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A Thesis

Entitled

The Synthesis and Biological Characterization of a Potential Hypoxic Cell Sensitizer

By

Amy C. Beickelman

Submitted as partial fulfillment of the requirements for

The Master of Pharmaceutical Sciences

____

Advisor: Miles P. Hacker, PhD

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College of Graduate Studies

The University of Toledo

December 2007

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Abstract

Hypoxia within a solid tumor is known to cause resistance to radiation therapy. It is the biggest problem that cancer patients face during radiation treatment. In this study, a potential hypoxic cell sensitizer, AB2, was synthesized and then investigated for its ability to sensitize hypoxic cells to radiotherapy. The study began with the chemical synthesis of AB2 in a three step process. The structure of AB2 contains a 2-nitroimidazole and an aza-anthracenedione which are both important to the predicted activity of AB2. Once the synthesis of AB2 was complete the biological characterization of the compound was studied. These experiments began by demonstrating that anoxia in 8226 multiple Myeloma cells caused resistance to radiotherapy.

Once resistance was demonstrated the effects of AB2 were tested. AB2 was administered to both anoxic and oxic 8226 multiple Myeloma cells and the cells were exposed to radiation. AB2 caused an increase in the cell death in anoxic cells as well as oxic cells that were irradiated. However the cell death in anoxic cells was higher than in oxic cells. Cell death also increased in anoxic and oxic cells that were not irradiated and were simply administered a dose of AB2. A dose response experiment showed that as the dose of AB2 increased the amount of cell death also increased. All of the data gathered from this study suggests that AB2 is a potential drug for cancer therapy. AB2 appears to have selectivity to anoxic cancer cells and improves the cell death from radiation exposure.

Further research is necessary to in order to gain a better understanding of the biological characteristics of AB2. More *in vitro* and *in vivo* studies should be done to examine the toxicity of the compound and find the most effective dose. The use of a radiosensitizer, such as AB2, in combination with radiotherapy in cancer patients could eliminate the resistance that hypoxia causes to radiotherapy. The data from this study shows that AB2 is a promising compound for improving cancer therapy.

December 2007

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I. Introduction

How Cancer Begins

Cancer is a disease that results from damage to the genome through mutations (Hanahan, 1978). Genome damage can occur from errors in DNA replication, attack of bases by free radicals, ionizing and UV radiation and chemical carcinogens. During DNA replication mutations can occur despite DNA's proofreading mechanism. If a mismatch in base pairs is not corrected by proofreading enzymes it can result in a spontaneous mutation. Biochemical process within the body, such as respiration, phagocytosis and inflammation, generate free radicals. When free radicals build up in the body they can cause the formation of DNA adducts and strand breaks. Ionizing radiation includes electron beams from x-ray machines, gamma radiation and neutron radiation. Carcinogenesis from ionizing radiation can result from direct ionization of DNA or indirectly from free radicals formed from the radiation. Ultraviolet light, emitted from the sun and fluorescent lights, is another form of radiation that can induce mutations in DNA. UV light can be absorbed directly into DNA causing an increase in the formation pyrimidines dimers. These dimers can result in mutations and increase the rate of carcinogenesis.

While all of the above mechanisms for DNA damage are considered to be potentially carcinogenic, the most commonly discussed carcinogens are chemical carcinogens which comprise a very large group of compounds that is growing larger every year. Chemical carcinogens have three main groups, alkylating agents, aralkylating

agents and arylhydroxylamines. Some chemical carcinogens can react with DNA and cause misincorporation of nucleotides. (McKinnell, 2006)

It is believed that the development of a tumor is a multiple step process that begins with damage to a gene that leads to a cancer cell that grows into a solid tumor which can then metastasize throughout the body. The initial mutation in the genome must alter either the proliferation or apoptosis of cells for cancer to develop. The first step in carcinogenesis is initiation which involves the mutated cell. Three important functions, metabolism, DNA repair and proliferation, are involved in initiation. Metabolism can cause the activation or inactivation of the carcinogen, DNA repair can correct mismatched bases or introduce a mismatch, and proliferation causes the mutation to become permanent in the genome. A cell that has undergone initiation is not a cancer cell and does not always progress into a tumor because they can die through apoptosis. Tumor promotion is the second step in carcinogenesis and is the clonal expansion of the initiated cell. There are numerous agents such as phenobarbital and tetradecanoyl phorbol acetate that are considered to be tumor promoters. Exposure to only a tumor promoter agent will not cause the development of a tumor because all steps in carcinogenesis are required. The final step in the development of cancer is tumor progression and begins when the tumor gains the ability to grow, invade other tissues and metastasize. (Tannock, 2005) If the genetic mutation increases protein activity to increase, then the gene is known as an oncogene. However, if the mutation decreases gene activity and allows increased tumor growth, the gene is called a tumor suppressor gene (Bertram, 2001).

Humans have a small subpopulation of cells, known as stem cells which enable the body to continually provide cells that replace dying cells. To help control this cell division the body has many molecular mechanisms that limit proliferation and apoptosis. If the mechanisms that limit proliferation and apoptosis are not functioning properly a neoplasm can develop and the risk of cancer increases because a neoplasm, an area of cell growth, exists (Bertram, 2001).

As previously stated, cancer requires genomic mutations, thus a neoplasm alone will not result in cancer. So what causes mutations to DNA? Damage alone to DNA does not directly cause a mutation to the genome. Replication and division of the damaged gene is necessary for a mutation to occur. Even once a mutation exists there are mechanisms that limit inappropriate cell division within stem cells and therefore several mutations are necessary for cancer cells to develop. *Cahill et al* demonstrated this in colon cancer, showing that at least five genes need to be mutated for disease to occur (Cahill, 2004).

Once cancer cells have developed, the growing tumor has mechanisms that help to ensure its survival. Cancer can use normal growth factors to insure constant proliferation which is what allows the tumor to grow and eventually metastasize. Multiple mechanisms are available to enable the proliferation. Cancer also has the ability to avoid apoptosis through ways such as having a modified FAS receptor. Normally FAS receptors bind ligand and induce apoptosis but tumor cells can contain a modified receptor that bind the ligand but do not undergo apoptosis (Cahill, 2004).

Biology of a Solid Tumor

Cancer is a group of diseases rather that one specific disease. There are five basic terms that are used to differentiate tumors of different origins:

Carcinoma – a tumor derived from epithelial cells.

Sarcoma – a tumor derived from muscle, bone, connective tissues, fat and/or cartilage.

Lymphoma – a cancer of the bone marrow that is derived from cells of the lymphatic system.

Leukemia - a cancer derived from white blood cells and/or their precursors.

Myeloma – a cancer involving B lymphocytes. (McKinnell, 2006)

As previously stated, the growth of a solid tumor involves various steps, beginning with hyperplasia in which mutated cell begins dividing uncontrollably and an excess of cells develops. These cells will be normal in appearance but there will be more of them than needed. Dysplasia begins, which involves the hyperplastic cells undergoing additional genetic mutations that cause even more abnormal growth. At this time, the cells have abnormal appearances and lack normal organization. The tumor now invades normal surrounding tissues. At this time, the tumor is considered to be formed, and is known as carcinoma *in situ*. Tumors, at this point in time, have not metastasized to tissues in the body but many do have the potential to be malignant. Cancer is defined by the presence of malignant tumor cells. Even tumors that aren't metastasizing are still considered to be malignant. (McKinnell, 2006)

A malignant tumor has several unique characteristics. A normal cell can only divide a certain number of times before it ages and dies but cancer cells can divide an

infinite number of times. The division of the cancer cells can occur without the normal signals that most cells require for division. Normal cells stop dividing when they come into contact with other cells or stop dividing through a process known as differentiation. Malignant tumor cells do not respond to these signals to stop division and begin differentiation. (McKinnell, 2006 & Tannock, 2005).

The growth of a tumor is dependent on an adequate amount of oxygen, necessary nutrients and waste removal. Solid tumors can obtain the necessary nutrients through simple diffusion via extracellular fluid until it is between two and three millimeters in size (Barton-Burke, 2006). For it to then continue to grow the delivery of nutrients needs to occur through the circulatory system because cells in the inner core of the solid tumor no longer receive nourishment through simple diffusion of extracellular fluid. A tumor therefore must be able to induce angiogenesis, the formation of blood vessels. Angiogenesis is an essential process in the development of an embryo and throughout adulthood. It is a tightly regulated process that is controlled by maintaining a balance between activation and inhibition of angiogenesis.

The mechanism of tumor angiogenesis is poorly understood but it is known that several growth factors play a role in the process. Oncogenes can drive the upregulation of several growth factors that stimulate angiogenesis, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 and placental growth factor (PIGF) (Tannock, 2005). VEGF binds to receptors on endothelial cells and activates them to begin dividing and producing enzymes that dissolve the basement membrane of the surrounding tissue. Integrins and metalloproteinases (MMPs) then help

the migration of endothelial cells and attach to the tumor. The blood vessels are then stabilized by muscle and blood flow to the tumor begins (Barton-Burke, 2006).

Oncogenes also have the ability to down regulate angiogenic inhibitors like thrombospondin-1 and interferon-α. A very common oncogene, Ras oncogene, is responsible for controlling the signal cascade of angiogenesis. Many other oncogenes, growth factors and tumor suppressor genes are believed have a role in tumor angiogenesis. For example the tumor suppressor genes, such as *p53,* reduce tumor angiogenesis when activated (Tannock, 2005.) Once a tumor is vascularized growth seems unimpeded and metastasis becomes an important threat since the blood vessels that are formed during angiogenesis allow the cancer cells access to reach distant sites of the body. (Fidler, 1978)

The process of metastasis involves a cascade of events beginning with the migration of cancer cells away from the primary tumor. Migration requires the rearrangement of the cytoskeleton so that the cells can attach to other cells and to the extracellular matrix through proteins on the plasma membrane. Once the basement membrane is reached, the cancer cells secrete enzymes to degrade the membrane and the cells move through. MMPs is one of the groups of enzymes that are found in tumors at elevated levels. They can degrade any of the proteins found in the extracellular matrix (ECM). Cathepsins is another group of enzymes that cause the hydrolysis of ECM proteins such as collagen, proteoglycan and laminin. Cathepsin B has been found in many solid tumors and contributes to the breakdown of the ECM. Plasmin is another enzyme that hydrolyzes ECM components and the basement membrane (McKinnell, 2006).

Destruction of the basement membrane provides the cancer cells access to blood vessels or access to lymphatic vessels. Lymphatic vessels differ from blood vessels in that they carry lymph instead of blood and are not under pressure. The lymphatic system moves lymph passively through vessels by a series of valves. The movement of the cancer cell into a vessel is called intravasation. Intravasation into a vessel can occur through diapedesis, digesting a hole into the wall of the vessel and allowing the cell to move in. After tumors cells are inside a vessel they then must move out of the vasculature, extravasation, and into a new tissue to divide and grow. Extravasation requires the cells to move between endothelial cells and through the basement membrane and into tissue (McKinnell, 2006). Once the cells reach different tissues and organ systems they can settle into the tissue and begin growing a tumor. The new tumor can also undergo metastasis and spread using the same cascade of events (Fidler, 1978).

Metastasis is considered to be a very inefficient process because so few migrating cancer cells actually form metastases. Cells can die when they detach from the tumor because they are no longer in close contact with other tumor cells and can't survive alone. Tumor cells can also die if they become damaged or stuck in the vessels they are traveling through due to their large size. They can also be recognized and killed by the immune system. Despite the inefficiency of metastasis it is still the main cause of death in those who have cancer. Millions of tumor cells can leave a tumor each day during metastasis and only a few of those cells need to survive to create a new tumor. (McKinnell, 2006)

Cancer Therapy

Cancer therapy dates as far back as the medieval period, 500 – 1000 A.D. with the use of caustic pastes and excision procedures (Burton-Burke, 2006). Today, cancer patients have many options for treatment including, surgery, chemotherapy, biologic therapies, molecular targeted therapy, stem-cell transplantations and radiotherapy (Tannock, 2005). The type of treatment used is based on many different factors such as the type of cancer, tumor size, malignancy, age of patient, and health of the patient.

When the tumor exists as a primary tumor, surgery can be an excellent treatment option. Removal of the tumor can render a person cancer free if all the cancerous cells are removed. Quite often surgical removal of a tumor is paired with chemotherapy treatments. This helps ensure that all of the cancer cells are killed and that disease is eradicated. Chemotherapy is the use of drugs to treat the disease and usually involves the use of a combination of drugs. The advantage of using a combination of drugs is they can act synergistically or have an additive effect. Since chemotherapeutic drugs require repeated doses in order to maximize the number of cells that are killed therapy can last several weeks. Combination therapies can also reduce the amount of toxicity because smaller doses of each drug can be used rather than higher doses necessary for single drug therapies.

Cancer chemotherapeutic drugs fall into several different groups including alkylating agents, antibiotics, antimetabolites, hormones, mitotic inhibitors (Burton-Burke, 2006). Each class of drugs act in a different way and targets different receptors and molecular sites. The main problem with chemotherapy is toxicity due to the lack of drug selectivity. Some of the more common side effects include nausea, vomiting, and

fatigue. The drugs can affect many different sites throughout the body including chemoreceptors in the brain stem, the gastrointestinal tract (Tannock, 2005).

Another large problem with chemotherapy is that tumors have the ability to gain resistance to drugs. Once resistance is gained against one drug it is likely that the tumor will be resistant to any drug within the same class. The mechanism of drug resistance is complex and as more is learned about it, chemotherapy will improve (McKinnell, 2006).

The term biological therapy refers to the use of the biological mediators to elicit an immune response. Biological therapy has been used against cancer for several decades and continues to improve as our knowledge of the immune system grows. Biological mediators are useful in cancer therapy because tumors have several ways that they can avoid being recognized by the immune system. Tumors can suppress growth factors that normally cause an immune response, have poor antigen processing and expression of tumor-associated antigens. Therefore the use of biological mediators can activate the immune system and an immune response against the tumor can occur.

There are several biological mediators that can be used in cancer therapy today. Interleukin-2 (IL-2) is a cytokine that is made by T cells and activates cytotoxic T cells, natural killer cells and macrophages which all attack tumor cells. Another cytokine used in cancer therapies is interferon-α (IFN-α) which can regulate angiogenesis through the inhibition of angiogenic promoters. Inhibiting angiogenesis can help prevent tumor metastasis and slow tumor growth. A biological therapy that holds great hope but has no proven efficacy as of yet is a cancer vaccine (Barton-Burke, 2006).

Molecularly targeted therapy is an attempt to selectively kill cancer cells. One approach has been through the use of monoclonal antibodies that target cell surface

markers commonly over expressed on cancer cell membranes. For example, B-cell lymphomas, multiple myelomas, and B-cell leukemia express the antigen CD20, chronic lymphocytic leukemia can express CD20 and CD52 and myelocytic leukemia can express CD33. Monoclonal antibodies are designed to recognize one specific antigen that the tumor cells expresses and bind exclusively to that antigen. Antibodies are made up of two parts, the Fc region and the Fab region. The Fc region is the signal for the immune systems killing of the antibody labeled cell where as the Fab region is recognized and binds to the antigen. Given the selectivity of monoclonal antibodies will only bind cells that express a specific antigen and kill cancer cells. There are several monoclonal antibodies on the market that are used for cancer treatments such as Alemtuzumab which targets CD52, Gemtuzumab ozogamicin which targets CD33 and Rituximab which targets CD20 (Barton-Burke, 2006).

Stem-cell transplantations include bone marrow transplants and the infusion of stem cells harvested from umbilical cord blood. The stem-cells used for this type of therapy can come from the cancer patient or from a donor.

Radiation

Radiation was first discovered in 1895, by Wilhelm Conrad Roentgen, and was very quickly applied medically as a treatment for various diseases. The concepts behind the mechanism of radiation were not even fully understood at that time but only months after its discovery radiotherapy against cancer was established. In January of 1896, Dr. Emil Grubbe, of Chicago, used radiation to treat advanced breast cancer. From that point the concept of radiotherapy became entrenched as a modality of cancer therapy. In 1913 the hot-cathode tube was invented which allowed control of the radiation dose given.

The method of exposure to radiation continued to be improved after the invention of the cathode tube. The electron linear accelerator was invented in the 1940's followed by the circular electron accelerator several years later. Both accelerators generated a higher dose of radiation to be given in a defined field. During the 1960's the use of a radioactive implant that contained a radio-neucleotide became a common therapy allowing for short sessions outpatient of therapy. Development of assays that allowed for the quantization of cell killing helped to give a better understanding of tumor cell sensitivity to radiation. The invention of the CT scan and MRIs in the 1970's improved the imaging of tumors. It became possible to know the exact location and size of a tumor so that better treatment plans could be made. Today it is possible to accurately deliver a beam of radiation to a tumor and even today we are still improving the efficiency and safety of this therapy (Bernier, 2004).

Radiation therapy uses ionizing radiation to kill tumor cells. A photon, electron or proton beam will either indirectly or directly damage the DNA and when the cancer cell tries to divide it is unable to and dies. The cellular response to the DNA damage from irradiation is divided into three categories; division delay, interphase death and reproductive failure. Division delay involves a disruption of the normal ratio of mitotic cells and results in a delayed G2 phase of the cell cycle. Interphase death occurs when cells that are in G1, S or G2 phase of the cell cycle are exposed to irradiation. Reproductive failure is the most common cellular response to radiation and involves the cells being limited to the number of divisions they can make. It is important to know that non-cancerous cells can repair the damage caused by radiation and do not always die from its exposure. Radiation can be given to specific localized areas of the body

allowing exposure to healthy cells to be minimized. One way that this is accomplished is through lead shields, known as collimators, which reduce leakage of radiation and help target the beam to a specific area. Multi-leaf collimators are used and help to control the amount of radiation reaching healthy tissue. The use of fractionated doses of radiation also contributes to exposing only localized areas. When smaller doses of ionizing radiation are given over time rather than one large dose the amount of surrounding tissues affected minimized (Bernier, 2004).

For the treatment of cancer there are two different types of radiation that can be used, external radiation therapy and internal radiation therapy. External radiation involves a beam of radiation that is directed from outside the body. X-rays and gamma rays are focused into a beam through collimators so that the tumor receives the majority of the radiation. High energy x-rays are used to treat tumors found deep in the body while electrons are used to treat superficial tumors. For internal radiation therapy the source of radiation is present within the body. The radiation source is implanted into the body either next to or directly into the tumor. Radioactive materials are used for this type of radiation therapy and include cesium, gold, and iridium. The effects that the radioactive material has on surrounding tissue are minimal (Washington, 2004). The type of radiation therapy used varies based on the type of cancer, location of the tumor and the patient's medical history but both have proven to be successful. (Bernier, 2004)

Radiation and Hypoxia

Radiation is one of the most widely used therapies to treat cancer. One of the major limitations is that there are cells within a tumor that are hypoxic, or deficient in oxygen. Tumor hypoxia can result from a variety of reasons including, low oxygen

tension in arterial blood, anemia, increased blood diffusion distances and decreased tissue perfusion. Oxygen concentrations in normal tissues are between 10 and 80 mm Hg while oxygen concentrations found in tumors can be less than 5 mm Hg. Hypoxia within a tumor can be either acute or chronic. Blood flow into the tumor does not occur at a constant rate and therefore hypoxic regions are not permanent. If the blood flow to the hypoxic region increases it is possible to reoxygenate the area. However, there are areas of the tumor that may never be exposed to oxygen and will always be hypoxic. Acute and chronic hypoxia can occur at the same time within a tumor and hypoxic regions are dispersed throughout the tumor rather than concentrated in one area (Tannock, 2005).

Hypoxic cells are less sensitize to radiotherapy because oxygen is required to maximize the damage to DNA through the production of free radicals. Therefore as hypoxic cells increase they increase the resistance to radiation. Hypoxia is also detrimental to cancer patients because the low oxygen levels within a tumor have been shown, to increase metastasis and cause a decrease in overall survival rate of patients (Harris, 2002). Under hypoxic conditions genes involved in anaerobic respiration, the production of growth factors and the modification of the oxygen carrying capacity of blood are upregulated (Tannock, 2005).

The upregulation of genes during hypoxia often involves the hypoxia response element (HRE). HRE is found in the promoter region of the gene and responds to hypoxia-inducible factor-1 (HIF-1). HIF-1 is involved in the HIF-1 pathway which is one response that cells have to hypoxic conditions. HIF-1 is a transcription factor that has two sub-units, HIF-1α and HIF-1β. HIF-1β is expressed in every cells but HIF-1α is only found in hypoxic conditions. When HIF-1α and HIF-1β dimerize the pathway is

activated and the production of growth factors and cell proliferation increases (Tannock, 2005).

Hypoxic tumor cells use the process of glycolysis to metabolize glucose rather than the tricarboxyclic-acid (TCA) cycle used in normoxic cells. Glycolysis produces intermediates that are required for synthesis of glycine, serine, purines and pyrimidines which contribute to cell growth. Glycolysis produces only 2 molecules of ATP compared to the 36 molecules that the TCA cycle produces from a glucose molecule. The process of glycolysis can result in acidosis within the tumor cells. Cancer cells are able to adapt to the lower pH and proteases are activated that help tumor metastasis (Harris, 2002). Hypoxic cells can use glycolysis to help promote proliferation and metastasis.

Hypoxia is toxic to cells over a prolonged period of time but tumor cells can have a mutated p53 gene that makes the cells resistant to hypoxia induced apoptosis. The presence of this mutation in p53 can therefore be viewed as a promoter of tumor progression (Tannock, 2005). Hypoxia decreases the ability of DNA repair proteins to correct mistakes made during DNA replication. Mutated p53 and impaired functioning of DNA repair proteins in combination with the HIF-1 pathway and glycolysis makes targeting hypoxic regions in a tumor an important therapy goal.

Reversal of hypoxic induced resistance to radiation is being studied by many researchers in the hope to improve cancer therapies. Various strategies have been studied clinically to increase oxygen delivery to the tumor, including blood transfusions and hyperbaric chambers. These approaches rely on the idea that if the oxygen level in the blood is higher then more oxygen can diffuse into the tumor. The use of anti-angiogenesis agents has also been studied to see if they reduce the amount of hypoxia in

the tumor. It is believed that these agents work by helping to regulate the vasculature (Tannock, 2005). Reoxygenation of tumor cells may be the most important part of radiotherapy in order to cure a patient. Many researchers are trying to solve this problem in the hope to improve the prognosis of cancer patients.

Hypoxic Cell Radiosensitizers

Radiotherapy could be greatly enhanced if drugs could sensitize hypoxic cells to ionizing radiation. There are several different types of drugs have been investigated including the nitroimidazoles, nitric oxide donor agents and bioreductive anticancer agents (Weinmann, 2003). Drugs that can sensitize hypoxic cells are considered to be selectively active within the body because it will only act where there are hypoxic cells. This selectivity helps to minimize the damage done to surrounding tissue and decreases the chance developing side effects. (Rauth, 1998)

Metronidazole, a 5-nitroimidazole, was one of the first nitroimidazoles studied in the 1970's (Weinmann, 2003). Nitroimidazoles are considered to be oxygen mimics and increase the sensitivity of hypoxic cells to radiation and the sensitivity to chemotherapeutic compounds. In some cases they are directly cytotoxic to hypoxic cells. Nitroimidazoles can diffuse further away from blood vessels than oxygen allowing it to reach more hypoxic tumors cells. Since the testing of metronidazole several more nitroimidazoles have been tested clinically including misonidazole, etanidazole, pimonidazole and nimorazole. Trials using radiation and misonidazole have not shown any significant effect on tumor cell death compared to using radiotherapy alone. Furthermore, these drugs cause an increase in neurotoxicity as well as other side effects in cancer patients (Weinmann, 2003). After misonidazole was found to not be an

effective radiosensitizer more nitroimidazoles were studied. Etanidazole and pimonidazole are both 2-nitroimidazoles that were found to have less neurotoxicity but it is still unclear if they are effective. Etanidazole is currently in phase II and III clinical trials and its efficacy is still being determined (Jin, 2007). Another 5-nitroimidazole, nimorazole, was also tested in clinical trials and has been found to have an increased effect on tumor cell death compared to radiation alone. Trials also showed increased patient survival with less toxicity and adverse effects than other previously tested nitroimidazoles (Weinmann, 2003). This group of compounds is continuing to be tested today to find the most appropriate nitroimidazole and the best dose that will minimize side effects and maximize the therapeutic benefits.

Nitric oxide is also capable of sensitizing hypoxic cells to radiotherapy. It is made within the body from L-arginine and has many different roles within the body. It can readily diffuse through cell membranes and have an effect on processes such as apoptosis, gene regulation and cytostasis. Nitric oxide reacts with superoxide radicals to make a peroxynitrate which is an oxidizing agent and effects bioactivity. Nitric oxide plays a variety of roles important in bioregulatory actions in mammalian cells including smooth muscle relaxation, neurotransmission and the inflammation process. However, in studies of radiosensitization nitric oxide donating compounds were used to create the nitric oxide as they produce far more nitric oxide in short periods of time. Common nitric oxide donor compounds include organic nitrates, nitrite esters, sodium nitroprusside and S-nitrosothiols (Al-Sa'doni, 2005). One study done by Mitchell *et al* used sodium trioxodinitrate, also known as Angeli's salt, to produce nitroxyl anions. Angeli's salt in

combination with an oxidizing agent was shown to cause radiosensitization in hypoxic cells but had no effect on cells with normal oxygen levels (Mitchell, 1998).

Studies have shown that inhibition of nitric oxide production by macrophages lowers anti-tumor responses. This finding supports the idea that nitric oxide produces cytostasis or cytotoxicity in tumors (Al-Sa'doni, 2005). Nitric oxide donor compounds could be an exciting possibility for targeting hypoxic cells within tumors and continues to be studied.

Bioreductive anticancer agents include several groups of compounds including mitomycins and benzotriazone di-N-oxides. These compounds are referred to as bioreductive agents since they require reduction reactions to generate cytotoxic metabolites. One such compound is mitomycin C (MMC) which has been shown to undergo reduction better in hypoxic cells than in oxic cells (Rauth, 1998). When MMC was used in combination with radiation in clinical trials, MMC improved the control of tumor growth and increase survival in patients with head and neck cancer, cervical cancer, and anal cancer compared to radiation alone (Weinmann, 2003). Tirapazamine, a benzotriazone di-N-oxide, is capable of inducing single and double strand DNA damage once it is metabolized to an active compound. Such activation occurs best in hypoxic cells since in the presence of oxygen the parent compound is reformed. Phase I and II clinical trials of tirapazamine combined with radiotherapy showed increased survival of patients with cancer of the head and neck. Unfortunately patients report muscle cramping and nausea and vomiting (Weinmann, 2003)

Drugs that target hypoxic tumor cells are showing promising results for future cancer therapies. Toxicity of the potential anti-cancer drug is one of the biggest problems

that researchers are facing. The search is still ongoing to find drugs that will selectively radiosensitize hypoxic cells while maintaining low levels of toxicity. Finding such a drug could dramatically change the treatment of cancer.

New Compound – AB2

The goal of this research was to design a compound that would selectively target hypoxic cells within a tumor. The compound created, AB2, (see Figure 1) for chemical structure) contains a 2-nitroimidazole and an aza-anthracenedione. The design of AB2 was based on several compounds that have previously been studied such as, metronidazole, etanidazole, misonidazole and nimorazole. Using the research of many nitroimidazole compounds the structure of AB2 was designed to have higher pharmacological activity and fewer adverse side effects than these compounds.

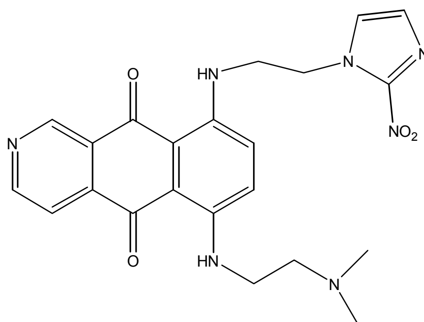


Figure 1:
Structure of AB1

Misonidazole, a 2-nitroimidazole, has been studied for many years as an anticancer drug. Its effects on hypoxic tumor cells have been shown in many different clinical trials. The results, in general, are disappointing and most trials do not show an advantage to using misonidazole in cancer therapy. One study included 331 cancer patients that were given either a dose of misonidazole, $12g/m^2$ applied over six weeks, or a placebo along with radiotherapy. Results showed that those patients who received misonidazole did not have a significant increase in survival rate (Weinmann, 2003). A phase III trial done in 306 patients with head and neck squamous carcinoma also showed

no significant advantage for the use of misonidazole. This trial compared patients who received fractionized radiotherapy and misonidazole (2.0g/m^2/week) and patients who just received fractionized radiotherapy alone. Misonidazole also been shown to have several adverse effects including nausea and vomiting which decreased patient compliance (Weinmann, 2003). These two clinical trials along with many other studies of misonidazole show that it is capable of sensitizing hypoxic tumor cells but not at a high enough level to have a therapeutic benefit to radiotherapy. One of the reasons that misonidazole is not a useful anti-cancer drug could be that it is unable to intercalate into DNA. Therefore it is hypothesized that creating a drug that intercalates would allow for a more selective targeting of hypoxic tumor cells leading to an increase in its anti-tumor activity.

Nimorazole is a 5-nitroimidazole that has been extensively studied for its ability to sensitize hypoxic cells to radiation. Early clinical trials done on patients with head and neck cancer showed promising results. When nimorazole was used in combination with continuous hyperfractionated accelerated radiation therapy (CHART) a slight increase in patient survival was observed (Weinmann, 2003). A phase II trial involving sixty one patients with head and neck cancer used nimorazole (1.2, 0.9 and 0.6 g/m^2) and CHART. All the patients in the study were followed up with for two years and the results showed a positive effect on the sensitization of hypoxic tumor cells. After two years the loco-regional control was 55%. However there were several potential adverse effects from the use of nimorazole such as a skin reaction, nausea, vomiting, peripheral neuropathy and encephalopathy (Henk, 2003). One Phase III trial involved 422 patients with invasive cancer of the larynx and pharynx. One group of patients received nimorazole and

conventional primary radiotherapy and the other group received a placebo and the same radiotherapy. Those who took the nimorazole had a loco-regional control rate of 49% versus the 33% control rate of those taking a placebo but the difference in the survival rate, between the two groups after ten years, 26% for nimorazole versus 16% for placebo, was found not to be statistically significant (Weinmann, 2003). While nimorazole has shown some promising results during clinical trials the survival rate of the patients is still not optimal. Our compound, AB2, is designed to have a high level of anti-tumor activity in addition to its ability to sensitize hypoxic cells.

Many of the radiosensitizers that have been studied against cancer have shown a high level of cardiotoxicity due to the combination of chemotherapy and radiotherapy. This was the main adverse effect that AB2 was designed to avoid. The structure of AB2 contains an aza-anthraquinone that is hypothesized to decrease the cardiotoxicity that other radiosensitizing compounds have shown. Clinical trials reported thus far have show there to be a decreased propensity of cardiotoxicity with the aza-anthracenedione Pixantrone (Beggiolin, 2001).

This study involves the synthesis of a new potential hypoxic cell sensitizer and its biological characterization. The synthesis is described as a three step process that ends with the structure of AB2. The structural analysis of AB2 is included within the results to confirm the predicted structure. The biological characterization includes a cytotoxicity assay to determine the best concentration of AB2 to administer to multiple myeloma cell lines. The drug was administered to hypoxic multiple myeloma cells and oxic multiple myeloma cells and differences in cell survival are measured. From these preliminary

studies AB2 demonstrates the potential to have therapeutic use against hypoxic tumor cells.

II. Materials and Methods

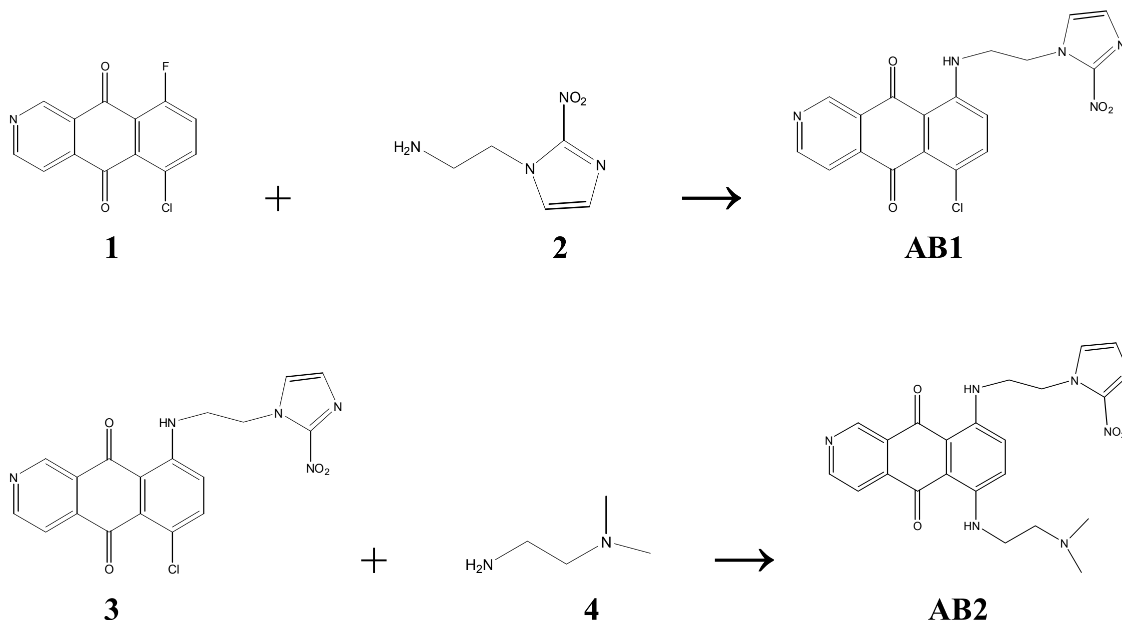


Figure 1. The chemical equations for the synthesis of AB1 and AB2.

Chemistry

2-(2-nitro-1H-imdazoyl)ethylphthalimide (2). Anhydrous potassium carbonate (3.05 g, 25.3 mmol) was added to anhydrous dimethylformamide (DMF) (75 mL) under nitrogen. No dissolution of the solid occurred. 2-nitroimidazole (2.5 g, 22 mmol) and 2-bromoethylphthalamide (5.9 g) were added to the suspension which then turned yellow in color. The mixture was then placed in a 110°C oil bath for 2 hours and was then cooled to room temperature. Solvent was removed under vacuum to yield a yellow solid. The solid was dispersed using distilled water (94.5 mL) and filtered from the aqueous phase. The solid was collected and dried and yielded 9.6 g of white solid.

Crystallization: All of the product was added to 150 mL of a mixture of methanol:water (3:1). The solution was heated to its boiling point for 10 minutes and left to cool and crystallize over night. The following day the small white crystals were collected through filtration and dried under vacuum.

The product (1.28 g) was mixed with absolute ethanol (24 mL) and heated to reflux. A solution of hydrazine hydrate (464 µL) in absolute ethanol (4 mL) was added to the suspension and refluxed for two hours. The mixture was cooled to room temperature and then placed into an ice bath for approximately 15 minutes. The precipitate was then removed by filtration, washing with absolute ethanol, which was then evaporated in vacuum to yield a yellow solid. The product was then applied to a silica gel quartz column containing approximately 30 g of silica gel with no TLC binder. The product was eluted out of the column with methylene chloride:methanol (1:1).

'H-NMR 600 mHz ($CDCL_3$): 7.16 (d, J=0.6 Hz, 2H); 4.48 (t, J=6 Hz, 3H); 3.16 (t, J=6 Hz, 3H); 1.246 (t, J=6.6, 5H)

Compound AB1. 6-Chloro-9-fluorobenzo[g]isoquinoline-5, 10-dione, **1**, (87.13 mg, 0.333 mmol) was combined with **2** (104.09 mg, 0.667 mmol) and triethylamine (46.4 µL, 0.333 mmol) in anhydrous tetrahydrofuran (THF) (1 mL). The solution was left at room temperature for approximately 72 hours and during that time the mixture changed from yellow to red in color. The product was dissolved in methylene chloride and applied to a silica gel quartz column containing approximately 25 g of silica gel with no TLC binder. The product was eluded out using methylene chloride as the solvent. The product was a reddish purple in color and weighted approximately 60 mg.

TLC (silica gel, methylene chloride:methanol (25:1, Rf= 5.9mm)

'H-NMR 400 mHz ($CDCL_3$): 9.51 (s, 1H); 9.05 (d, J=4.8 Hz, 1H); 8.01 (d, J=5.2 Hz, 1H); 7.61 (d, J=9.2 Hz, 1H); 7.18 (d, J=3.2 Hz, 2H); 7.07 (d, J=9.2 Hz, 1H); 4.76 (t, J=6 Hz, 2H); 3.98 (q, J=6 Hz, 2H)

Mass Spec. (Electrospray) 398.2$[M+1]^+$ (predicted M=397)

Mp = 232-232.8ºC

Compound AB2. A mixture of **3** (39 mg, 0.1mmol) and 1, 2-ethanediamine, N,N-dimethyl-, **4**, (110.2 µL) in anhydrous pyridine (1 mL) was placed in a 90°C oil bath for four hours. The solvent was evaporated under vacuum and the product rinsed several times with ethanol, evaporating the solvent off after each rinse. The product was then applied to a chromatography column containing approximately 25 g of silica gel with no TLC binder. The product was eluded from the column using methylene chloride:methanol (25:1) yielding 70 mg and was blue in color.

TLC (silica gel, methylene chloride:methanol (25:1), Rf= 5.3mm)

'H-NMR 400 mHz ($CDCL_3$): 9.58 (s, 1H); 8.96 (d, J=4.8 Hz, 1H); 8.14 (d, J=5.2 Hz, 1H); 7.21 (d, J=2.4 Hz, 2H); 7.14 (d, J=7.6 Hz, 2H); 4.72 (t, J=6 Hz, 2H); 3.98 (q, J=6.4 Hz, 2H); 3.53 (q, J=5.6 Hz, 2H); 2.697 (t, 2H)

Mass Spec. (Electrospray) 450.6$[M+1]]^+$ (predicted M=449.6)

Other Chemistry Procedures

Synthesis of 2-[2-(dimethyl(amino)ethyl]-5-(chloro)indazolo-[4,3gh]isoquinolon-6(2H)-one. A mixture of 2-dimethyl(amino)ethyl hydrazine (1.5 g) and anhydrous THF (5 mL) was added drop-wise, over a 30 minute period, to an aza-anthraquinone (1.26 g) and diisopropylethylamine (0.84 mL) in anhydrous THF (25 mL). The solution was stirred at room temperature for one hour. The solution was added to water (75 mL) and then extracted with methylene chloride 5 times using 25 mL of solvent. The organic layers were collected and dried with sodium sulfate and evaporated under vacuum. The product was applied to a chromatography column and methylene chloride:methanol (10:1) was used to elute the product from the column. The product obtained was a yellow solid that weighed 1.9 g.

Synthesis of 2-amino-imidazole. *O*-methyl-isourea-hemisulfate (44.37 g, 0.258 mol) was placed under nitrogen and deionized water (75 mL) and 2-amino-acetaldehyde-diethylacetal (54.6 g, 0.41 mol) were added. The reaction mixture was stirred under nitrogen for 4 hours at 50ºC. The reaction mixture was then cooled to 20°C and concentrated sulfuric acid (3.5 mL) was added. The mixture was then placed in a 100°C oil bath with stirring for 2 hours, was cooled to 20°C and added drop-wise to ice cold ethanol (0-5°C, 1500 mL). After 1 hour stirring at 0-5°C, the product was filtered, washed with ice cold ethanol (25 mL) and dried under vacuum (Weinmann *et al, 2002*).

Synthesis of 2-nitroimidazole. 2-aminoimidazolium sulfate (1.57g, 5.94 mmol) and 50% fluoboric acid (7 mL) was dissolved into distilled water (10 mL) and placed into a -

20ºC ice bath to cool. A solution of sodium nitrite (4.1 g, 59.4 mmol) in distilled water (10 mL) was added drop-wise to the 2-aminoimidazolium sulfate solution and then stirred for 30 minutes in a -10ºC ice bath. The mixture was then added to a solution of cupric sulfate-5H2O (29.7 g, 119 mmol) and dimethylaminophenol (DMAP) (1 g) in distilled water (200 mL). Sodium nitrite (4.1 g, 59.4 mmol) was then added and the solution was stirred at room temperature for 2 hours. The pH of the solution was adjusted to pH=2 using nitric acid and was then extracted several times with ethyl acetate (100 mL portions) and the ethyl acetate layers pooled. The product was dried over sodium sulfate overnight and solvent was removed under vacuum (Agrawal *et al, 1979*).

Biological Characterization Methods

Preparation of Media

8226 multiple myeloma cell were grown in suspension, RPMI (500mL), supplemented with 10% (v/v) fetal bovine serum and 5% penicillin-streptomycin solution. The media was stored at 4°C but warmed before use.

Cell Maintenance

The 8226 multiple myeloma cell cultures were maintained in T75 flasks with approximately 20 mL of media. Media was changed every four to five days. The cell culture was placed into a 50 mL test tube and centrifuged for five minutes at 3000 rpm. After centrifugation the media was removed by aspiration. Fresh media, 5 mL, was added to the test tube and the cell pellet dispersed. Half of the cell suspension, 2.5 mL, was placed into one T75 flask and 17.5 mL of media added. The other portion was either placed into a second T75 flask or disposed of properly depending on the need for cell

cultures. The media was then placed back into the refrigerator and the cell cultures were returned to the incubator.

Cell Enumeration

After the cell pellet is broken up a portion of the cell suspension is placed into a hemocytometer. A hemocytometer is a piece of laboratory equipment that is used to determine the number of cells per unit volume of a cell suspension (Figure 2). The cell suspension was placed into the hemocytometer by

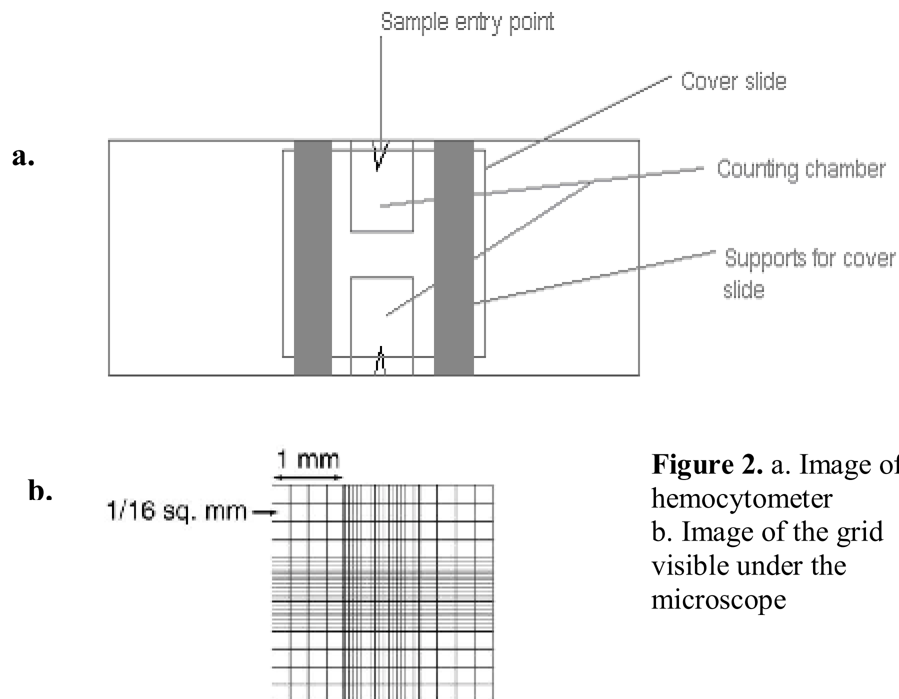


Figure 2. a. Image of a hemocytometer
b. Image of the grid visible under the microscope

using a 10 µL pipet and placing the pipet tip into the sample introduction point and dispensing the suspension from the pipet. Capillary action pulls the cell suspension into the hemocytometer until the entire area was filled. Three or four of the 1 mm x 1 mm squares were counted and the number of cells counted averaged. Any excess cell had 25% bleach solution added to it to kill the cells and dispose of them properly.

Cytotoxicity Tests of AB2

A solution of 2 mg of AB2 and 0.2 mL of PBS were mixed together to have a concentration of 500 µg/mL. A series of serial dilutions were then done using 20 µL of drug concentrations and adding 180 µL of PBS to get four more concentrations, 50 µg/mL, 5 µg/mL, 0.5 µg/mL and 0.05 µg/mL. Next flasks of 8226 multiple myeloma cells were counted and diluted to 50,000 cells/mL using fresh media. Three wells in eight rows of a 96 well plate were filled with 190 µL of cell suspension and placed in the incubator overnight. The next day the cell suspensions in each well were dosed with 10 µL or 25 µL of the various concentrations of AB2 or PBS as a control.

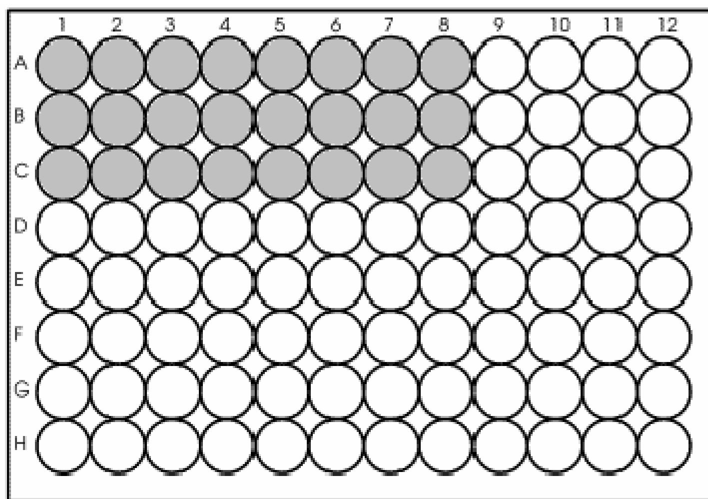


Figure 3. Diagram of a 96 well plate. The wells that are gray are the wells that were filled with cell suspension.

Determining the Time of Radiation Exposure for Cell Cultures

8226 multiple myeloma cells were diluted with media to 500,000 cells/mL. Five test tubes were filled with 2 mL of the cell suspension and exposed to 260 rads/min for various time periods (Figure 11a & 12a). After radiation exposure each of the five cell suspensions were diluted 1:10 using fresh media. Each of the five suspensions were split

into three test tubes containing 3mL so that there were 3 test tubes for each of the radiation exposure times. All 15 test tubes of cells were placed into the incubator. After four days cell counts were performed (Figure 11 & 12).

Determining the Effect of Anoxia on Cell Survival after Radiation

Counted 8226 multiple myeloma cells from a T75 flask and diluted with media to have 500,000 cells/mL. 1 mL of the cell suspension was placed into 10 test tubes, 5 labeled anoxic and 5 labeled oxic. The anoxic tubes were exposed to a stream of nitrogen for 5 minutes, and capped tightly. All 10 test tubes were placed into the incubator for 4 hours. Each of the test tubes were exposed to 260 rads/min for varying times (Figures 13). After radiation exposure diluted each of the 10 cell suspensions 1:10 using fresh media. Split the suspensions into three test tubes containing 3mL so that there were 3 test tubes for each of the radiation exposure times. All 30 of the test tubes were placed into the incubator. Cell counts were done six days later (Figures 13).

Determining the Effect of AB2 on both Anoxic and Oxic Cells after Radiation

Counted 8226 multiple myeloma cells from a T75 flask and diluted with media to have 500,000 cells/mL. 1 mL of the cell suspension was placed into 24 test tubes, 12 labeled anoxic and 12 labeled oxic. Six of the anoxic tubes and six of the oxic tubes were administered 10 μL of AB2. The 12 anoxic tubes were exposed to a stream of nitrogen for 5 minutes and capped tightly. All test tubes were then placed into the incubator for 4 hours. Each of the test tubes of cell suspension were exposed to 260 rads/min for varying times (Figures 17 & 18). After radiation exposure diluted each of the 24 cell suspensions 1:10 using fresh media. Split the suspensions into three test tubes containing 3mL so that

there were 3 test tubes for each of the radiation exposure times. All 72 of the test tubes were placed into the incubator. Cell counts were done five days later (Figure 17 & 18).

Dose Response Curves for AB2 with and without Radiation Exposure

Mixed 2 mg of AB2 into 200 μL of sterile PBS until completely dissolved to get a drug solution of 10 mg/mL. Took 100 μL of this solution and added it to 900 μL of PBS to make a 1 mg/mL drug solution. Took 50 μL of the 10mg/mL drug solution and added it to 950 μL of PBS to make a 0.5 mg/mL drug solution. Took 100 μL of the 1mg/mL drug solution and added it to 900 μL of PBS to get a 0.1 mg/mL drug solution.

Counted 8226 multiple myeloma cells from a T75 flask and diluted with media to have 500,000 cells/mL. Placed 0.7 mL of cell suspension into 16 small test tubes, 8 labeled anoxic and 8 labeled oxic. 33 μL of each drug solution concentration was administered into 2 anoxic tubes and 2 oxic tubes and 33 μL of PBS was administered to 2 anoxic and 2 oxic tubes. The 8 anoxic tubes were exposed to a stream of nitrogen for 5 minutes. All test tubes were then placed into the incubator for 4 hours. Four of the anoxic tubes and 4 of the oxic test tubes (1 of each drug solution concentration) were exposed to 260 rads/min for 2 minutes (Figure 19 & 20). After radiation exposure diluted all of the 16 cell suspensions 1:10 using fresh media. Split the suspensions into 3 test tubes containing 1mL each. All 48 of the test tubes were placed into the incubator. Cell counts were done seven days later (Figure 19 & 20).

III. Results

Our initial target compound was 2-[2-(dimethyl(amino)ethyl]-5-(chloro)indazolo-[4,3gh]isoquinolon-6(2H)-one, also called MH1, and MH2 (Figure 4). MH1 was successfully synthesized but MH2 was never made. Several attempts were made to add 2-(2-nitro-1H-imdazoyl)ethylphthalimide (Figure 5) to the chlorine on MH1 but none succeeded. The target compound was then changed from MH2 to AB2 (Figure 5) and synthesis began (see material and methods for procedures). AB1 was made in a multiple step process beginning with a replacement reaction of the fluoride molecule with 6-chloro-9-fluorobenzo-[g]isoquinoline-5, 10-dione. 2-Ethanediamine, N,N-dimethyl- was then added onto AB1 by replacing the chloride molecule with it. Mass spectrometry and NMR results (Figures 6-9) all confirmed that the synthesis of AB1 and AB2 was successful.

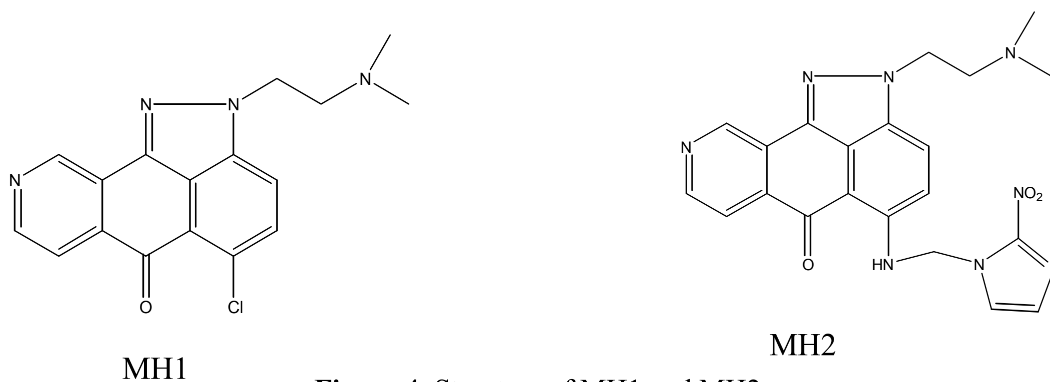


Figure 4. Structure of MH1 and MH2.

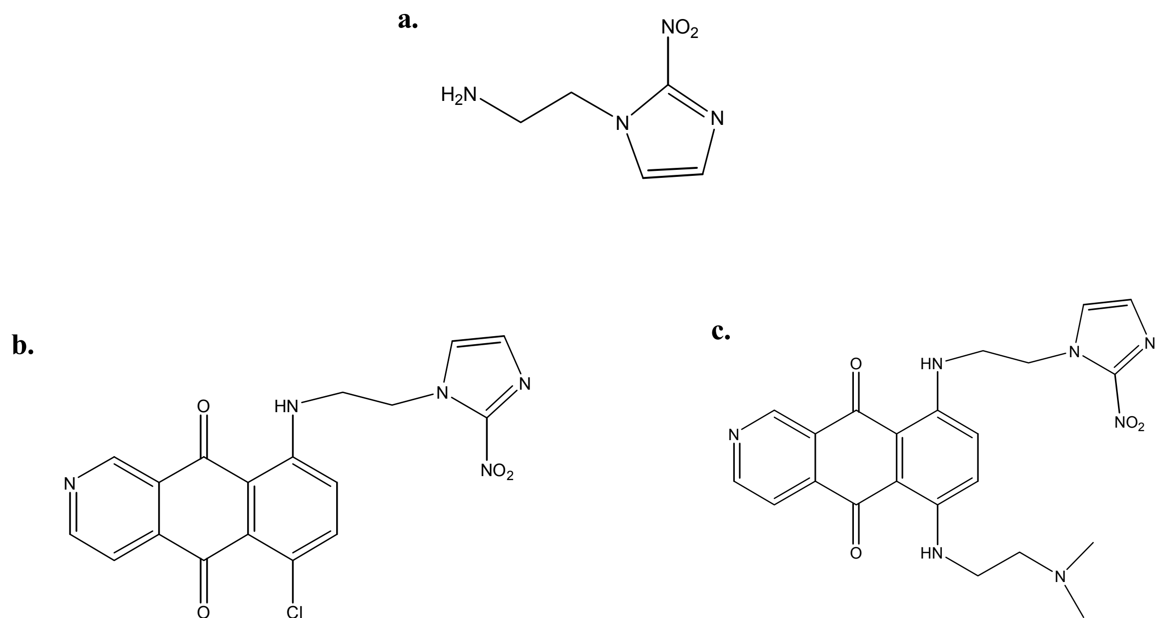


Figure 5. a. Structure of 2-(2-nitro-1H-imdazoyl)ethylphthalimide.
b. Structure of AB1. c. Structure of AB2.

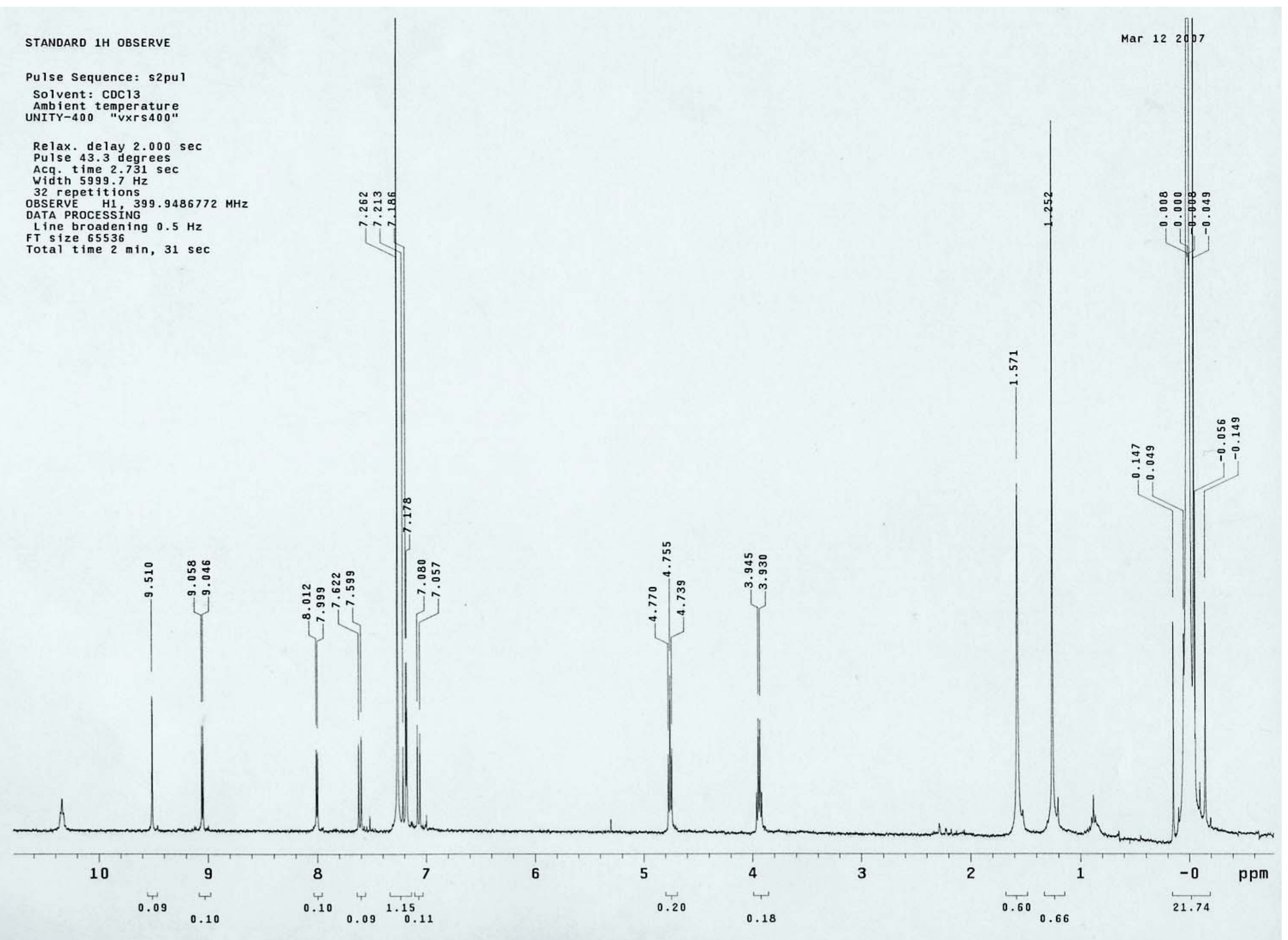


Figure 6 ¹H-NMR of AB1 in $CDCl_3$.

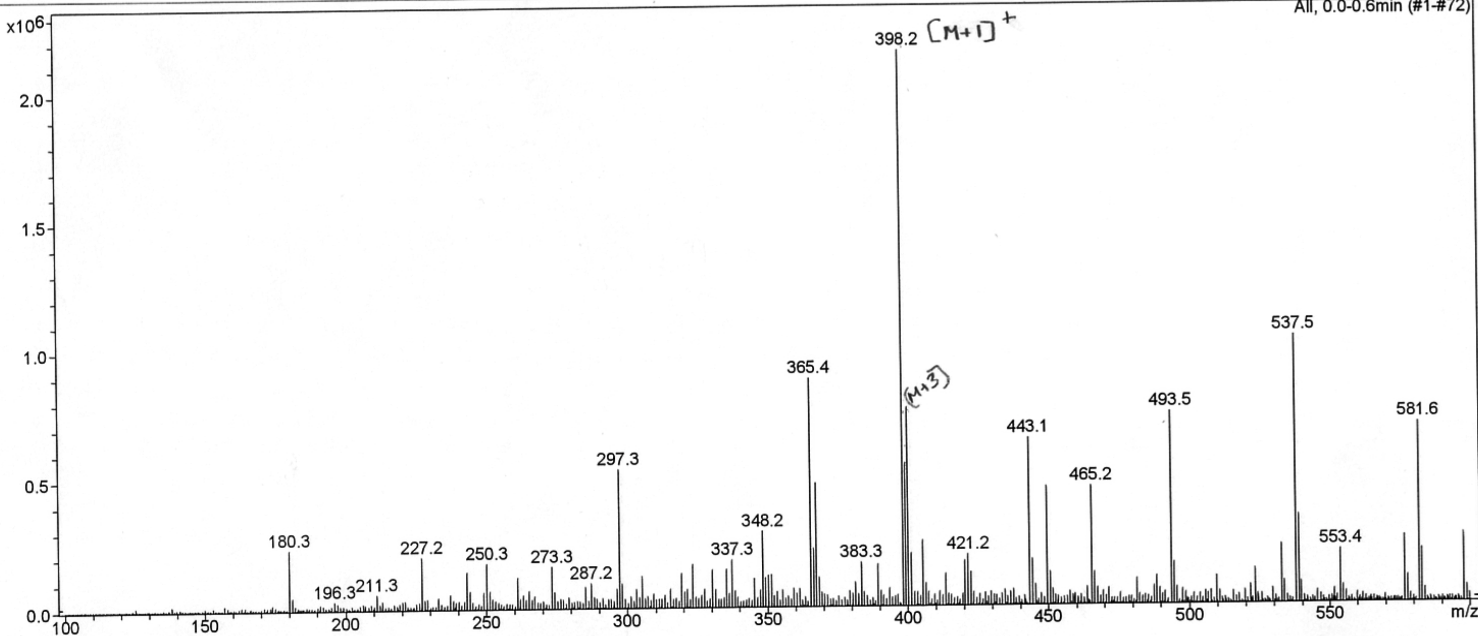


Figure 7. Mass spectrometry of AB1.

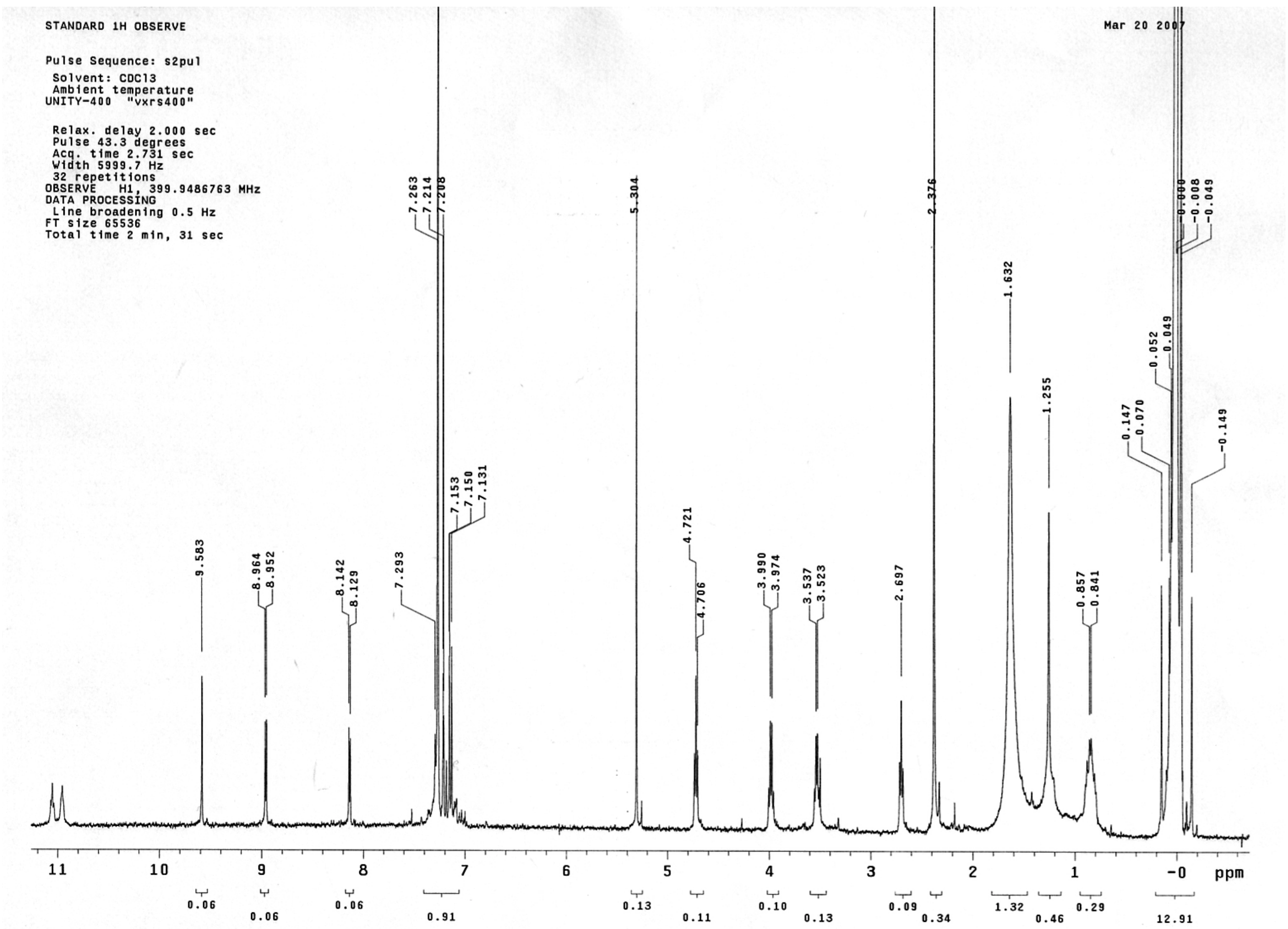


Figure 8. ^{1}H-NMR of AB2 in $CDCl_3$.

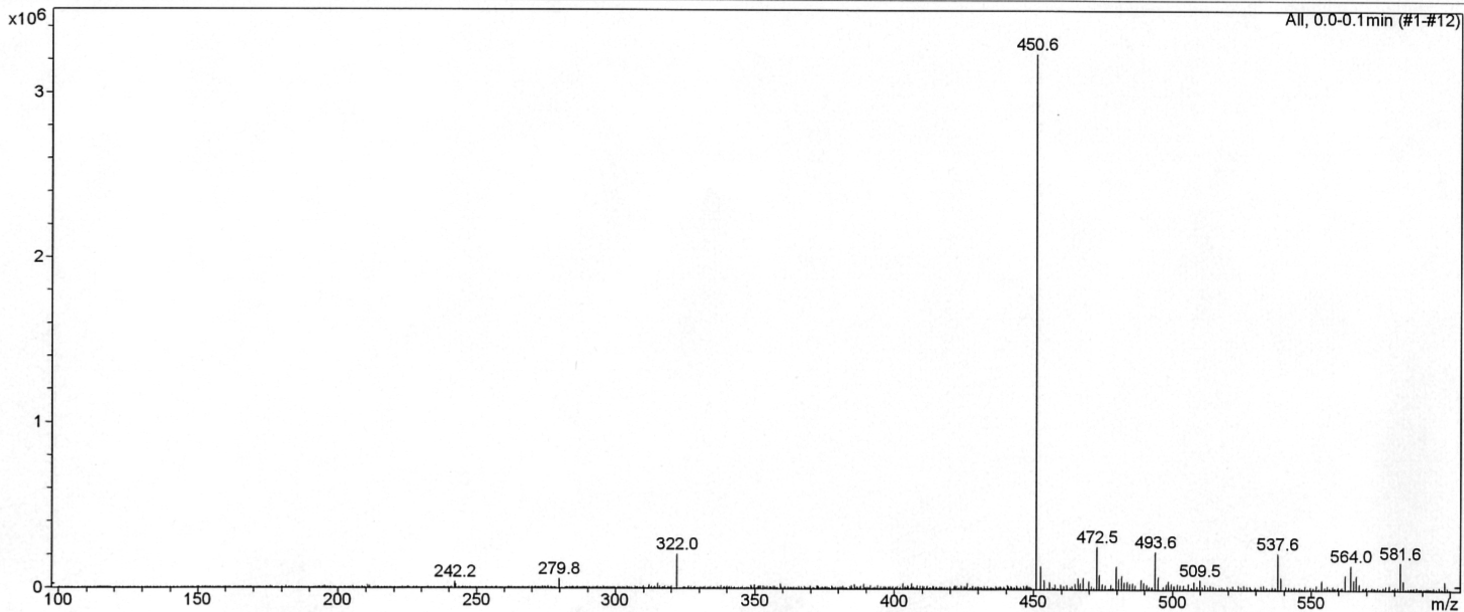


Figure 9. Mass spectrometry of AB2.

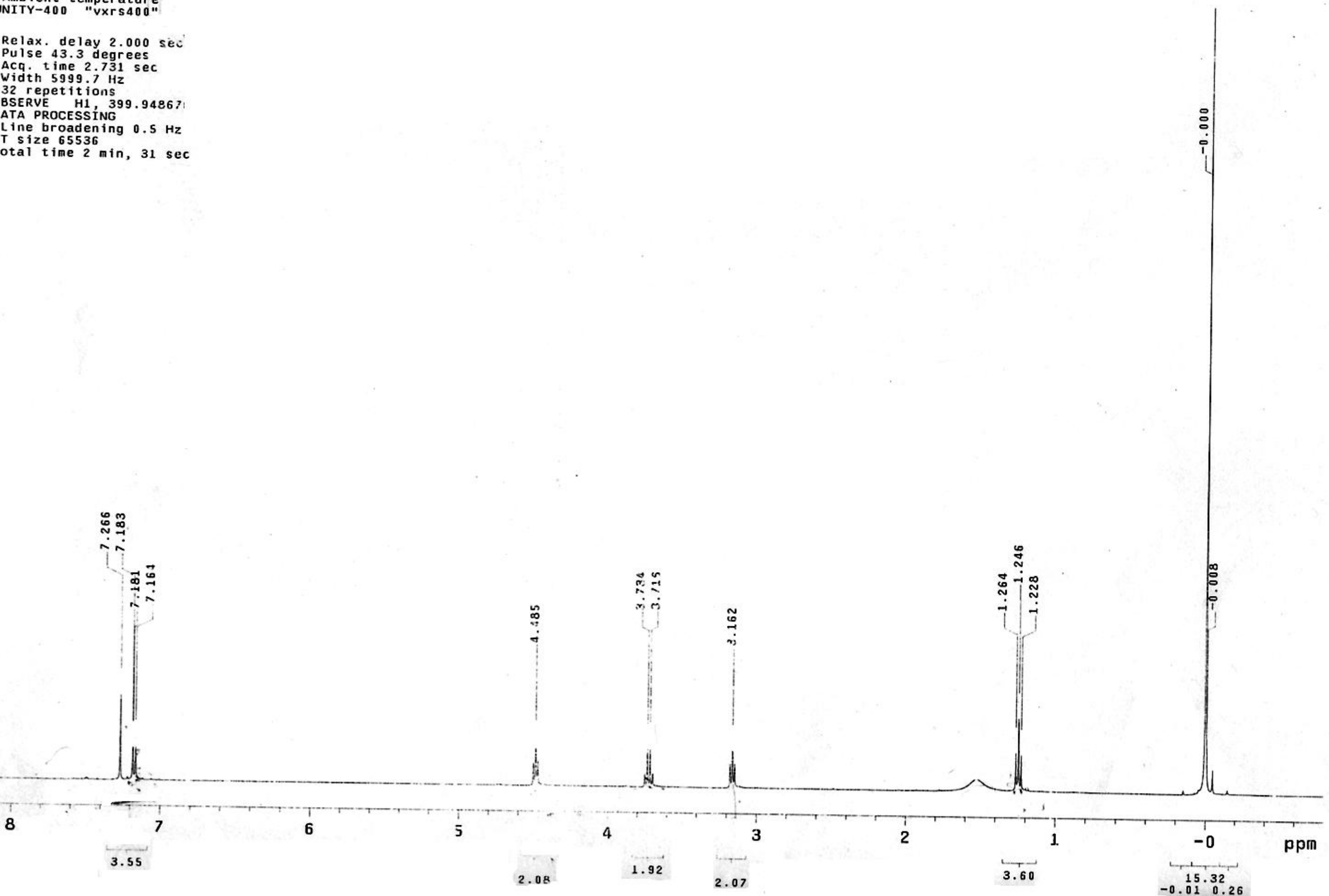


Figure 10. ‘H-NMR of 2-(2-nitro-1H-imdazoyl)ethylphthalimide in $CDCl_3$.

Cytotoxicity of AB2 was studied by setting up two 96 well plates containing 8226 cells. The following concentrations of AB2 were in the cell suspensions of the plate wells, 0 µg, 0.0095 µg, 0.095 µg and 0.95 µg. Neither of the plates had cell growth inhibition (Figure 11).

a.

Drug Conc. Added to Wells (µg/mL)	Drug Conc. (µg)	Average Number of Cells for Plate 1 (cells/mL)	Average Number of Cells for Plate 2 (cells/mL)	Average Number of Cells for Plate 1 & 2 (cells/mL)
0	0	903,000	953,000	928,000
0.05	0.0005	805,000	901,000	853,000
0.05	0.00125	793,000	892,000	842,500
0.5	0.005	770,000	860,000	815,000
5	0.05	788,000	875,000	831,500

b.

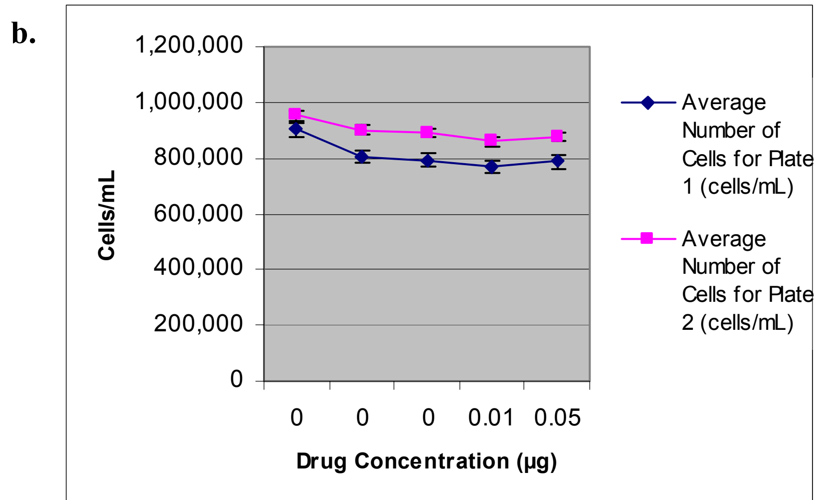
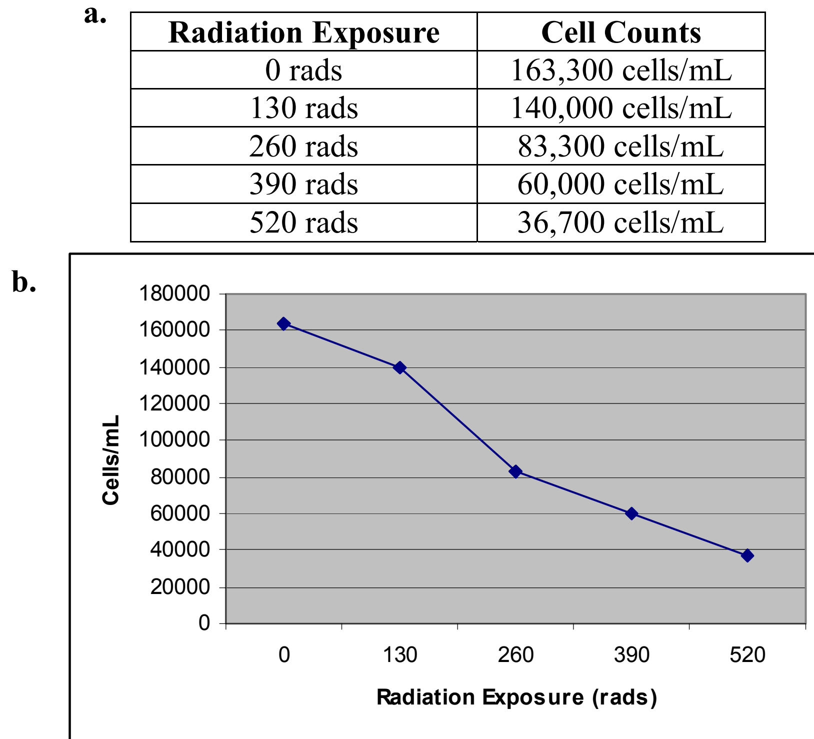


Figure 11. a. Cell counts for 8226 cells and the corresponding drug concentration administered. b. Graph of the cell counts.

Radiation exposure time was determined through a set of experiments using 8226 multiple myeloma cells. The radiation machine used emitted 260 rads/min. The exposures that were tested 0 rads, 130 rads, 260 rads, 390 rads and 520 rads. The cell

counts obtained four days later showed an increase in the amount of cell death and a consistent increase in cell death with increased exposure time (Figure 12).



a.

Radiation Exposure	Cell Counts
0 rads	163,300 cells/mL
130 rads	140,000 cells/mL
260 rads	83,300 cells/mL
390 rads	60,000 cells/mL
520 rads	36,700 cells/mL

Figure 12. a. Table of 8226 multiple Myeloma cell counts and radiation exposures. b. Graph of the results found in the table.

The next set of experiments was done to determine the effect of anoxia on the cell survival of 8226 multiple myeloma cells when exposed to radiation (Figure 13). A significant increase in cell survival of the anoxic cell suspension was seen at 520 rads, 390 rads and 260 rads.

a.

Tube	Radiation Exposure (rads)	Cell Counts for oxic (cells/mL)	Cell counts for anoxic (cells/mL)
1	520	153000	240,000
2	390	183000	283000
3	260	246700	336700
4	130	350000	366700
5	0	406700	400000

b.

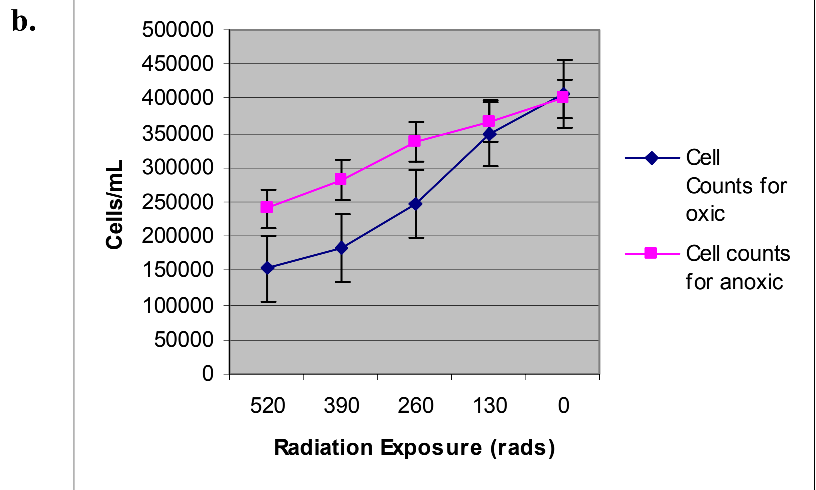


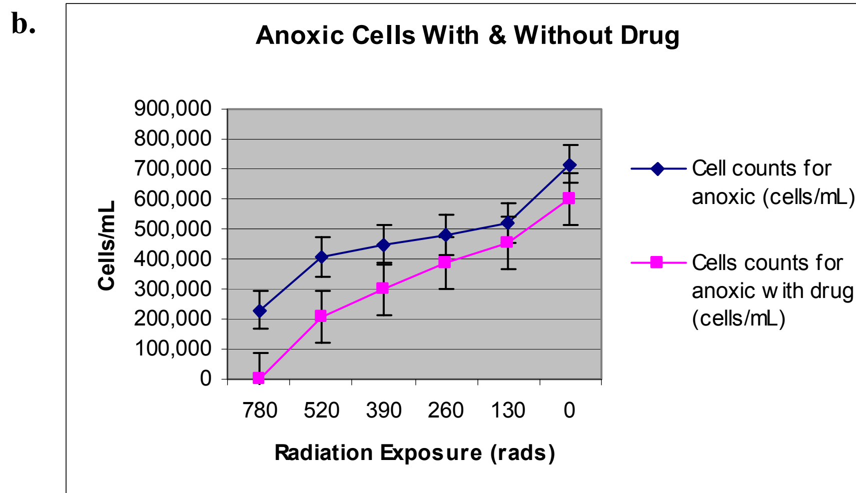
Figure 13. a. Table of 8226 multiple Myeloma cell counts for oxic suspensions and anoxic suspensions and radiation exposures. b. Graph of the results found in the table.

The effect of AB2 on 8226 multiple myeloma anoxic cells and oxic cells exposed to radiation for various periods of time was then tested. Anoxic versus oxic cells showed that the anoxic cells had a higher amount of cell survival after radiation exposure (Figure 15b). The anoxic cells that were administered 5µg/mL AB2 exhibited more cell death than the anoxic cells that had no AB2 administered (Figure 14b & Figure 15c). Oxic cells that were administered the same dose of AB2 as the anoxic cells showed the same pattern. There was less cell survival in the oxic cells that received AB2 than those that did not (Figure 14c & Figure 15d).

a.

Tube	Radiation Exposure (rads)	Cell Counts for oxic (cells/mL)	Cell Counts for oxic with drug (cells/mL)	Cell counts for anoxic (cells/mL)	Cells counts for anoxic with drug (cells/mL)
1	780	253,000	135,000	230,000	0
2	520	330,000	240,000	406,700	206,700
3	390	365,000	233,000	450,000	296,700
4	260	400,000	280,000	480,000	386,700
5	130	480,000	310,000	520,000	453,000
6	0	566,700	476,700	715,000	600,000

b.



c.

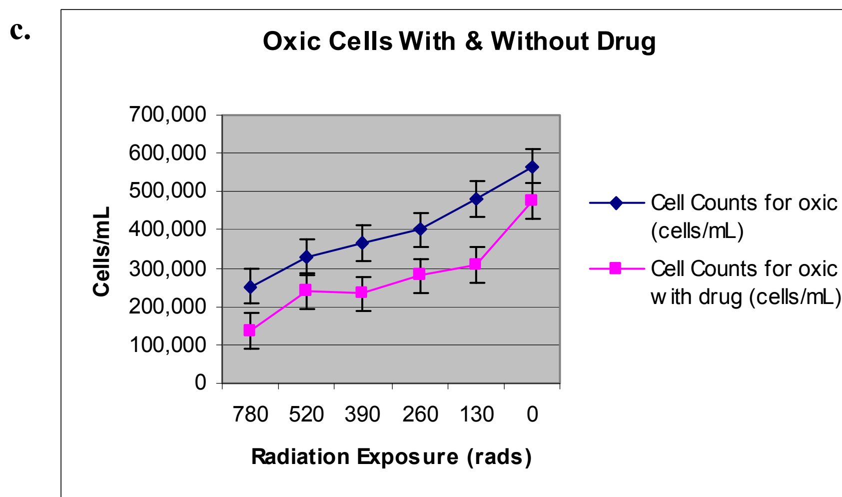
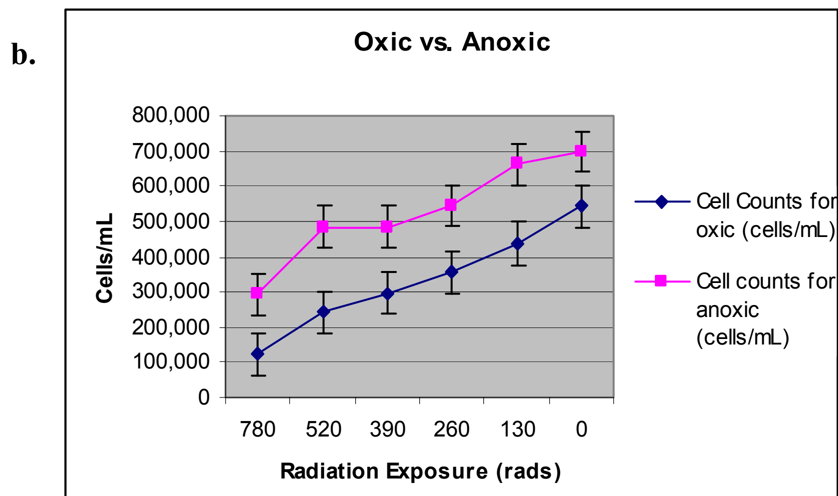


Figure 14. a. Table of 8226 multiple Myeloma cell counts for oxic cells with & without a dose of AB2 and for anoxic cells with & without a dose of AB2. b. Graph showing anoxic cells vs. anoxic cells with a dose of AB2 growth after radiation exposure. c. Graph showing oxic cells vs. oxic cells with a dose of AB2 growth after radiation exposure. (see next page)

a.

Tube	Radiation Exposure (rads)	Cell Counts for oxic (cells/mL)	Cell Counts for oxic with drug (cells/mL)	Cell counts for anoxic (cells/mL)	Cells counts for anoxic with drug (cells/mL)
1	780	123,000	158,900	294,300	337,700
2	520	243,000	266,700	484,300	417,800
3	390	296,700	291,000	483,200	350,000
4	260	356,700	322,000	544,500	501,000
5	130	436,700	333,400	661,000	581,000
6	0	543,000	446,600	697,800	682,000

b.



c.

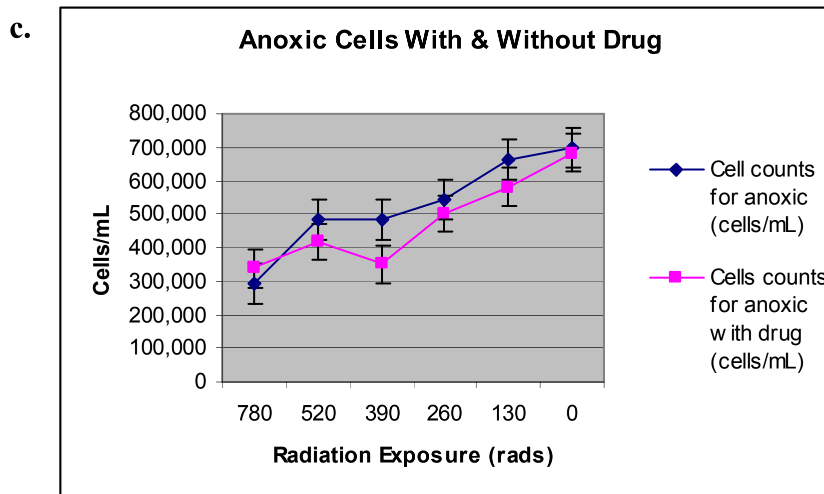
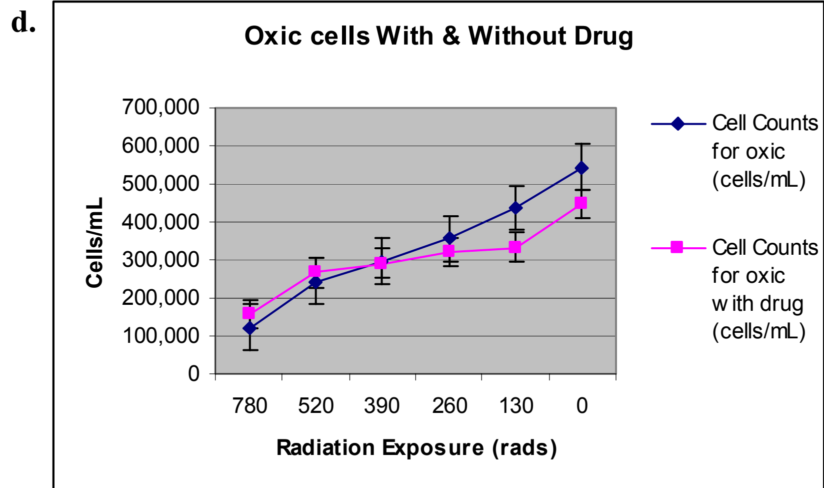


Figure 15. a. Table of 8226 multiple Myeloma cell counts for oxic cells with & without a dose of AB2 and for anoxic cells with & without a dose of AB2. b. Graph showing anoxic vs. oxic cell growth after radiation exposure. c. Graph showing anoxic cells vs. anoxic cells with a dose of AB2 growth after radiation exposure. d. Graph showing oxic cells vs. oxic cells with a dose of AB2 growth after radiation exposure. (see next page)

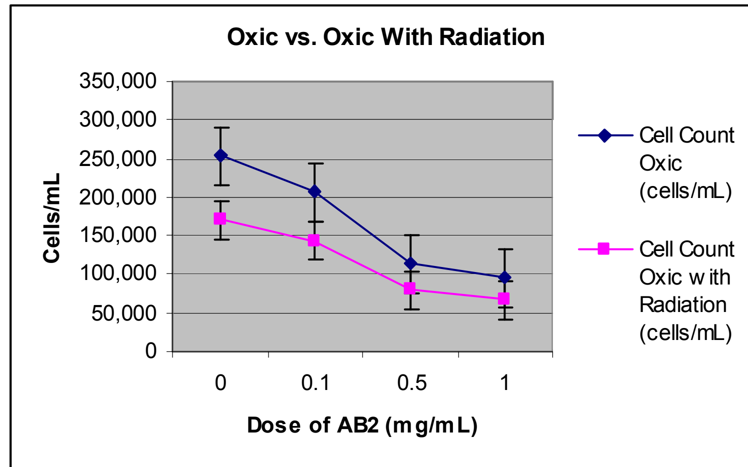


The dose response of AB2 on 8226 multiple myeloma cells was tested with radiation exposure (520 rads) and without radiation exposure. The concentrations of drug solutions of AB2 tested were 0 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1mg/mL. The anoxic cells that were exposed to radiation showed a small decrease in cell survival compared to the anoxic cells that were not irradiated (Figure 16) and the same results were seen with the oxic cells. However, the results are not statistically significant with the exception of the results in Figure 1b. All of the cell suspensions showed a decrease in cell survival as the concentration of AB2 increased whether the cells were oxic or anoxic (Figure 16). Results of the oxic cells that were exposed to radiation versus the anoxic cells that were exposed to radiation show that at the highest concentration of AB2 the cell survival is statistically the same (Figure 17).

a.

Tube	Dose of AB2 (mg/mL)	Cell Count Oxic (cells/mL)	Cell Count Oxic with Radiation (cells/mL)	Cell Count Anoxic (cells/mL)	Cell Count Anoxic With Radiation (cells/mL)
1	0	253,000	170,800	336,700	236,700
2	0.1	206,700	143,000	178,000	106,000
3	0.5	113,000	80,000	163,000	103,000
4	1	95,000	67,000	60,000	60,000

b.



c.

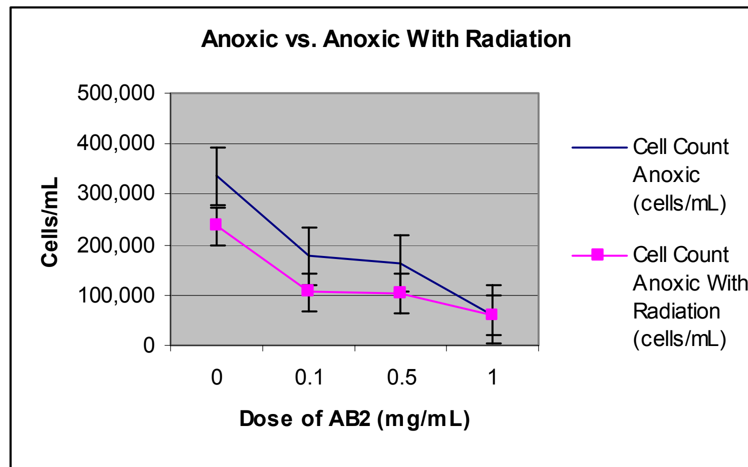


Figure 16. a. Table of 8226 multiple Myeloma cell counts of anoxic and oxic cells, with & without radiation exposure. b. Graph of the cell growth of oxic cells vs. oxic cells with radiation exposure. c. Graph of the cell growth of anoxic cells vs. anoxic cells with radiation exposure.

a.

Tube	Dose of AB2 (mg/mL)	Cell Count Oxic with Radiation (cells/mL)	Cell Count Anoxic With Radiation (cells/mL)
1	0	195,000	218,300
2	0.1	145,800	155,000
3	0.5	45,000	13,300
4	1	32,500	5,000

b.

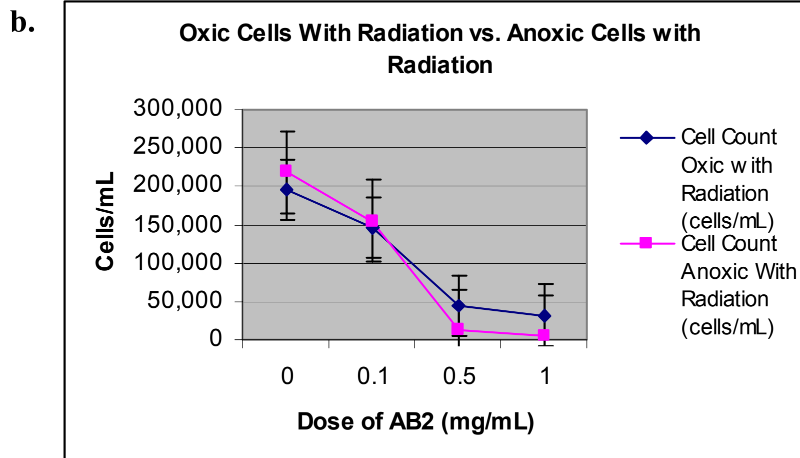


Figure 17. a. Table of 8226 multiple Myeloma cell counts for oxic and anoxic cells exposed to radiation. b. Graph of the cell growth.

IV. Discussion

The goal of the present study was to develop a new class of radiosensitizers that intercalates DNA and targets areas of hypoxia within solid tumors. Tumor hypoxia is a major problem in the therapy of cancer patients. Oxygen is required during radiation therapy in order to maximize the damage to DNA through the production of free radicals. Hypoxia is also detrimental because low oxygen levels within a tumor have been shown to increase metastasis and cause a decrease in overall survival rate of patients (Harris, 2002). Hypoxia occurs in tumors for various reasons including low oxygen tension in arterial blood, anemia, increased blood diffusion distances and decreased tissue perfusion. If blood flow into the tumor is not adequate or constant areas of hypoxic will occur. This type of hypoxia is considered reversible and will only persist until blood flow increases back into the area. However, some areas within a tumor can be permanently hypoxic serves as a significant toad block to radiation therapy. Reversal of hypoxic induced resistance to radiation would greatly increase the effectiveness of radiotherapy in cancer patients either through enhanced oxygen delivery or the use of a radiosensitizing drugs.

The amount of hypoxia within a tumor is directly related to the outcome of radiotherapy and the prognosis of patients undergoing radiotherapy (Denko, 2003 & Ljungkvist, 2005). Several different methods have been studied in attempt to overcome the resistance to radiation that hypoxia causes. The use of various radiosensitizers is one of those methods (Ljungkvist, 2005). Nitroimidazoles, considered to be oxygen mimics,

have been studied extensively and many show great potential as therapeutic drugs (Weinmann, 2003). They are a direct substitute for oxygen molecules in the radical reduction pathway that results from DNA oxidation, resulting in breaks in DNA strands. Nitroimidazole compounds diffuse across cell membranes and undergo a cytoplasmic reduction reaction and form a radical. When nitroimidazole compounds are in cells that have normal oxygen levels it reacts with the radical anion to form a non-charged nitroimidazole. This non-charged nitroimidazole can then diffuse out of the cell. However, if the nitroimidazole compound is in a hypoxic cell the nitroimidazole radical anion undergoes further reduction to form nitrous compounds. These nitrous compounds combine covalently with macromolecules in the cytoplasm and become trapped inside the cell (Weinmann, 2003).

Tumor hypoxia can be found in the majority of cancers and is easily mimicked *in vitro* by depriving the cells of oxygen for an extended period of time. For this study the 8226 multiple myeloma cells were deprived of oxygen by exposing them to a stream of nitrogen gas for several minutes and then closing them off from the environment. The data shows that the 8,226 multiple myeloma cells that were exposed to the nitrogen gas do in fact have resistance to radiotherapy, indicating that tumor hypoxia was being mimicked *in vitro*. Even at the highest level of radiation tested, 520 rads, the cell survival in anoxic cells was higher than in oxic cells. Sensitizing hypoxic cells to radiation is necessary to kill all of the cells during radiotherapy. However, it is worth noting that when there was no radiation exposure to hypoxic cells and oxic cells the amount of cell survival was equivalent. This shows that radiation does play an important role in the death of tumor cells.

Sensitizing hypoxic areas within a solid tumor to radiation has several different beneficial effects that AB2 can contribute to. In this study, the sensitization of hypoxic 8226 multiple myeloma cells is due to the 2-nitroimidazole within the structure of AB2. An increase in cell death after the administration of AB2 to the hypoxic cells was shown, indicating radiosensitization of the hypoxic cells. This sensitization of hypoxic cells does not only increase cell death in tumor cells. AB2 may cause a reduction in the rate of metastasis because the sensitivity of the hypoxic cells to radiation increases. Low oxygen levels within tumors have been shown to increase metastasis and AB2 may help counteract this (Harris, 2002). When AB2 acts as an oxygen mimic due to the 2-nitroimidazole and moves into hypoxic cells it becomes an oxygen molecule substitute in the radical reduction pathway and causes DNA strands breaks ultimately sensitizing hypoxic cells to radiation. This sensitization to radiation kills hypoxic tumor cells and therefore reduces the amount of cells that can metastasize. Hypoxia also causes genes involved in anaerobic respiration to be up-regulated which increases growth factors (Tannock, 2005). Sensitizing hypoxic cells with the use of AB2 may help stop the increase in growth factors within the tumor. Glycolysis is also affected by hypoxia resulting in the production of intermediates that activate proteases and contribute to cell growth. It is probable that AB2 also plays on role in the decrease of glycolysis within the tumor through the sensitization of hypoxic cells. *In vivo* studies will help to determine whether AB2 is causing a significant decrease to these hypoxia-related events.

When hypoxia occurs in normal healthy tissues toxicity is more chronic in nature and can result in apoptosis of cells. This is not the case in tumor cells. Tumor cells can have a mutated p53 gene which makes the cells resistant to hypoxia induced apoptosis.

Mutation in p53 gene of cancer cells can therefore be viewed as a promoter of tumor progression (Tannock, 2005). Metastasis is promoted in tumor cells that have this mutation and survival of the patient decreases. The use of AB2 as a part of cancer therapy would help to counter act the resistance to cell death that a mutated p53 gene provides because it increases cell death in hypoxic cells. AB2 does not directly affect the mutated p53 gene or the resistance to apoptosis that it causes but sensitizing hypoxic cells to radiation and causing cell death would counter act the affects of a mutated p53 gene.

Nitroimidazoles have been studied as radiosensitizers and the effectiveness of many is unclear. Misonidazole, a 2-nitroimidazole, shows no significant increase in patient survival and several adverse effects but is capable of sensitizing hypoxic cells to radiotherapy (Weinmann, 2003). While misonidazole lacks therapeutic benefits, AB2 may solve this problem. The use of a 2-nitroimidazole in the structure of AB2 causes an increase in the sensitivity of anoxic cells to radiation (Weinmann, 2003). By combining the 2-nitroimidazole with an aza-anthraquinone, the compound is able to intercalate into the DNA of the tumor cells, resulting in better efficacy. At a dose of 5 μg/mL cell death increases in the anoxic cells at high levels of radiation which indicates that sensitization to radiation in occurring.

A dose response experiment produced data about the cytotoxicity of AB2. The data shows that as the dose of AB2 increased the amount of cell death increases in both anoxic and oxic cells that were exposed to radiation. Even when the anoxic and oxic 8226 multiple myeloma cells were not exposed to any radiation after receiving increasing doses of AB2 cell death still increased. This suggests that AB2 has toxicity within cancer cells even without radiation exposure. If radiation exposure is not required to produce cell

death in the 8226 multiple myeloma cells it may suggest that high doses may cause toxicity *in vivo*. Further studies are needed to determine the best dose that does not produce high levels of toxicity.

Several experiments were done to compare the effects on cell death of anoxic and oxic cells that were exposed to radiation with and without a dose of AB2. The data indicates that AB2 has some effect on the cell death of oxic 8226 multiple myeloma cells. The data from one of the experiments showed the oxic cells that received a dose of AB2 showed more cell death than the oxic cells that that were not dosed with AB2. However, the cell death that occurs in the oxic cells that were administered AB2 was not as high as the cell death seen in anoxic cells. This suggests that AB2 has selectivity to hypoxic cancer cells rather than oxic cancer cells. Selectivity to hypoxic cells helps to decrease adverse effects seen *in vivo* and decrease the amount of damage to surrounding tissues making therapy more tolerable to the cancer patient.

This study gave preliminary data suggesting that AB2 may be a beneficial anti-cancer drug. AB2 is a new compound and therefore more information is needed to fully characterize the compound. However, it appears that this compound has activity within hypoxic tumor cells. Sensitization of hypoxic cells to radiation was the goal when AB2 was designed and this study supports this concept. However, more research needs to be done to closely examine AB2's mechanism of action in tumor cells. AB2's exact mechanism of action is currently unknown even though this study demonstrates that AB2 has a great potential as a new therapeutic drug in the fight against cancer.

Further research is needed in order to complete AB2's biological characterization and fully understand the potential therapeutic effects as an anti-cancer drug. An increase

in the purity of the drug is needed therefore crystallization of AB2 should be done. Crystallization will allow for the optimal dose to be determined without the need for alterations of the dose due to impurities. Once a pure sample of AB2 is obtained more *in vitro* studies should be done that investigate several more doses of AB2. The results can then be compared to the results from this study and correlations can be made. After *in vitro* studies are complete research can move into *in vivo* studies to evaluate AB2's effect in living animals. *In vivo* studies will also evaluate if there is any toxicity, specifically whether cardiotoxicity occurs. The major adverse effect many anti-cancer drugs is cardiotoxicity. AB2 was designed to avoid this by incorporating. The aza-anthracenedione backbone in the structure of AB2 to help decrease the cardiotoxicity, as has been reported clinically for the aza-anthracenedione Pixantrone.

Cancer is a disease that kills millions of people each year and therefore it is important to continue researching various ways to improve current treatments. Hypoxia within tumor cells has become the biggest problem encountered during radiation therapy. It results in a less effective response of the cancer cells to therapy and more patient death. The use of radiosensitizers is a promising option as a pharmaceutical agent to aide in the improvement of radiotherapy. This study synthesized a novel compound, AB2, and was able to show that it is capable of sensitizing cancer cells to radiation. More research needs to be done on AB2 to expand further on the data reported in this study but AB2 has the potential to be a beneficial anti-cancer drug. The use of a radiosensitizer in combination with radiotherapy in cancer patients could eliminate the problem that hypoxia causes within tumors. AB2 may be the compound that is able to overcome the resistance to radiation that hypoxic tumor cells cause.

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