-hydroxyisoquinoline was formed (scheme II-7a) and used in place N-methyl-4-hydroxyisoquinoline for cycloaddition reaction (scheme II-8b).



Scheme II-7a Synthesis of N-ethyl naphthoquinone adduct



Scheme II-7b Synthesis of N-ethyl naphthoquinone adduct

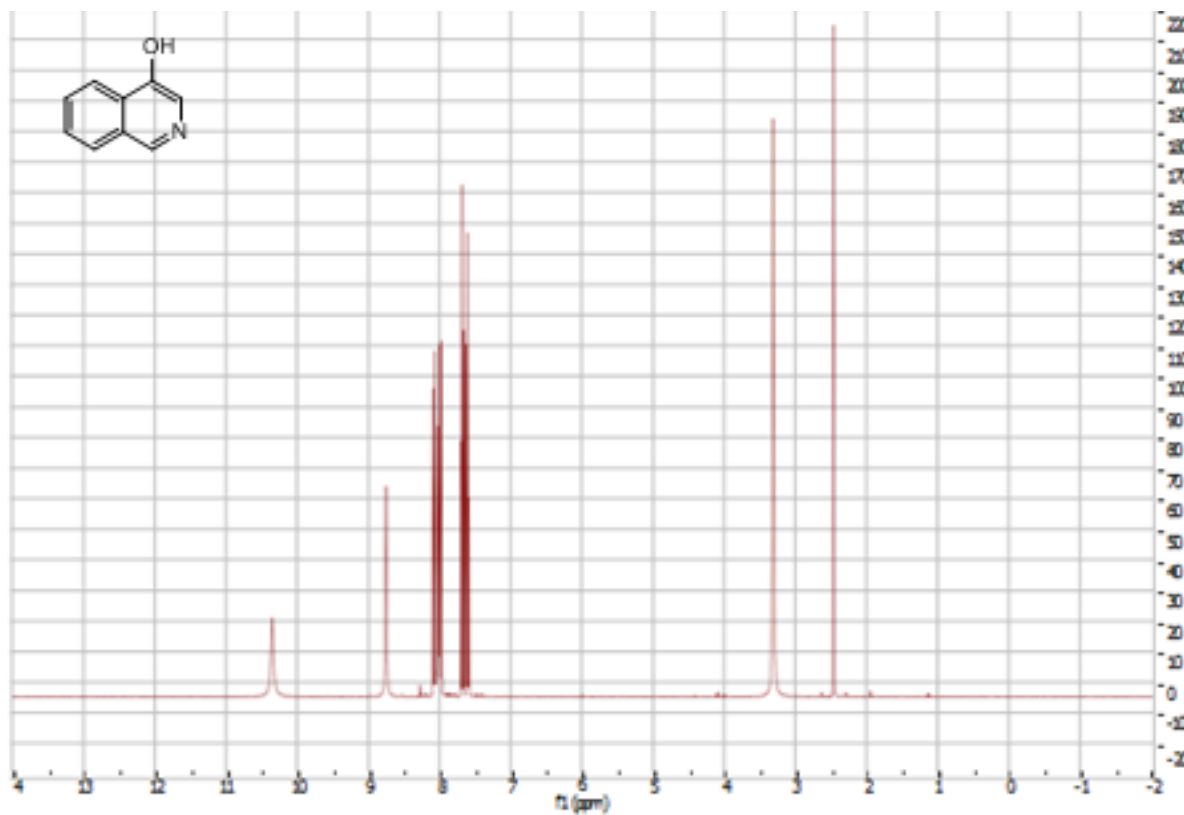


Figure II-5a ¹H NMR of 4-hydroxyisoquinoline

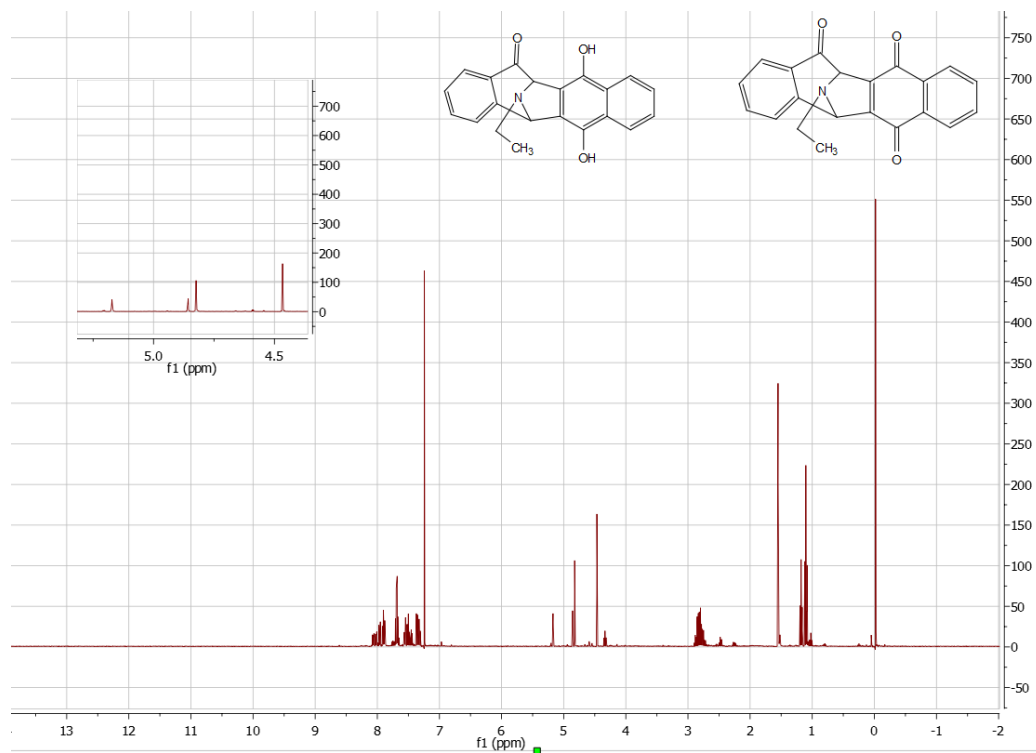


Figure II-5b ^1H NMR of mixed product

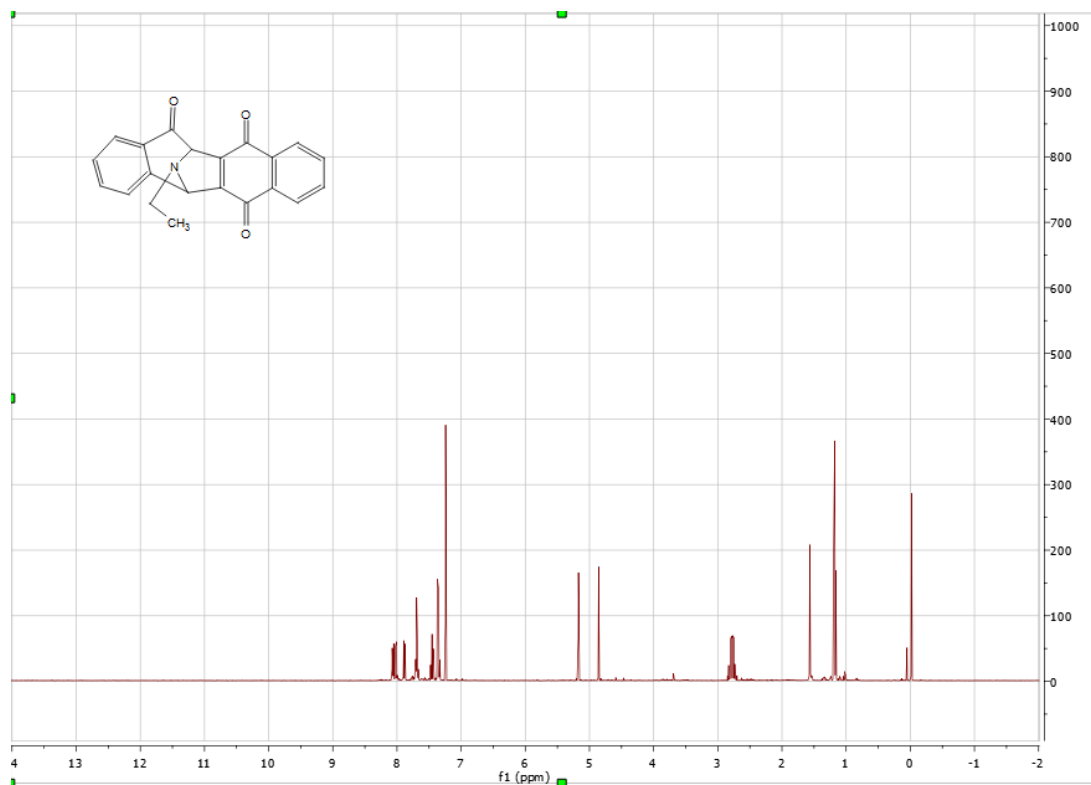


Figure II-5c ^1H NMR of *N*-ethyl naphthoquinone adduct (Quinone)

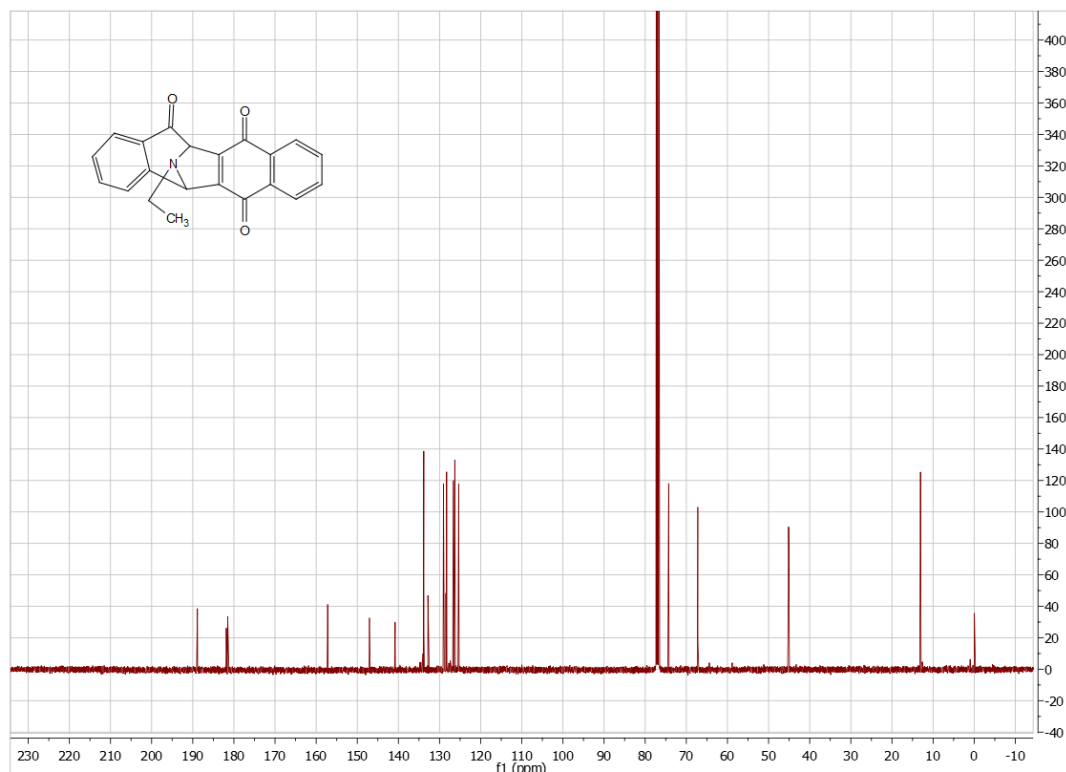
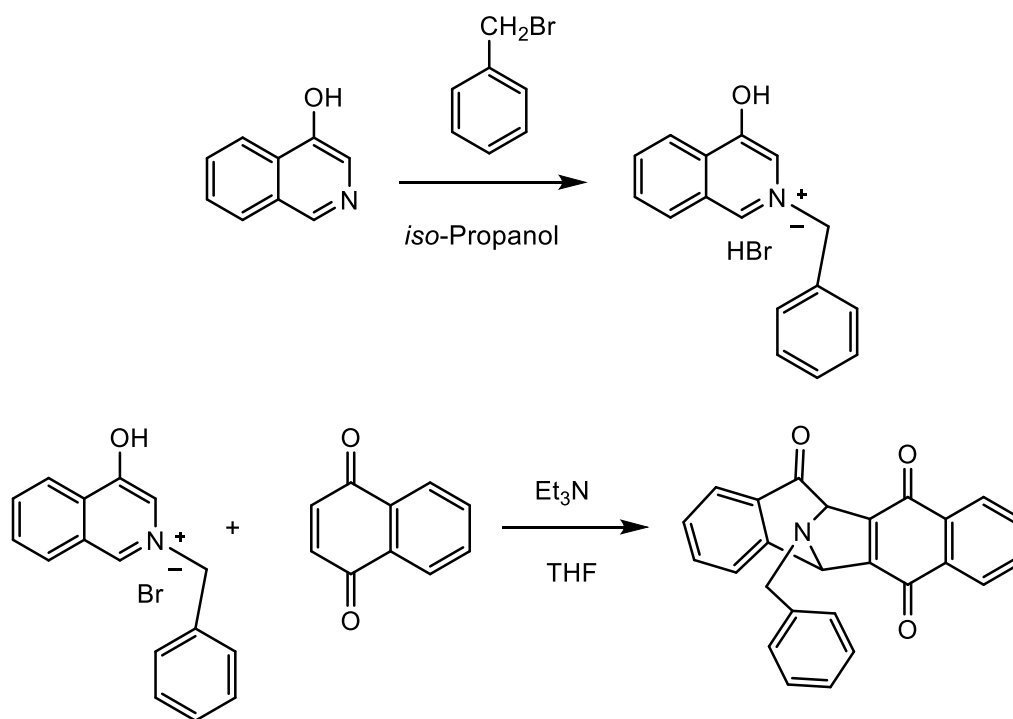


Figure II-5d ^{13}C NMR of *N*-ethyl naphthoquinone adduct (Quinone)

Interestingly, after 19 hours of monitoring with TLC, there was no sign of product formation. We allowed the reaction to proceed longer; apparently, still no product formed. We worked it up to see what was happening and surprisingly, NMR showed the presence of two products (figure II-5b)- one in the quinone form and the other in the hydroquinone form at a ratio of 60:40 respectively. Attempts to separate these products using flash column chromatography proved elusive. The RF was so close that different solvent combinations couldn't give a clean separation of the lagging spot- quinone adduct. With chloroform/methanol (99.5:0.50) we were able to get a small quantity of the lagging spot sufficient for evaluation (figure 5c and d). The reason for partial oxidation of the product still remains a mystery. Repeated experiments still showed partial oxidation of only 60% of the product. We tried to use CAN to bring about the oxidation of the hydroquinone but to no avail.

In addition, we equally substituted a benzyl group for the methyl group at the nitrogen. The reason was to see the effect of a more bulky substituent on the nitrogen head. The scheme of reaction is shown in scheme II-8. This reaction gave a single oxidized product with a yield of 82%



Scheme II-8. Synthesis of N-benzyl naphthoquinone adduct

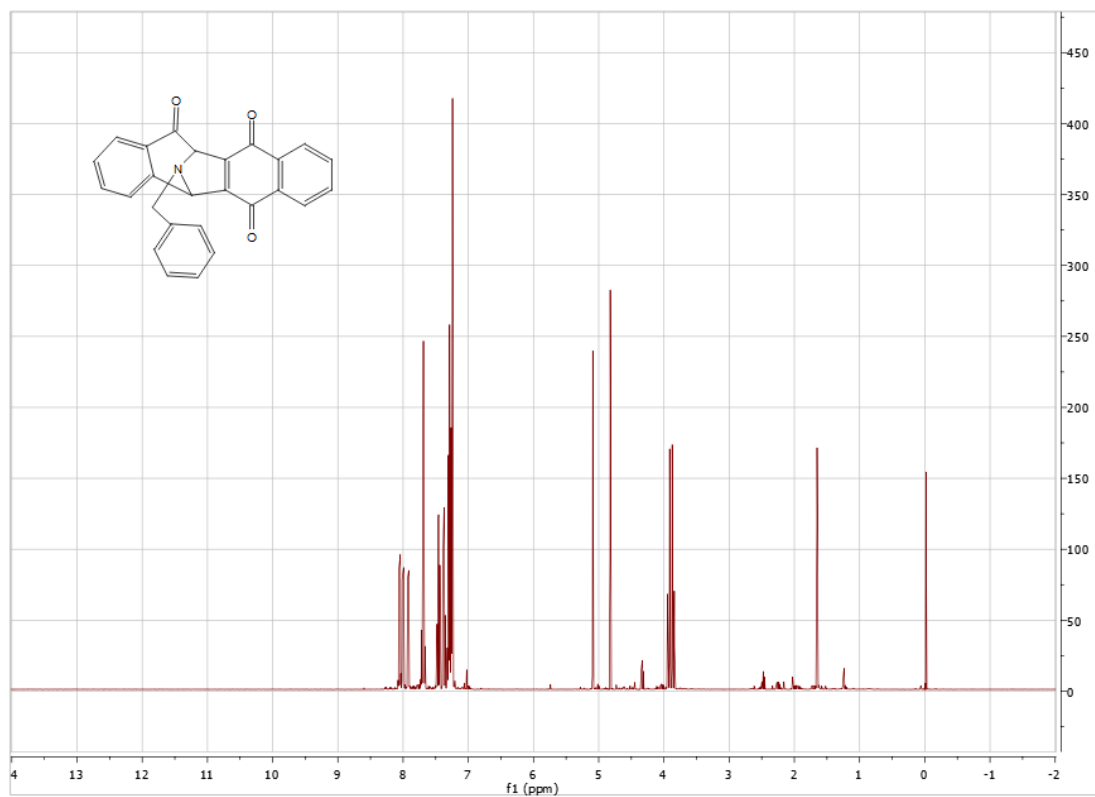


Figure II-6b ^1H NMR of N-benzyl naphthoquinone adduct

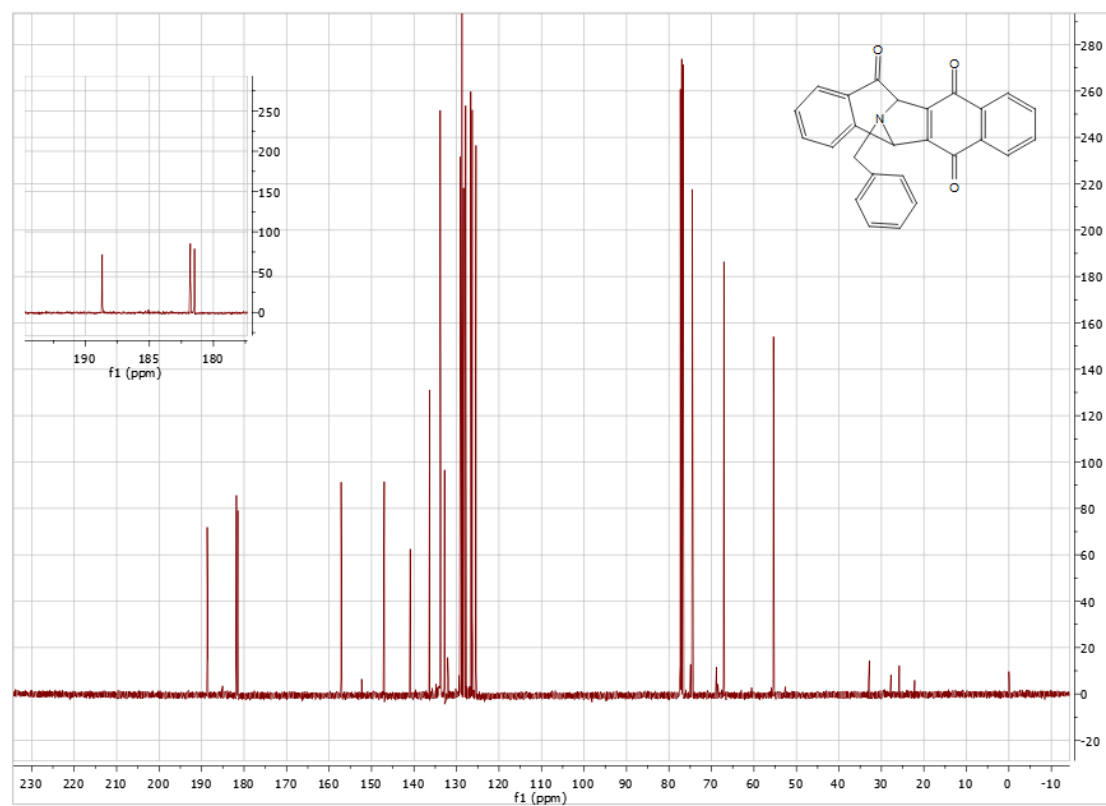
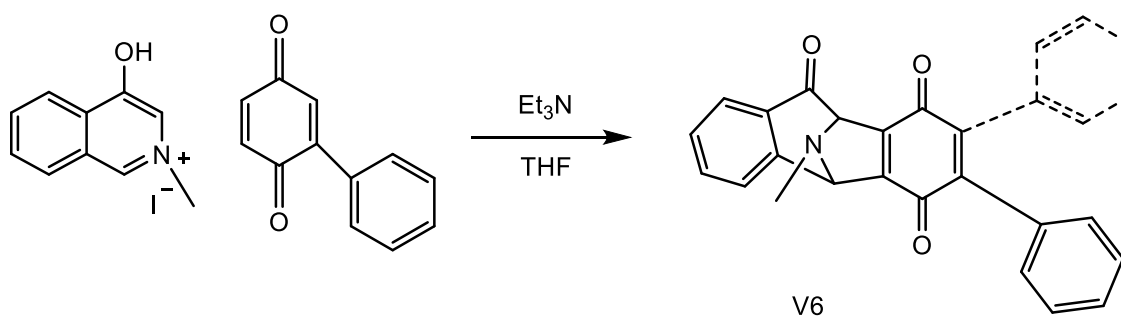


Figure II-6b ^{13}C NMR of N-benzyl naphthoquinone adduct

It would be ideal if we can have a product that does not have any substituent on the nitrogen. This would give us an idea of the importance of the substituents on the nitrogen. Attempts were made to get such an adduct product but were not successful.

2.6 p-Phenylbenzoquinone adduct

Another interesting scheme we thought might impact activity of TU100 was having ring “d” mobile instead of fused. So we used commercially available p-phenyl benzoquinone and reacted it with N- methyl-4-hydroxyisoquinoline (scheme II-9). Because the reaction is not regioselective, we expected 2 products. The initial reaction gave an oxidized product (figure II-7a) but a repeat of the experiment has been stopping at the hydroquinone stage (figure II-7b).



Scheme II-9. Synthesis of N-ethyl naphthoquinone adduct

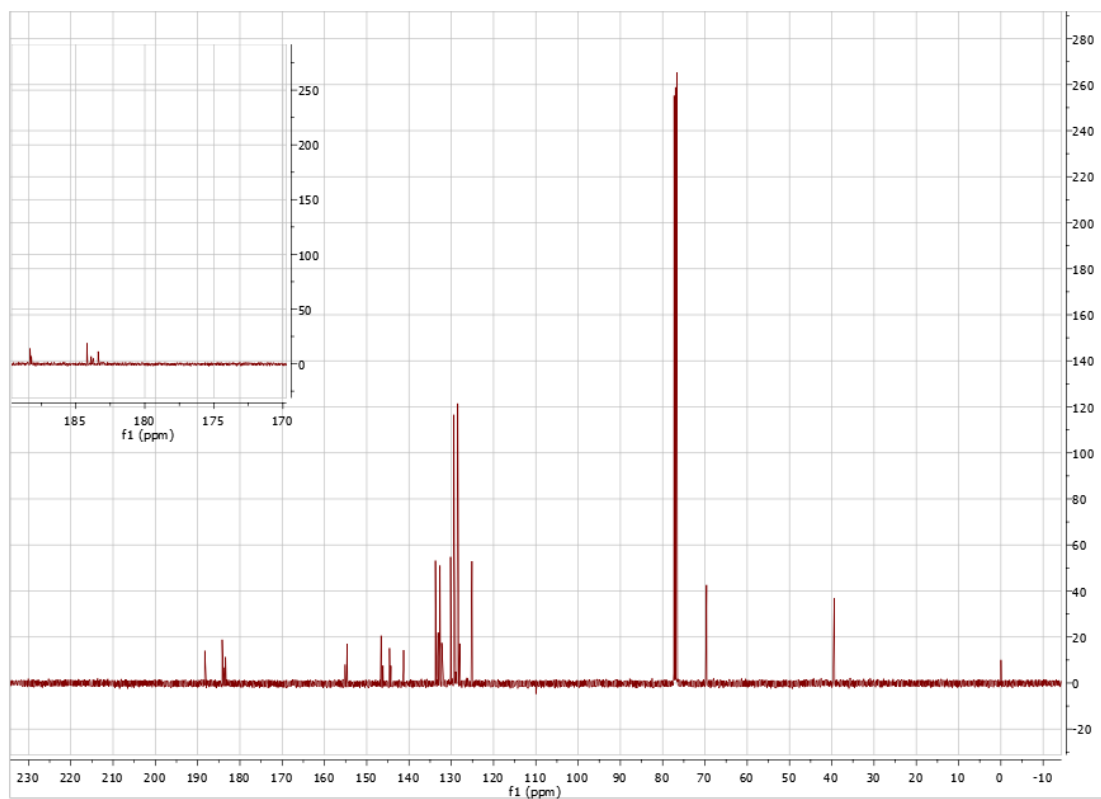


Figure II-7a ^1H NMR *p*-Phenylbenzoquinone adduct

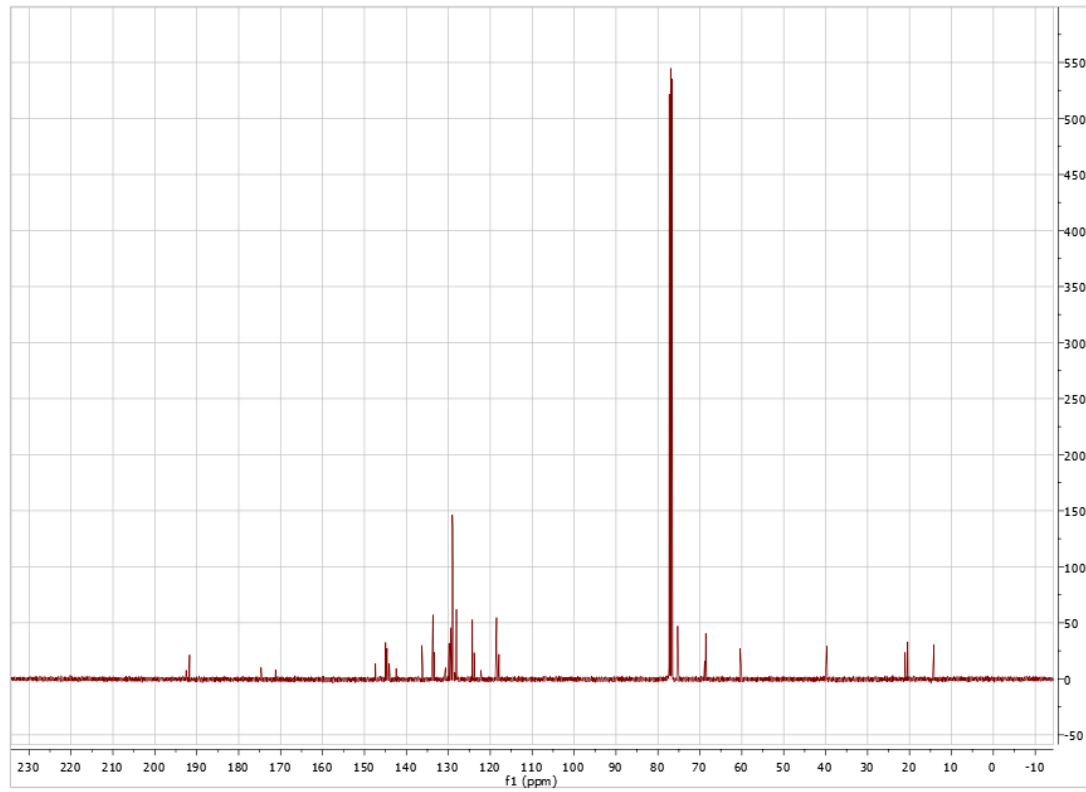


Figure II-7b ^1H NMR *p*-Phenylbenzoquinone adduct (hydroquinone)

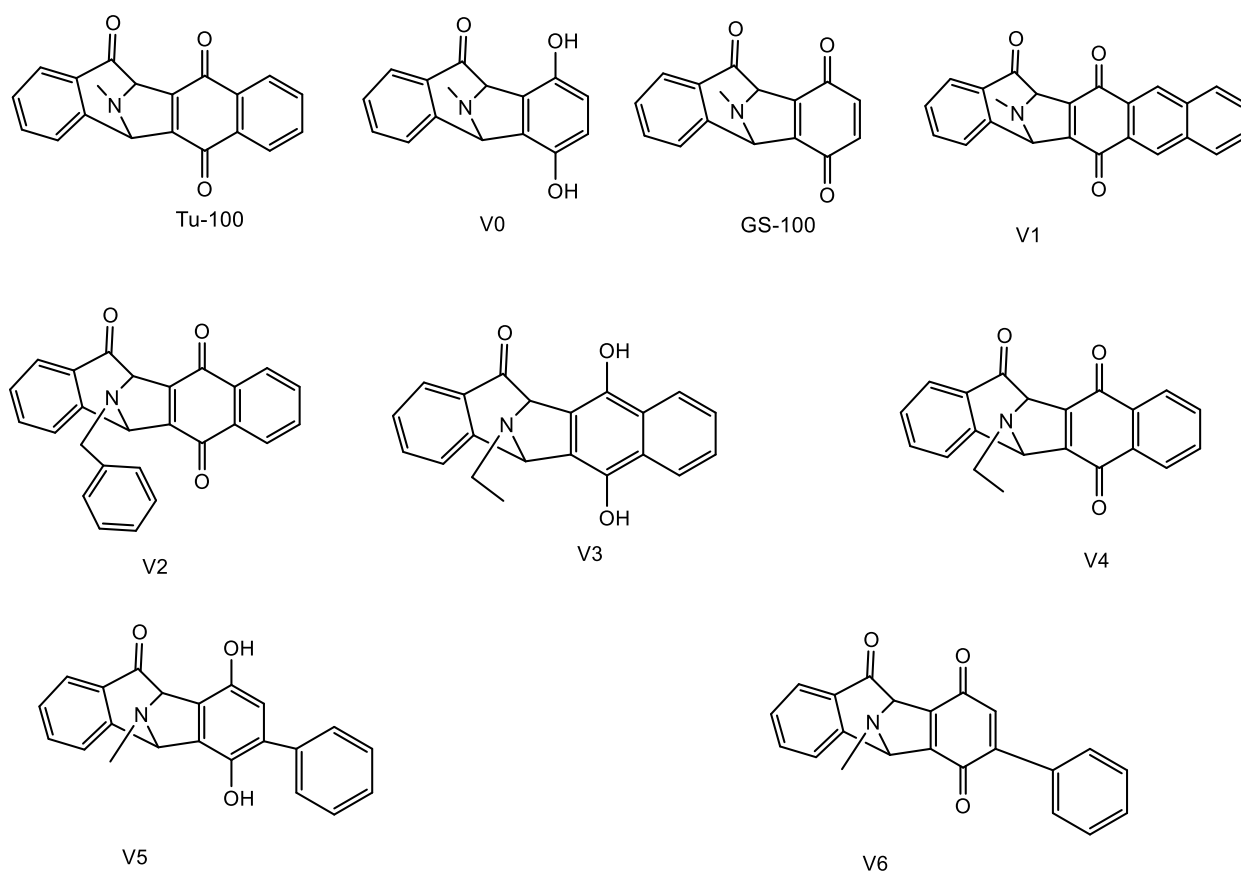


Figure II-7. Summary of the compounds that have been successfully synthesized

2.7 Antitumor toxicity

Structural modifications of TU100 at predetermined regions give novel compounds (figure II-7) with different antitumor profiles. For the majority of the new compounds synthesized, cell viability analysis was done to evaluate their biological activity against cancer cell lines relative to TU100. The first compound evaluated for biological activity was GSU100. Cytotoxicity profile evaluation was based upon the conversion of resazurin to the fluorescent resorufin by live cells[23]. Both TU100 and GS100 caused dose-dependent cell death in multiple cell lines (NIH3T3, H293, and HDFs, BxPC3, MCF7, MDA-MB, PC3, SKBr3 & T47D). IC50s varies between cell lines and the compounds (Figure II-8a). TU100 has better kill kinetics than

GS100 in all the cell lines tested and across all concentrations (Figure II-8a-e). This shows that oxidizing the benzoquinone adduct does not significantly increase the antitumor activity even though it helps increase the antitumor effect as compared to benzoquinone adduct (data not shown).

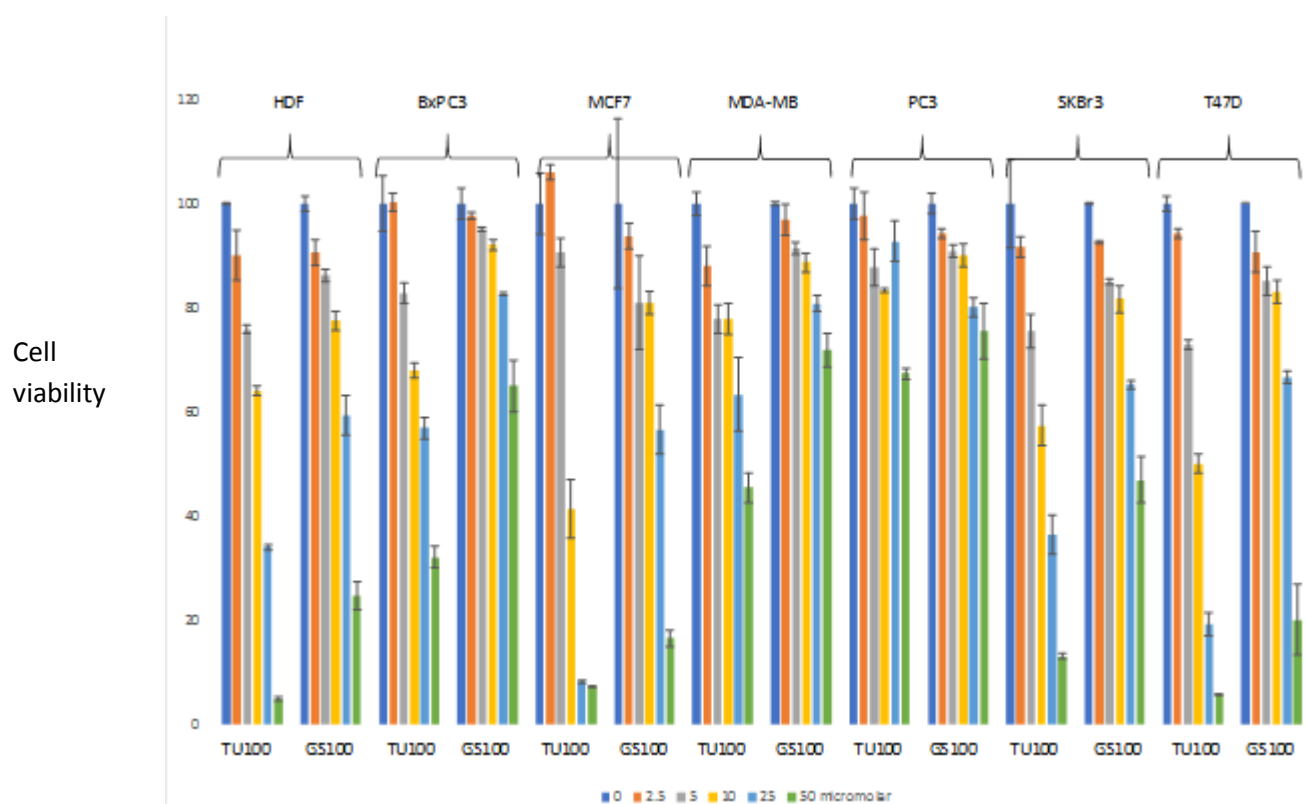


Figure II- 8a comparison of TU100 and GSU100 effects on cancer cell viability

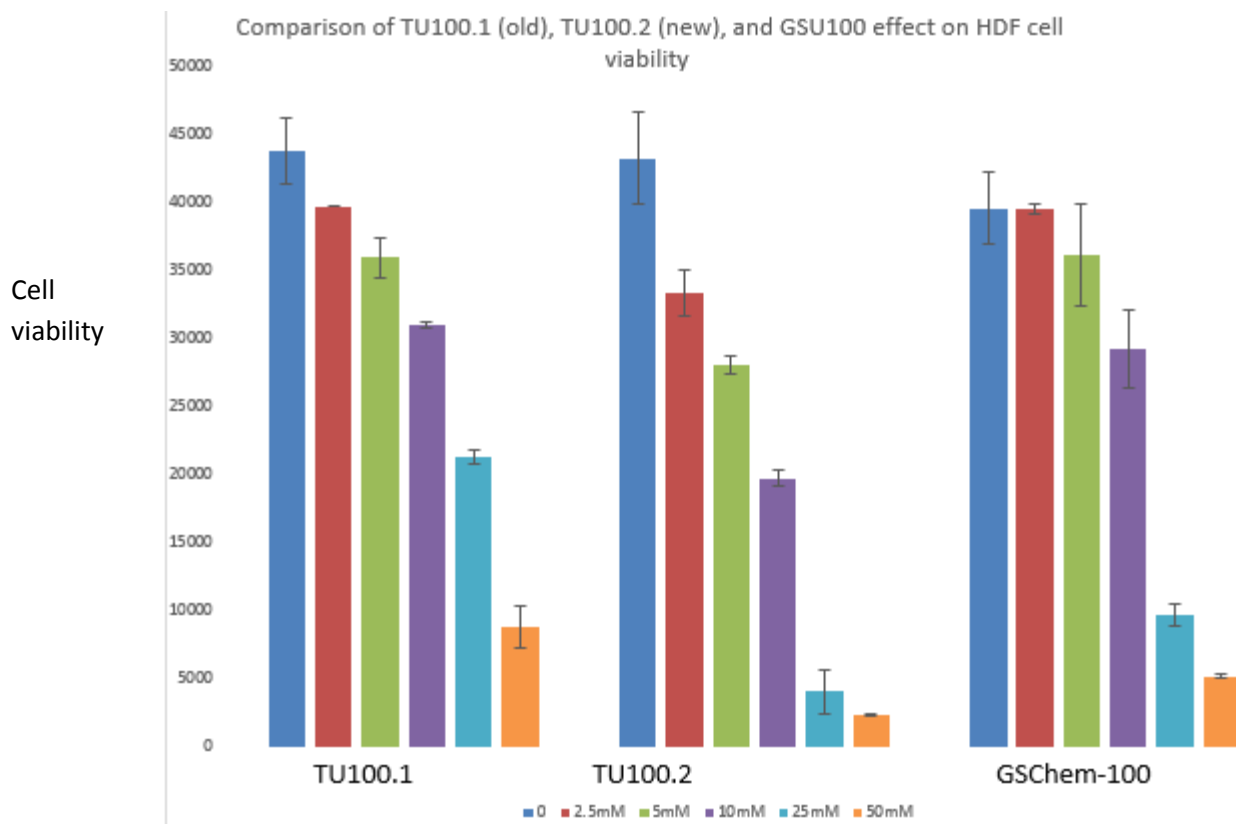


Figure II-8b comparison of TU100 and GSU100 effects on HDF cell viability

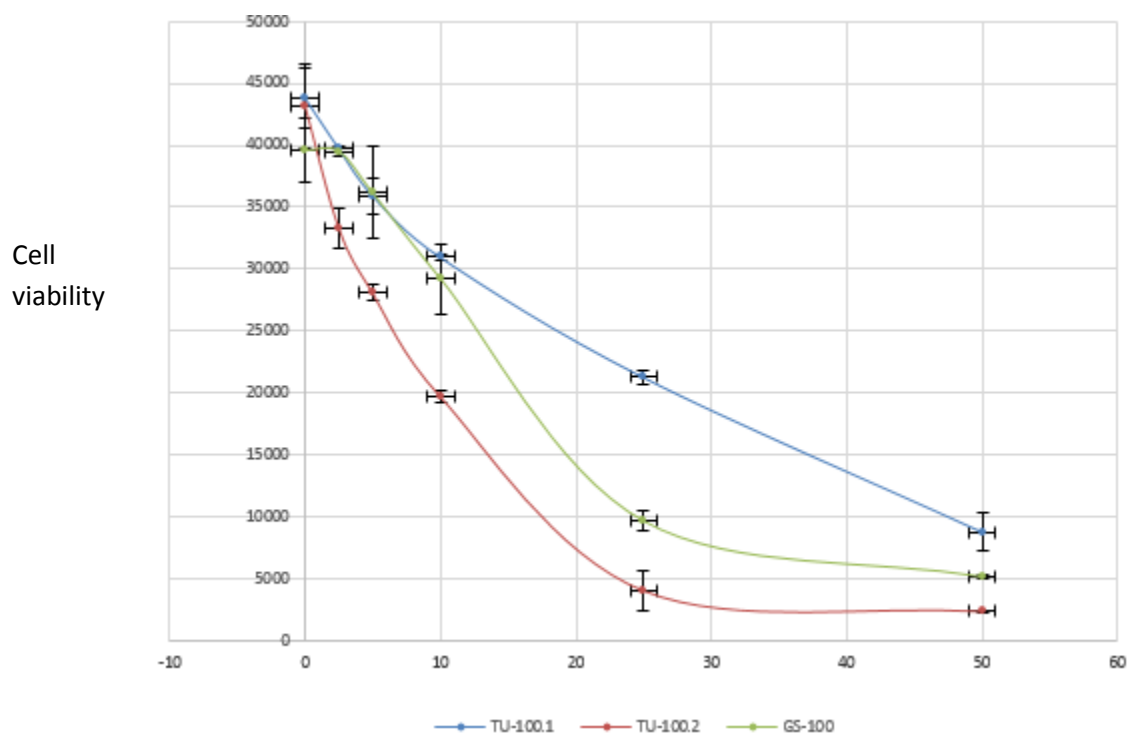
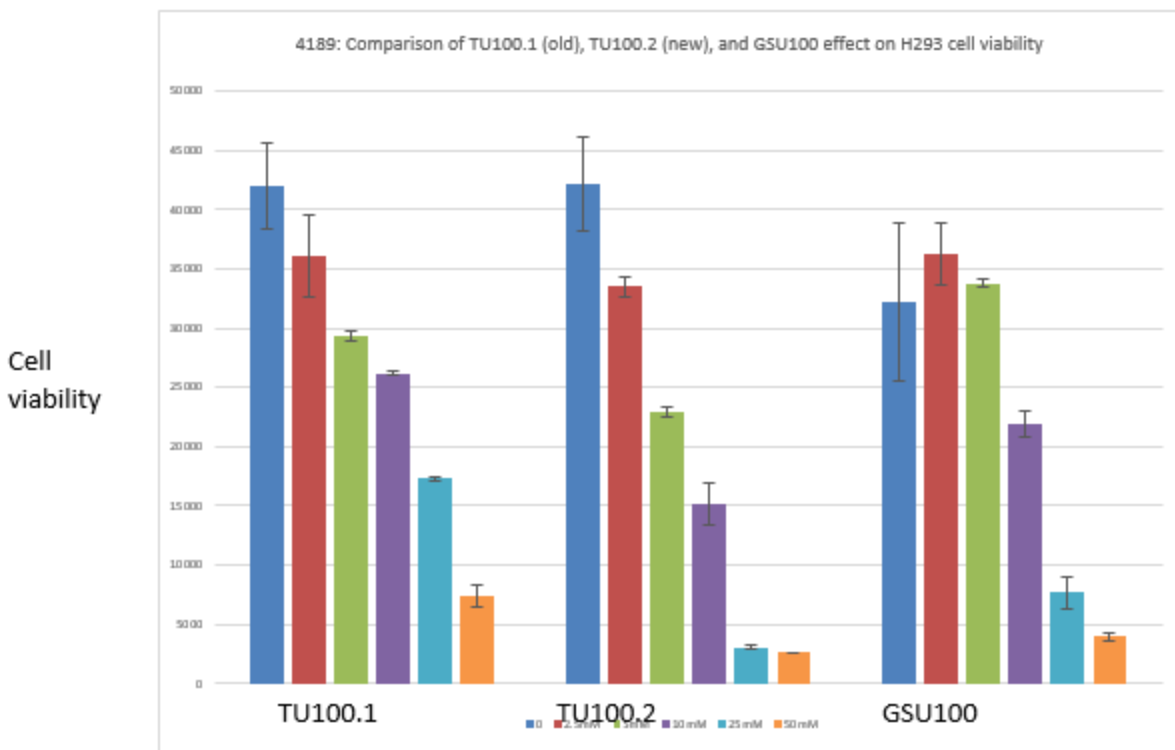


Figure II-8c Re-plot of 8b data to highlight differences



FigureII-8d comparison of TU100 and GSU100 effect on H293 cell viability

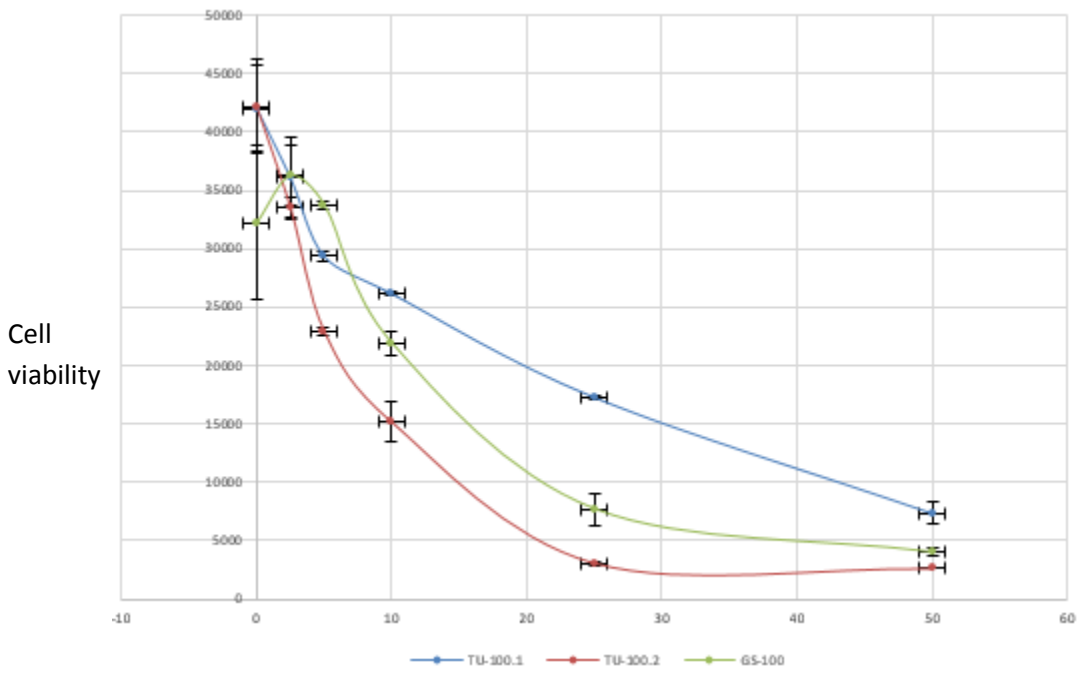


Figure II-8e Re-plot of 8c data to highlight differences

Notes:

- *HDF= immortalized human diploid fibroblasts; not transformed. Model of a normal cell.*
- *H293= kidney epithelial cells, transformed (ie cancer).*
- *Cell viability measured by CTB assay living cells convert resazurin to fluorescent resorufin.*
- *cells plated overnight and then treated with drug in DMSO (1% final) for ~15hrs.*
- *Looks like similar overall pattern of cytotoxicity: TU100.2>GSU100>TU100.1.*
- *TU100.1= TU100 batch synthesized in 2011 used for preliminary studies*
- *Tu100.2= recently synthesized TU100.*

Once the next batch of compounds were synthesized, V1, V2, V3 and V4, they were sent for biological activity evaluation. Figure II.9 shows the result of different concentrations of the compounds on h293 cell lines. H293 cells are derived from kidney epithelial cell and later transformed to a cancerous cell. We chose this because the lead compound, TU100, shows high sensitivity to kidney cell lines. DMSO was used as a control because the compounds were dissolved in DMSO. The degree of inhibition of cancer cell proliferation and viability varies among compounds. Considering the biological activity of V0 to GSU100 and V3 to V4, it can be safely inferred that oxidation of the hydroquinone is crucial for activity. This might be related to a decrease in the ability of the un-oxidized products to generate reactive oxygen species which previous studies had shown is partly responsible for the activity of TU100. Removal of the ring “d” (TU100 vs GS100) causes a noticeable decrease in activity while the addition of the “e” ring (TU100 vs V1) increases activity. Hence, it seems increasing the number of rings increase the activity.

Equally, N- terminal substitution seems to influence biological activity with N-ethyl substitution displaying the highest capability of ATP reduction in fibroblast cells, closely followed by N-benzyl and then N-methyl. Both N-ethyl and N-benzyl substitution produces compounds that are more potent than TU100. ATP production is crucial to cells viability. Reduction of ATP production shows a decrease in the number of viable cells in the medium. This suggests the cells are undergoing apoptosis either via the intrinsic or extrinsic pathway. Extrinsic pathway for induction of apoptosis involves the degradation of the powerhouse, mitochondria, of the cells in order to release Caspase 3. Interestingly, the effect of the compounds on ATP levels show that TU100 has a modest effect on ATP, but only at higher concentration. GSU100 has no effect on ATP, consistent with the relative lack of cytotoxicity on H293s. V3 is similar to GSU100. V1, 2, 4 all decrease ATP, but with somewhat different kinetics. This generally fits with their increased cytotoxicity. Given the different cytotoxicity profiles on H293s and the differential effects on ATP levels, it seemed likely the compounds might display specificity for particular types of cancer cells and that they might have different mechanism of actions. This is consistent with NCI suggestion that TU100 might act via more than one mechanism.

Looking at the modifications, none of the alterations resulted in total loss of antitumor activity. However, activities/ potency do vary with modification. It seems like modification might either favor a particular mechanism or reduce it. Each of the regions seem to act or contributes independently to overall cytotoxicity.

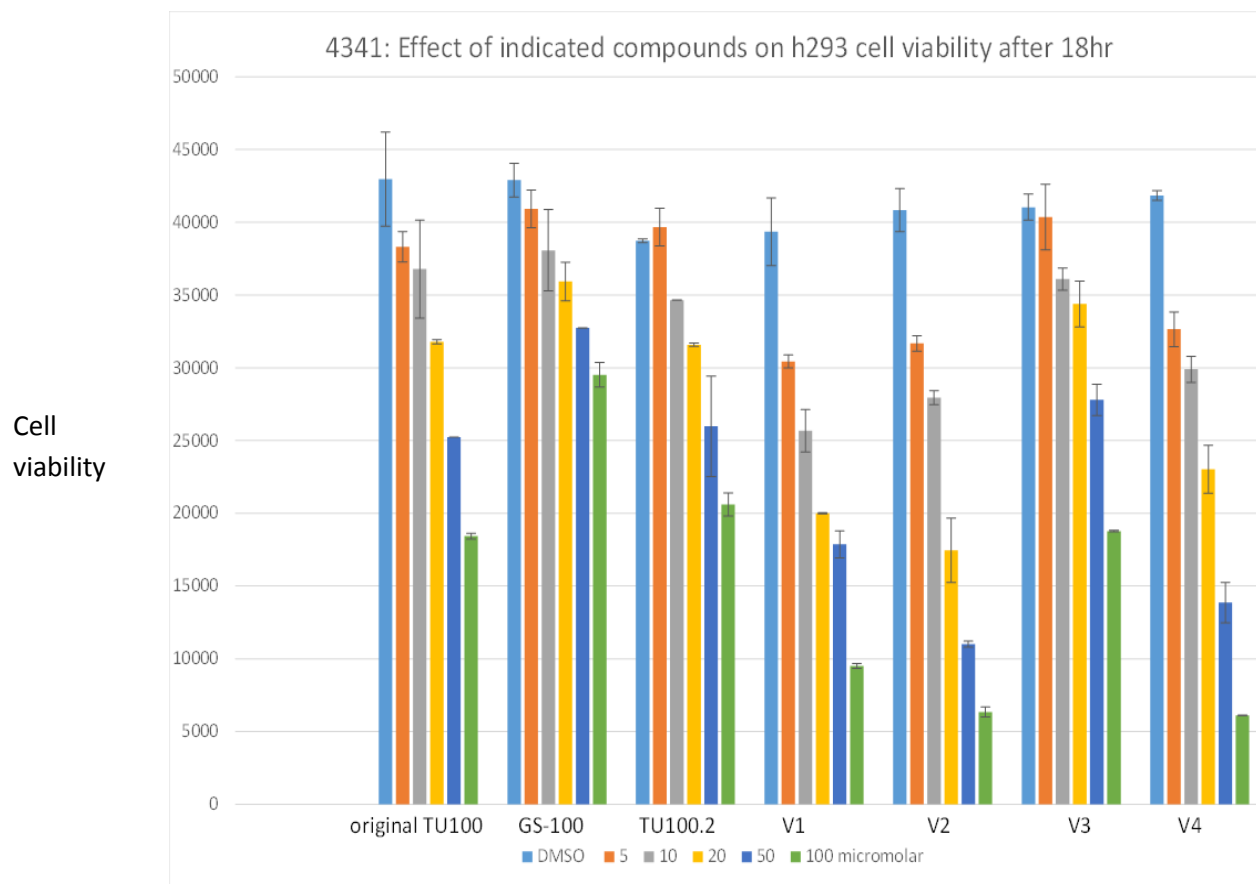


Figure II-9 Effect of indicated compounds on h293 cell viability

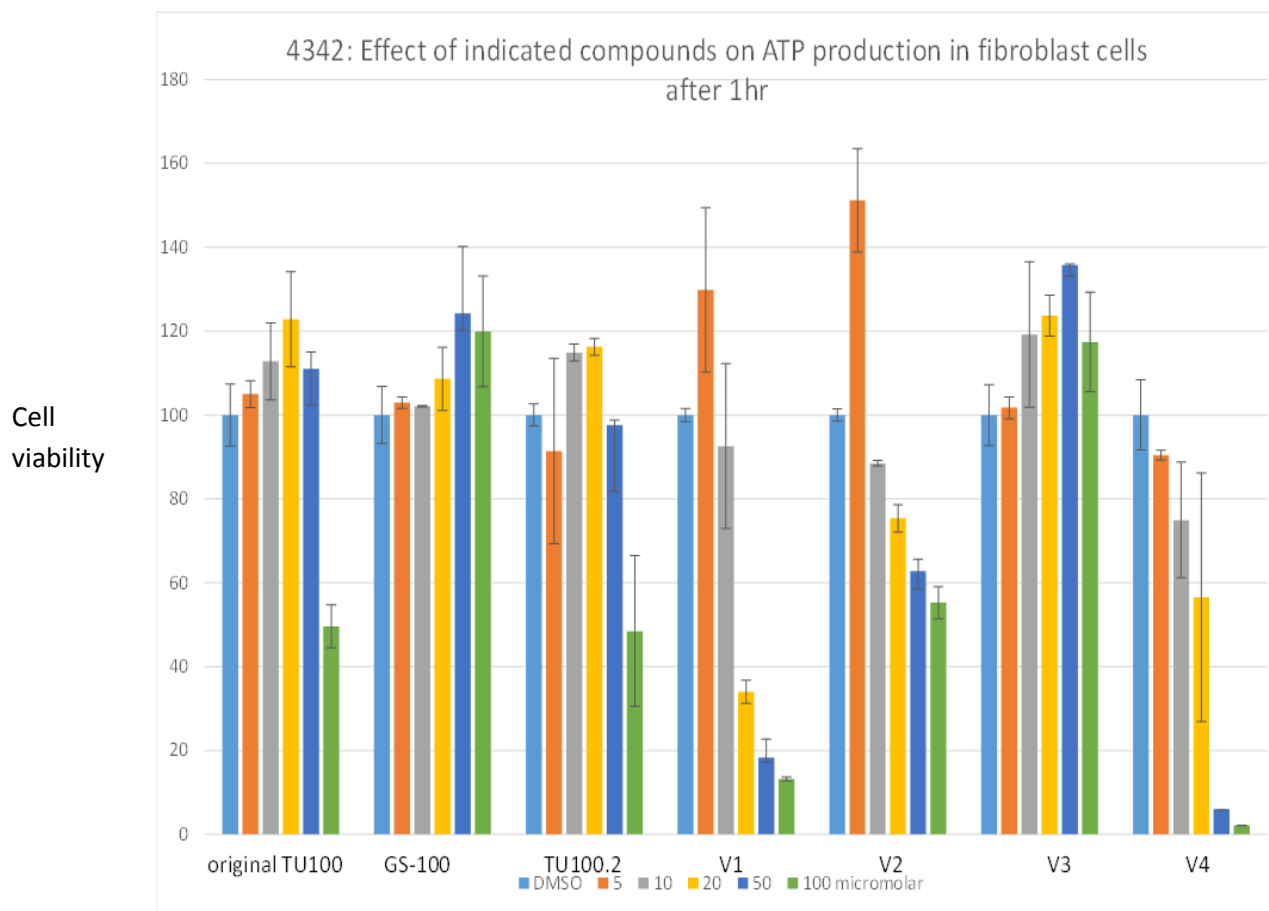


Figure II-10 Effect of indicated compounds on ATP production in fibroblast cell

These data confirm that GSU100 is overall less cytotoxic, and TU100 has differing efficacies against different types of cancer cell lines. This implies the mechanism of cytotoxicity may be somewhat specific and agrees with the original NCI data suggesting specificity.

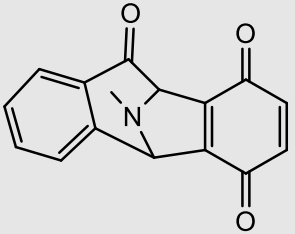
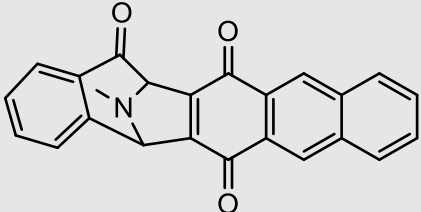
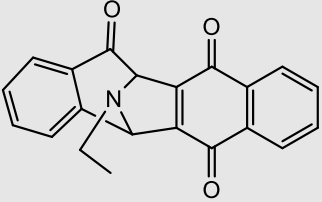
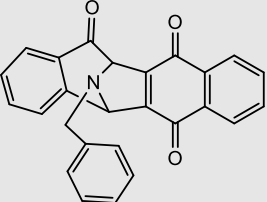
Compound	Corresponding effect vs TU100
 <p data-bbox="509 485 623 516">GSU100</p>	<p data-bbox="711 268 1219 369">Decrease activity vs TU100 Lower ability to inhibit ATP production Less effective on different cell lines</p>
 <p data-bbox="201 789 240 821">V1</p>	<p data-bbox="711 552 1211 621">Best Cytotoxic profile Better ability to inhibit ATP production</p>
 <p data-bbox="537 1035 576 1066">V4</p>	<p data-bbox="711 850 1206 919">Better Cytotoxic profile vs TU100 Excellent inhibition of ATP production</p>
 <p data-bbox="482 1287 521 1318">V2</p>	<p data-bbox="711 1096 1011 1165">Good Cytotoxic profile Good ATP Inhibition</p>

Figure II-10 Summary of chemical modification and their resulting effect

In Summary, synthesis of TU100 has resulted in a unique class of compound that rapidly and irreversibly inducing apoptotic cell death. The various derivatives alter the ability to induce cell death (figure II-10), suggesting they may have different mechanisms of action. For example, removal of a ring, GSU100, causes a decrease in activity, lowers ability to inhibit ATP production, and is less effective on different cell lines as compared to TU100. The Anthraquinone adduct gives the best cytotoxic profile and has a better effect on ATP production

inhibition than TU100. The N-ethyl naphthoquinone adduct give the best effect on inhibition of ATP production. N-benzyl naphthoquinone adduct also gives a better Cytotoxicity and ATP inhibition profile vs TU100. For *p*-Phenylbenzoquinone adduct, we have not been able to get sufficient data on its biological activity profile yet. Also, we have not been able to successfully synthesize the N-methylPyridinium adduct. We hypothesize that when synthesized, there might be drastic loss of antitumor activity.

2.8 Future Work

We noticed that both modification of the rings and the nitrogen substituents elicit observable differences in the biological profile. We have started synthesizing compounds that combined modification in these regions to see if we will have a synergistic effect, additive effect or antagonistic effect. Similarly, attempts will be made to synthesize the N-pyridinium adduct. Furthermore, work is still ongoing on getting more biological activity data on these compounds. Eventually, we will get to a stage where structural analogues will focus on substitutions on the rings such having methyl, methoxy and hydroxyl substituent on the ring. Further structural analogues will be based on biological results from previous analogue which is a common practice in biological activity relationship when one is interested in potency and selectivity enhancement.

Our overall goal is to develop a structural activity relationship for this class of compound and in so doing, get a novel compound with excellent antitumor profile as well as lesser toxicity on normal cells

CHAPTER 3

EXPERIMENTALS

1,4-naphthoquinone

The starting material 1,4-naphthoquinone hydrate was sourced commercially from Aldrich Chemical Company with a purity of 97%. Because of the nature of our experiment, the naphthoquinone was purified by sublimation. The greenish black compound was placed in a cold finger sublimation apparatus and placed in an oil bath at a temperature of 100°C under vacuum.

Preparation of 4-hydroxy Isoquinoline

4-hydroxy-N-methylisoquinoline iodide is not commercially available, as a result, this was synthesized from commercially available 4-bromoisoquinoline using the method described by K. Constable and Carroll.[24] 4-bromoisoquinoline was methoxylated with sodium methoxide using copper(1) iodide in refluxing xylene to give 4-methoxyisoquinoline with a yield of 91%. In more precise terms, 50g(0.24moles) of 4-bromoisoquinoline, was methoxylated with 54g (0.84moles) of sodium methoxide using a total of 91.4g(0.48moles) of activated copper(1)oxide in refluxing *p*-xylene(850mL) and methanol(250mL). The copper(1)iodide was activated by placing it in an oven overnight and added in batches after an initial addition of 22.892g (0.12 moles). It should be noted that *p*-xylene was purified and water free methanol was added via cannula. After a yield of 91% was attained, the reaction mixture was filtered using about 0.5 inches Celite filled Buchnenchen funnel. The products were extracted from the filtrate using aqueous washing before reacting with hydrobromic acid. The resulting mixture now contains 91% 4-methoxyisoquinoline and 9% 4-bromoisoquinoline (35.05g) and was treated with 500mL

of 48% HBr at 80°C for 24 hours to get 4-hydroxyisoquinoline; this was extracted by adding sodium hydroxide to the solution until the product precipitates out at the isoelectric point of 6.5.

Synthesis of methyl-3-oxypyridyl-betaine hydroiodide

Under nitrogen, a solution of 3-hydroxypyridine (9.5 g, 0.1mol) and methyl iodide (14.2 g, 0.1mol) was allowed to reflux in 50 mL of 50:50 1-propanol/acetone for 19 hours. After 19 hours, the solution was concentrated under reduced pressure to yield a darkbrown solid. This solid was further purified by recrystallizing in acetone and ethyl ether. The product purity was confirmed by ¹H NMR and ¹³C NMR. ¹H NMR (400 MHz, DMSO-d₆) δ 8.45 – 8.37 (m, 2H), 7.91 – 7.80 (m, 2H), 5.87 (s, 1H), 4.24 (s, 3H).

Synthesis Of 4-hydroxy-N-ethylisoquinoline

Under nitrogen, a solution of 4-hydroxyisoquinolinium (14.5 g, 10mmol) and bromoethane (10.9 g, 10mmol) was allowed to reflux in 50 mL of 1-propanol for 19hours. After 19 hours, the solution was concentrated under reduced pressure to yield a white solid. This solid was further purified by dissolved in boiling acetone followed by addition of ethyl ether to precipitate the pure 4-hydroxy-*N*-methylisoquinolinium. Product purity was confirmed by ¹H NMR and ¹³C NMR. ¹H NMR (400 MHz, DMSO-d₆) δ 9.55 (s, 1H), 8.40 – 8.31 (m, 2H), 8.14 (ddd, J = 8.4, 7.0, 1.2 Hz, 1H), 8.09 – 7.97 (m, 3H), 4.66 (q, J = 7.3 Hz, 2H), 1.57 (t, J = 7.3 Hz, 3H).

Synthesis of 4-hydroxy-N-benzylisoquinoline

To a stirred solution of 4-hydroxyisoquinoline(0.725g, 5mmol) in 1-Propanol(25mL), commercially sourced benzyl bromide(0.855g, 5mmol) was added and allowed to reflux for

48 hours to give 4-hydroxy-N-benzylisoquinoline. The solution was concentrated under reduced pressure to yield a white solid. This solid was further purified by dissolving in boiling acetone followed by addition of ethyl ether to precipitate a pure 4-hydroxy-N-benzylisoquinoline. Synthesis and purity was confirmed by both ^1H NMR and ^{13}C NMR. ^1H NMR (400 MHz, Chloroform-*d*) δ 9.32 (s, 1H), 8.73 (d, $J = 1.4$ Hz, 1H), 8.25 (d, $J = 7.9$ Hz, 1H), 8.17 (d, $J = 8.3$ Hz, 1H), 7.88 (ddd, $J = 8.3, 7.0, 1.2$ Hz, 1H), 7.76 (ddd, $J = 8.2, 7.0, 1.1$ Hz, 1H), 7.55 – 7.48 (m, 2H), 7.36 – 7.28 (m, 2H), 5.82 (s, 1H), 0.92 (t, $J = 7.4$ Hz, 1H).

Synthesis of TU100

TU100 was synthesized as described by DiCesare et al [12]. Under nitrogen, a solution of 4-hydroxy-*N*-methylisoquinolinium iodide (0.500 g, 1.73 mmol) and 1,4-naphthoquinone (0.320 g, 2.04 mmol) was stirred in 20 mL of dry tetrahydrofuran followed by the addition of triethylamine (0.28 mL, 2.04 mmol). The solution was stirred for 19 hours at room temperature followed by dilution with ethyl acetate (200 mL) and washed with 20 mL water followed by 20 mL brine. The product was dried (magnesium sulphate) and concentrated under reduced pressure to yield a dark brown solid. This solid was further purified by flash column chromatography using silica gel and chloroform. The initial hydroquinone product undergoes rapid air oxidation upon reaction workup and purification protocols to the quinone, which was confirmed by structural characterization. Structural characterization by ^1H NMR (400 MHz, Chloroform-*d*) gave the following values: δ 2.62 (3H, s), 4.75 (1H, s), 5.06 (1H, s), 7.35–7.50 (3H, m), 7.70–7.76 (2H, m), 7.91 (1H, d, $J = 7.0$ Hz), 8.03–8.11 (2H, m).

Synthesis of benzoquinone adduct(V0)

This was synthesis as described by DiCesare et al [12]. Under nitrogen, a solution of 4-hydroxy-*N*-methylisoquinolinium iodide (0.500 g, 1.73 mmol) and 1,4-benzoquinone (0.220 g, 2.04 mmol) was stirred in 20 mL of dry tetrahydrofuran followed by the addition of triethylamine (0.28 mL, 2.04 mmol). The solution was stirred for 19 hours at room temperature followed by dilution with ethyl acetate (200 mL) and washed with 20 mL water followed by 20 mL brine. The product was dried (magnesium sulphate) and concentrated under reduced pressure to yield a dark brown solid. This solid was further purified by flash column chromatography using silica gel and ethyl acetate: hexane 70:30. Purity and synthesis was confirmed by NMR. Structural characterization by ¹H NMR (400 MHz, Chloroform-*d*) gives the following values: δ 2.51 (3H, s), 4.48 (1H, s), 4.95 (1H, s), 6.50(2H, s), 7.23–7.30 (3H, m), 7.83 (1H, d, *J* = 7.5 Hz), 8.33(1H, s), 8.33 (1H, s), 8.47 (1H, s).

Oxidation of benzoquinone adduct (GSU100)

After several unsuccessful attempts to oxidize the hydroquinone adduct of the reaction between 4-hydroxyl-*N*-methylisoquinone, we found that reacting with ceric ammonium nitrate (CAN) gave the intended oxidized product. Under nitrogen, 0.156g (0.5mmoles) of benzoquinone adduct in 10mL of acetonitrile was stirred with 0.648g (1.182mmoles) of CAN for 2 hours. The resulting solution was diluted with ethyl acetate (200 mL) and washed with 20 mL of water followed by 20 mL brine. The product was dried (magnesium sulphate) and concentrated under reduced pressure to yield a dark brown solid. This solid was further purified by flash column chromatography using silica gel and ethyl acetate: hexane 70:30. Purity and synthesis was confirmed by NMR. Structural characterization by ¹H NMR (400 MHz, Chloroform-*d*) δ 7.89 (ddt, *J* = 7.6, 1.5, 0.5 Hz, 1H), 7.45 (td, *J* = 7.5, 1.5 Hz, 1H), 7.36 (td, *J* =

7.6, 1.3 Hz, 1H), 7.28 (dt, $J = 7.4, 0.8$ Hz, 1H), 6.69 (d, $J = 10.1$ Hz, 1H), 6.65 (d, $J = 10.1$ Hz, 1H), 4.89 (s, 1H), 4.57 (d, $J = 0.7$ Hz, 1H), 2.55 (s, 3H).

^{13}C NMR (101 MHz, Chloroform- d) δ 188.15, 184.18, 183.89, 154.67, 144.29, 141.11, 136.77, 136.31, 133.77, 129.14, 128.45, 127.90, 125.06, 76.48, 69.56, 39.31.

Synthesis of Anthraquinone Adduct (V1)

Anthraquinone adduct was synthesized by substituting 1,4-naphthoquinone with 1,4-anthraquinone as the starting material. Under nitrogen, a solution of 4-hydroxy-*N*-methylisoquinolinium iodide (1.00 g, 3.48 mmol) and 1,4-anthraquinone (0.871 g, 4.18 mmol) was stirred in 40 mL of dry tetrahydrofuran followed by the addition of triethylamine (0.582 mL, 4.18 mmol). The solution was stirred for 19 hours at room temperature followed by dilution with ethyl acetate (200 mL) and washed with 20 mL water followed by 20 mL brine. The product was dried (magnesium sulphate) and concentrated under reduced pressure. This solid was further purified by flash column chromatography using silica gel and chloroform: methanol 99:1. Purity and synthesis was confirmed by NMR. Structural characterization ^1H NMR (400 MHz, Chloroform- d) δ 8.56 (d, $J = 19.0$ Hz, 2H), 8.05 – 7.98 (m, 2H), 7.91 (dd, $J = 7.7, 1.4$ Hz, 1H), 7.68 – 7.63 (m, 2H), 7.47 (td, $J = 7.5, 1.4$ Hz, 1H), 7.42 – 7.32 (m, 2H), 5.09 (s, 1H), 4.79 (d, $J = 0.7$ Hz, 1H), 2.62 (s, 3H).

^{13}C NMR (101 MHz, Chloroform- d) δ 188.48, 181.63, 181.33, 158.12, 147.94, 141.42, 136.43, 134.73, 134.59, 133.73, 130.33, 130.24, 129.78, 129.72, 129.52, 129.47, 129.22, 129.05, 128.93, 128.42, 128.16, 125.32, 77.02, 69.98, 39.42

Synthesis of *N*-ethylnaphthoquinone adduct (V3 & V4)

To 0.245g (1.0mmol) of the 4-hydroxy-N-Ethylisoquinoline previously synthesized described above, 0.1738g (1.1mmol) of purified naphthoquinone and 0.1531 ml of triethylamine was added to 10ml of dry tetrahydrofuran. The solution was stirred for 19 hours at room temperature followed by dilution with ethyl acetate (200 mL) and washed with 20 mL water followed by 20 mL brine. The product was dried (magnesium sulphate) and concentrated under reduced pressure. This solid was further purified by flash column chromatography using silica gel and chloroform:methanol 99:1. Purity and synthesis was confirm by NMR. Structural characterization by NMR (400 MHz, Chloroform-d) gives the following values; ¹H NMR (400 MHz, Chloroform-d) δ 8.10 – 7.96 (m, 2H), 7.89 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.73 – 7.67 (m, 2H), 7.50 – 7.39 (m, 1H), 7.35 (dd, *J* = 7.9, 6.7 Hz, 2H), 5.17 (s, 1H), 4.86 (d, *J* = 0.7 Hz, 1H), 2.87 – 2.66 (m, 2H), 1.18 (t, *J* = 7.2 Hz, 3H).

¹³C NMR (101 MHz, Chloroform-d) δ 188.87, 181.85, 181.51, 157.19, 147.03, 140.80, 134.02, 133.83, 133.81, 132.83, 132.65, 129.05, 128.48, 128.27, 126.64, 126.29, 125.35, 77.18, 74.29, 67.20, 45.13, 13.07.

Synthesis of N-benyl naphthoquinone adduct (V2)

To 0.316g(1.0mmol) of the 4-hydroxy-N-benzylisoquinoline previously synthesized has described above, 0.1896g(1.2mmol) of purified naphthoquinone and 0.1531ml(1.2mmol) of triethylamine was added to 10ml of dry tetrahydrofuran. The solution was stirred for 19 hours at room temperature followed by dilution with ethyl acetate (200 mL) and washed with 20 mL water followed by 20 mL brine. The product was dried (magnesium sulphate) and concentrated under reduced pressure. This solid was further purified by flash column chromatography using silica gel and chlorform:methanol 99:1. Purity and synthesis was confirm by NMR. Structural

characterization by ^1H NMR (400 MHz, Chloroform-*d*) gives the following values; ^1H NMR (400 MHz, Chloroform-*d*) δ 8.08 – 8.03 (m, 1H), 8.03 – 7.97 (m, 1H), 7.92 (dd, $J = 7.6, 1.5$ Hz, 1H), 7.74 – 7.65 (m, 2H), 7.46 (td, $J = 7.5, 1.5$ Hz, 1H), 7.37 (td, $J = 7.6, 1.3$ Hz, 1H), 7.33 – 7.22 (m, 6H), 5.09 (s, 1H), 4.82 (d, $J = 0.7$ Hz, 1H), 4.00 – 3.80 (m, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 188.69, 181.80, 181.48, 157.17, 147.00, 140.91, 136.35, 134.03, 133.86, 133.84, 133.82, 132.83, 132.66, 129.09, 128.91, 128.69, 128.55, 128.35, 127.83, 126.63, 126.29, 125.40, 77.21, 74.49, 67.08, 55.41.

Cell culture

Cells were obtained from the American Tissue Culture Collection (ATCC) and maintained in DMEM (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) unless specified otherwise. H293 are kidney epithelial cells, NIH3T3s are mouse embryo fibroblasts, HeLa are cervical cancer cells, and HDFs are human diploid fibroblasts immortalized by telomerase expression. Cultures were maintained in a 37 °C water jacketed incubator with 5% CO₂. Serum starvation was carried out by removing media, washing with PBS, and then adding DMEM with 0.1% FBS. For experiments in 96-well plates (BD Falcon, Bedford, MA, USA), proliferating cells were removed from the stock plate using trypsin (Fisher Scientific) and counted using trypan blue and a hemocytometer. Typically between 1000 and 2500 cells were plated in each well containing 100 μL DMEM plus 10% FBS. For flow cytometry, proliferating cells were plated in 60 mm tissue culture plates (Nunc, Rochester, NY, USA) and then processed. In general, Cell viability analysis was carried out using various untransformed and transformed tissue culture cell lines to evaluate the cytotoxicity profile of the compounds. Cell death was quantified using the Cell Titer-Blue Cell Viability

Assay® (Promega, Madison, WI, USA), which is based upon the conversion of resazurin to the fluorescent resorufin by live cells[23]. Proliferating cells were plated in 96-well plates and treated with the indicated concentrations of compounds for 24 h. Resazurin was then added for 2–4 h and fluorescence measured at Ex560/Em590.

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